The Scale-up Production of Human Brain Organoids (HBOs) on a Pillar Plate *via* Spheroid Transfer

This standard operating procedure (SOP) provides step-by-step methods for the scale-up generation of human brain organoids (HBOs) on a 36PillarPlate by transferring embryoid bodies (EBs) in an ultralow attachment (ULA) 384-well plate to the pillar plate and differentiating them in a 384DeepWellPlate for static culture (or 36PerfusionPlate for dynamic culture). Please read the protocol carefully before performing experiments.

1. Materials:

1.1 Materials for maintenance and passage of iPSCs

- Costar® 6-well clear TC-treated multiple well plates, individually wrapped, sterile (Corning, Cat. no. 3516)
- EDi029-A, a male human iPSC line (Cedar Sinai Biomanufacturing Center, USA)
- Matrigel® growth factor reduced (GFR) basement membrane matrix (Corning, Cat. no. 354230)
- DPBS, no calcium, no magnesium ($dPBS^{-/-}$; Gibco, Cat. no. 14190)
- Accutase™ cell dissociation reagent (Gibco, Cat. no. A1110501)
- **Complete mTeSR™ plus medium:** Mix the mTeSR™ plus basal medium with the 5x supplement (StemCell Technologies, Cat. no. 100-0276) at room temperature.
  Note: *Thaw the frozen 5x supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C. Do not thaw in a water bath at 37°C. Once thawed, use the supplement immediately or aliquot and store at - 20°C for up to 3 months.*
- StemPro™ EZPassage™ tool (Gibco, Cat. no. 23181-010)
- ReLeSR™ human pluripotent stem cell passaging reagent (StemCell Technologies, Cat. no. 100-0483)
- CryoStor® CS10 cell freezing medium (StemCell Technologies, Cat. no. 07959)
- Mr. Frosty™ freezing container (Thermo Scientific, Cat. no. 5100-0001)

1.2 Materials for iPSC differentiation into HBOs on the pillar plate

- 36PillarPlate (Bioprinting Laboratories Inc., Cat. no. 36-01-00)
- LoadingPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- 36PetriLid (Bioprinting Laboratories Inc., Cat. no. 36-03-00)
- 36PerfusionPlate (Bioprinting Laboratories Inc., Cat. no. 36-02-00)
- 384DeepWellPlate (Bioprinting Laboratories Inc., Cat. no. 384-02-00)
- Ultralow attachment (ULA) 384-well plate (S-BIO, Cat. no. MS-9384UZ, or FaCellitate, Cat. no. F224384)
- Matrigel (Corning, Cat. no. 354234) for plate coating
- Deep petri dish, 100 mm x 20 mm (Corning, Cat. no. 70165-102)
- CEPT cocktail consisting of 50 nM chroman 1 (R&D systems, Cat. no. 7163), 5 µM emricasan (Selleckchem, Cat. no. S7775), 0.1%(v/v) polyamine supplement (Sigma Aldrich, Cat. no. P8482), and 0.7 µM trans-ISRIB (R&D systems, Cat. no. 5284)
- DMEM/F-12 (Fisher Scientific, Cat. no. 11-320-033)
- Gibco™ KnockOut™ Serum Replacement (Fisher Scientific, Cat. no. 10-828-010)
- ESC-quality FBS (ThermoFisher, Cat. no. 10-439-001)
- Gibco™ GlutaMAX™ Supplement (Fisher Scientific, Cat. no. 35-050-061)
- MEM non-essential amino acid (MEM-NEAA) solution (100x) (Fisher Scientific, Cat. no.11-140-

050)
- 2-Mercaptoethanol (Fisher Scientific, Cat. no. 50-114-7851)
- Recombinant human basic fibroblast growth factor (bFGF) (R&D systems, Cat. no. 233-FB-010)
- Gibco™ Neurobasal™ Medium (Fisher Scientific, Cat. no. 21-103-049)
- Gibco™ N-2 Supplement (100X) (Fisher Scientific, Cat. no. 17-502-001)
- B27 supplement without vitamin A (Fisher Scientific, Cat. no. 12-587-010)
- Insulin (MilliporeSigma, Cat. no. I9278)
- Heparin (MilliporeSigma, Cat. no. H3149)
- Gibco™ Penicillin-Streptomycin (Fisher Scientific, Cat. no 15-140-122)
- L-Ascorbic acid (MilliporeSigma, Cat. no. A4544)
- B27 supplement with vitamin A (Fisher Scientific, Cat. no. 17-504-044)
- **hESC medium:** To prepare 500 mL of the hESC medium for iPSC culture, mix 400 mL of DMEM/F-12, 100 mL of KnockOut™ Serum Replacement (KOSR), 15 mL of ESC-quality FBS, 5 mL of GlutaMAX, 5 mL of MEM-NEAA, and 3.5 µL of 2-mercaptoethanol. Filter the hESC medium using a vacuum-driven 0.2-µm Stericup filter unit (MilliporeSigma, Cat. no. Z259969). This medium can be stored for up to 2 weeks at 2 - 8°C. Add bFGF to a final concentration of 4 ng/mL (**Table 1**).
- **Neural induction medium (NIM):** To prepare 50 mL of the NIM, mix 48.5 mL of DMEM/F-12, 500 µL each of N-2 supplement, GlutaMAX supplement, and MEM-NEAA. Add heparin to a final concentration of 1 µg/mL and then filter the NIM using a vacuum-driven 0.2-µm Stericup filter unit. Store the medium at 2 - 8°C for up to 2 weeks.
- **Cerebral differentiation and maturation medium without vitamin A (CDM - vit A):** To prepare 250 mL of the CDM - vit A, mix 125 mL of DMEM/F-12, 125 mL of Neurobasal medium, 1.25 mL of N-2 supplement, 62.5 µL of insulin, 2.5 mL of GlutaMAX supplement, 1.25 mL of MEM-NEAA, and 2.5 mL of Penicillin-Streptomycin. Prepare a 1:100 dilution of 2-mercaptoethanol in DMEM/F-12 and add 87.5 µL of 100-fold diluted 2-mercaptoethanol to the medium. Add 2.5 mL of B27 supplement. Filter the CDM - vit A using a vacuum-driven 0.2-µm Stericup filter unit and store it at 2 - 8°C for up to 2 weeks.
- **Cerebral differentiation and maturation medium with vitamin A (CDM + vit A):** To prepare 50 mL of the CDM + vit A, mix 25 mL of DMEM/F-12, 25 mL of Neurobasal medium, 0.25 mL of N-2 supplement, 12.5 µL of insulin, 0.5 mL of GlutaMAX supplement, 0.25 mL of MEM-NEAA, 0.5 mL of Penicillin-Streptomycin, 0.5 mL of 400 µM ascorbic acid, 0.35 µL of 2-mercaptoethanol, and 1 mL of B27 supplement with vitamin A. Filter the CDM + vit A using a vacuum-driven 0.2-µm Stericup filter unit and store it at 2 - 8°C for up to 2 weeks.

1.3 Materials for cell viability assay of HBOs on the pillar plate

- Fisherbrand™ 384-well polystyrene plates, white (Fisher Scientific, Cat. no. 12-566-623)
- CellTiter-Glo® 3D cell viability assay (Promega, Cat. no. G9683)

1.4 Materials for immunofluorescence staining of HBOs on the pillar plate

- Shallow petri dish, 90 mm x 15 mm (VWR, Cat. no. 75799-946)
- 36PillarPlate (Bioprinting Laboratories Inc., Cat. no. 36-01-00)
- 384DeepWellPlate (Bioprinting Laboratories Inc., Cat. no. 384-03-00)
- Fisherbrand™ 384-well polystyrene plates, clear (Fisher Scientific, Cat. no. 12-566-625)
- Phosphate-buffered saline (PBS, 1x) sterile-filtered (Fisher Scientific, Cat. no. AAJ61196AP)
- 4% Paraformaldehyde (Fisher Scientific, Cat. no. AAJ19943K2)
- Sodium borohydride (Sigma Aldrich, Cat. no. 452882)

- Triton X-100 (Fisher Scientific, Cat. no. MTX15681)
- Tween 20 (Sigma Aldrich, Cat. no. P1379)
- Normal donkey serum (Sigma Aldrich, Cat. no. S30-M)
- Primary antibodies to target protein of interest
- Secondary antibodies based on the host of primary antibodies
- DAPI (Fisher Scientific, Cat. no. D1306)
- Visikol Histo-M tissue clearing reagent (Visikol, Cat. no. HM-30)
- RapiClear 1.52 (Sunjin Lab, Taiwan)
- Fisherbrand™ premium cover glass (Fisher Scientific, Cat. no. 125485J)

1.5 Materials for RT-qPCR analysis of HBOs on the pillar plate

- 384DeepWellPlate (Bioprinting Laboratories Inc., Cat. no. 384-02-00)
- Cultrex™ organoid harvesting solution (R&D Systems, Cat. no. 3700-100-01)
- Phosphate-buffered saline (PBS, 1x) sterile-filtered (Fisher Scientific, Cat. no. AAJ61196AP)
- Benchtop plate centrifuge (Hermle Benchmark, Cat. no. Z-287 A)
- 4°C Refrigerator
- Sterile, RNase-free pipet tips (Fisher Scientific, Cat. no. 21-377-45, 10-320-700, and 13-611-127)
- Cultrex™ organoid harvesting solution (R&D Systems, Cat. no. 3700-100-01)
- 70% ethanol in water
- Forward and reverse primers for the genes of interest
- Forward and reverse primers for the house-keeping gene
- RNeasy plus mini kit (Qiagen, Cat. no. 74134)
- High-capacity cDNA reverse transcription kit (ThermoFisher, Cat. no. 4368814)
- PowerTrack™ SYBR green master mix (ThermoFisher, Cat. no. A46110)
- QuantStudio™ 5 Real-Time PCR System (ThermoFisher, Cat. no. A28574)

Table 1. Preparation of the stock solution of additives and the final working concentrations in cell differentiation media

Additives	Solvent	Stock conc.	Working conc.	Dilution factor
Chroman 1	DMSO	10 mM	50 nM	200,000x
Emricasan	DMSO	50 mM	5 µM	10,000x
trans-ISRIB	Warm DMSO	10 mM	0.7 µM	14,286x
FGF basic/FGF2/bFGF	$dPBS^{-/-}$ containing at least 0.1% (w/v) BSA	10 µg/mL	4 ng/mL	2,500x
Heparin	dPBS	50 mg/mL	1 µg/mL	50,000x
L-ascorbic acid	DMEM/F12	40 mM	400 µM	100x

2. Methods

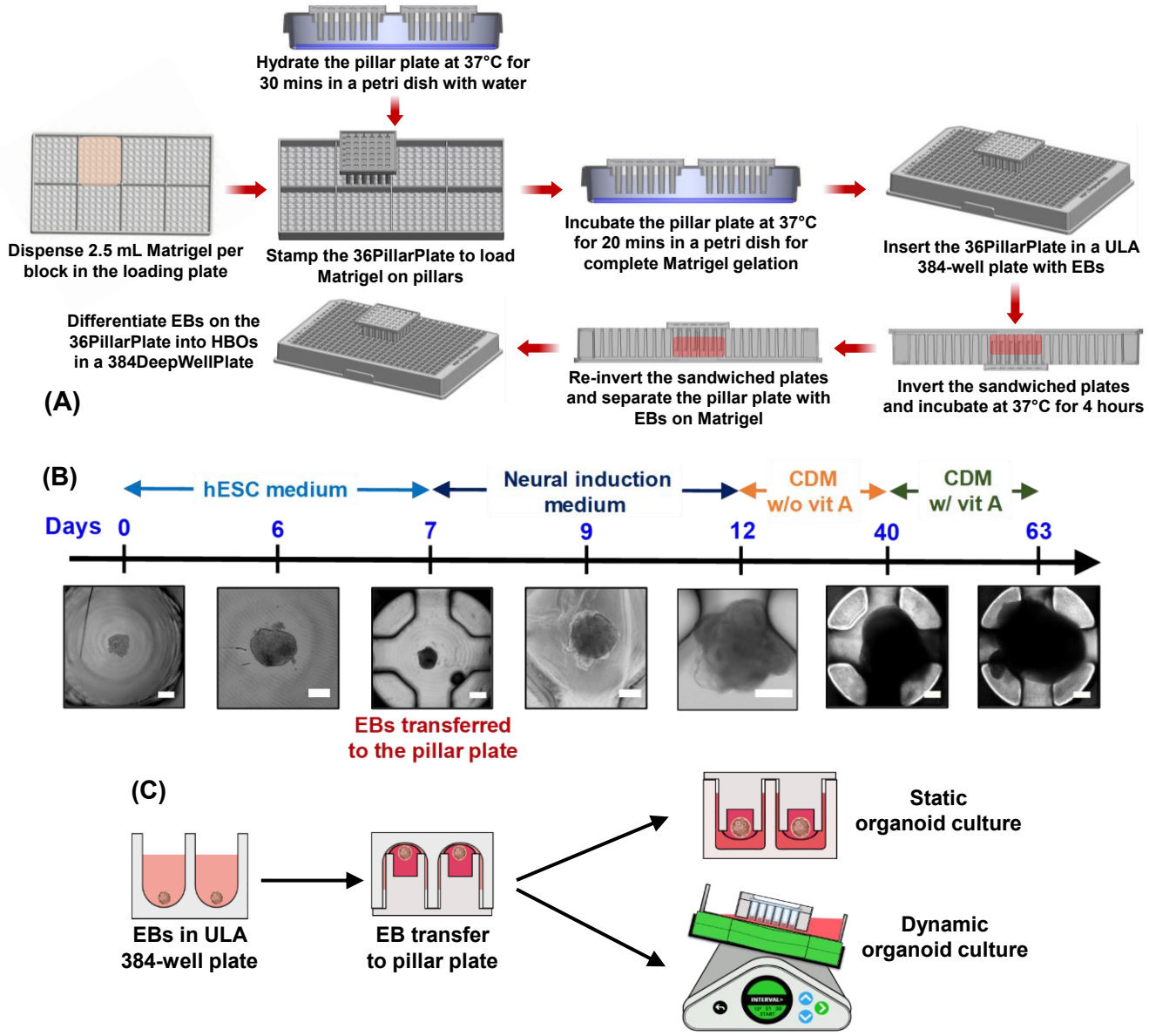


Figure 1. (A) Standard operating procedures (SOPs) for the scale-up production of human brain organoids (HBOs) using a pillar plate through transfer of embryoid bodies (EBs) in Matrigel and static culture in a 384DeepWellPlate. **(B)** The differentiation protocol of iPSCs into cerebral organoids. Scale bar: 200 µm (days 0, 7, 40, and 63), 250 µm (days 6 and 9), and 400 µm (day 12). **(C)** Transfer of EBs from an ultralow attachment (ULA) 384-well plate to a pillar plate as well as static and dynamic culture of cerebral organoids on the pillar plate.

2.1 Maintenance and passage of iPSCs

1. Thaw growth factor reduced (GFR) Matrigel overnight by incubating the vial in a refrigerator at 4°C.
2. Confirm the concentration of GFR Matrigel by checking the lot number of the Matrigel vial and prepare the aliquot of 0.5 mg GFR Matrigel using the following calculation:

$$\frac{0.5\ \text{mg}}{\text{Stock concentration of GFR Matrigel in mg/mL}} = \text{Volume (mL) for aliquote}$$

Suspend each aliquot of 0.5 mg GFR Matrigel in 6 mL of cold DMEM/F-12 to coat 6 wells of a 6-well plate.

3. Dispense 1 mL of the diluted GFR Matrigel solution in cold DMEM/F-12 in each well of a 6-well plate and swirl the 6-well plate to cover the surface of each well with Matrigel evenly. Incubate the 6-well plate with Matrigel at room temperature for at least 1 hour.
   Note: *Place the Matrigel solution on ice while handling. Make sure not to dry the Matrigel-coated wells. Matrigel-coated well plates can be stored in a 4°C refrigerator for up to 1 week by wrapping it with parafilm, if not used immediately.*
4. Prepare 50 mL of the complete mTeSR plus medium by adding 10 mL of 5x supplement in 40 mL of the mTeSR plus basal medium.
5. Thaw the frozen iPSCs quickly in a 37°C water bath until only a small ice crystal remains. Using a 1 mL pipette, slowly add the cells to 9 mL of the complete mTeSR plus medium in a sterile 15 mL conical tube. Gently mix the cells with the medium and centrifuge the conical tube for 3 minutes at 200 rcf. Discard the supernatant and resuspend the cells in 2 mL of the fresh complete mTeSR plus medium.
6. Remove the Matrigel solution from the 6-well plate by aspiration and then dispense 2 mL of the iPSC suspension into the Matrigel-coated wells.
   Note: *Typically, one cryovial of iPSCs is seeded into a single well of the Matrigel-coated 6-well plate.*
7. Gently rock the 6-well plate with iPSCs in all directions to ensure even distribution of the cells within the wells and then place the plate in a 5% CO_2 incubator at 37°C.
8. After a 1-day incubation, observe the plate under a bright-field microscope to confirm cell attachment on the surface. If minimal attachment is observed, continue to incubate for an additional day without changing the medium to allow more time for cell attachment.
9. After cell attachment, change the medium daily until the cells are ready for use or passage. Monitor colony formation under the microscope and remove any differentiated areas of iPSCs using a sterile cleaning tool (**Fig. 2**).
   Note: *The sterile cleaning tool can be prepared by bending a glass Pasteur pipette using an alcohol lamp.*

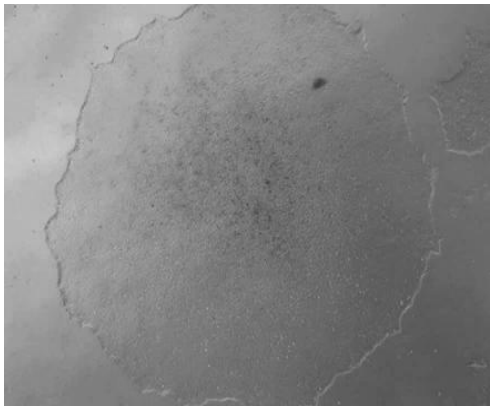
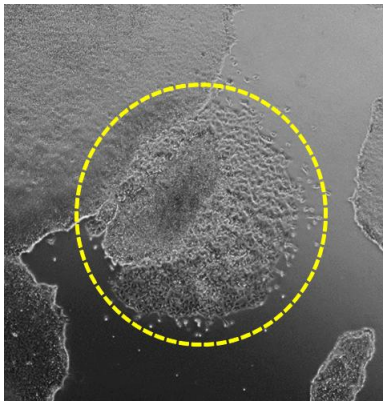
(A)  (B) 

Figure 2. Morphology of **(A)** an undifferentiated colony of iPSCs and **(B)** a differentiated colony of iPSCs, the latter encircled by a yellow dashed line.

10. Prepare a new 6-well plate with Matrigel coating before iPSC passage.
11. Passage iPSCs with either the EZPassage tool or the ReLeSR enzyme-free passaging reagent and seed the cell suspension in the new Matrigel-coated 6-well plate.

11.1. When passaging iPSCs using the EZPassage tool, first aspirate the spent medium and replace it with fresh mTeSR plus medium. Next, cut the iPSC colonies into small squares by rolling the EZPassage tool across the entire 6-well in one direction, applying uniform pressure. After rotating the 6-well plate 90 degrees, repeat the cutting process. Check the 6-well plate under a microscope to confirm that the colonies have been properly sectioned. Then, using a 5 mL serological pipette, aspirate the medium from the 6-well plate and gently scrape the surface to dislodge the colonies, slowly releasing the medium as you do so. Finally, transfer the cell suspension to a new Matrigel-coated 6-well plate containing 2 mL of complete mTeSR plus medium per well.

11.2. When passaging iPSCs using ReLeSR, first remove the spent medium by aspiration and then add 1 mL of the ReLeSR reagent. After incubation for 30 - 45 seconds, aspirate the ReLeSR from the well plate and incubate the well plate for 4 - 5 minutes in a 5% CO_2 incubator at 37°C. Then, add 1 mL of the complete mTeSR plus medium and pipette gently to dislodge the cells. Finally, transfer the cell suspension at a desired density (typically 100 µL of the cell suspension for a 1:10 split ratio) to a new Matrigel-coated 6-well plate containing 2 mL of the complete mTeSR plus medium per well.

12. After 24-hour incubation, observe the plate under the microscope to confirm cell attachment on the surface.
13. Change the medium daily until the cells are ready for use or passage.
14. For cryopreservation, harvest the iPSC suspension using either the EZPassage tool or the ReLeSR reagent into a 15 mL conical tube and centrifuge the tube for 3 minutes at 200 rcf. Discard the supernatant and resuspend the cell pellet with 1 mL of CryoStor® cryoprotectant reagent. Transfer the cell suspension into a labeled cryovial, place the vial into a Mr. Frosty container, and store it in a -80°C freezer overnight (**Fig. 3**). Finally, transfer the cryovial containing the cell suspension into a liquid nitrogen dewar for long-term storage of the cells.

Figure 3. Freezing cryovials containing iPSCs in the Mr. Frosty container in a - 80°C freezer.

2.2 Embryoid body (EB) formation in a ultralow attachment (ULA) 384-well plate

1. Inspect iPSC colonies under a bright-field microscope and remove any differentiated iPSCs using a sterile cleaning tool. Remove the spent medium, rinse the iPSCs with dPBS once, and dispense 1 mL of Accutase into a single well of the 6-well plate. Incubate the plate in a 5% CO_2 incubator at 37°C for 5 minutes.
   Note: *For optimal outcomes, it is crucial to initiate the differentiation process with high-quality iPSCs. Therefore, early passage numbers iPSCs, typically ranging between 10 to 20, stored in frozen vials, are suitable for generating cerebral organoids. Additionally, it is important to begin cell passage at 70 - 80% confluency. This meticulous preparation is essential for successful iPSC differentiation.*
2. After iPSC detachment, transfer the cell suspension into a 15 mL conical tube containing 9 mL of the complete mTeSR plus medium using a 1 mL pipette, centrifuge the tube at 200 rcf for 3 minutes, carefully discard the supernatant, and resuspend the cells in 1 mL of the hESC medium.

3. Count the viable cells using trypan blue exclusion and calculate the number of viable cells per mL in the cell suspension.
4. Prepare cell suspension in the hESC medium supplemented with the CEPT cocktail and 4 ng/mL bFGF.
   Note: *The CEPT cocktail and bFGF are added in the hESC medium only on the day of cell seeding.*
5. Seed the cells at a density of 1,000 cells per 70 µL of the cell suspension in each well of an ultralow attachment (ULA) 384-well plate.
6. Incubate the ULA 384-well plate with iPSCs in a 5% CO_2 incubator at 37°C for 48 hours.
7. After 48 hours of incubation, change the medium to the hESC medium without the CEPT cocktail and bFGF. Change the medium every alternate day until day 7.

2.3 EBs transfer from the ULA 384-well plate to a pillar plate and neuroectoderm generation

1. Inspect EBs in the ULA 384-well plate under a brightfield microscope prior to EB transfer to the pillar plate. The typical size of EBs to be transferred is 300 - 400 µm.
2. Remove 30 µL of growth medium from each well of the ULA 384-well plate prior to the spheroid transfer.
   Note: *The optimum volume of cell culture media in the ULA 384-well plate required for EB transfer is 35 - 40 µL per well. Excess volume of cell culture media in the ULA 384-well plate will lead to the overflow of the media after the pillar plate sandwiching onto the ULA 384-well plate.*
3. Thaw Matrigel® stock overnight by submerging the unopened bottle in a bucket of ice placed in a 4°C refrigerator. Prepare 500 µL aliquots of Matrigel and store them at - 20°C for future use.
4. Thaw Matrigel® aliquots overnight in a 4°C refrigerator prior to spheroid transfer.
   Note: *It is important to thaw Matrigel aliquots in advance in a 4°C refrigerator and maintain Matrigel chilled on ice during use since Matrigel starts to solidify above 10°C. Do not freeze and thaw Matrigel aliquots.*
5. For static cerebral organoid culture in a 384DeepWellPlate, dispense 70 µL/well of a neural induction medium (NIM) in the 384DeepWellPlate, cover with a well plate lid, and place it in a 5% CO_2 incubator at 37°C for at least 1 hour to warm up the medium and avoid air bubble formation in the deep wells.
6. Hydrate the surface of the pillar plate by inserting two 36PillarPlates into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile distilled water. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 30 minutes prior to hydrogel loading (**Fig. 4**).
   Note: *Hydrating the surface of the pillar plate in a humid environment is necessary to increase surface hydrophilicity and minimize air bubble entrapment on the pillars after Matrigel loading. Ensure that the pillars are not immersed in water when transferring the assembly to the CO_2 incubator, as excess water on the pillars may interfere with uniform hydration.*

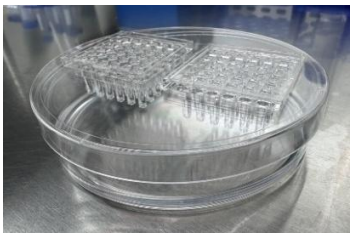


Figure 4. Hydration of the pillar plate surface in a 100 x 20 mm petri dish with 5 mL of sterile, distilled water to minimize air bubble entrapment.

7. Gently mix 1.5 mL of warm cell culture medium with 1.5 mL of cold Matrigel to obtain a final concentration of 4 - 6 mg/mL Matrigel (i.e., 50% Matrigel)

Note: *Use diluted Matrigel immediately. If not used right away, keep it on ice. Do not reuse thawed or diluted Matrigel.*

8. If bubbles form on the pillar plate after hydrogel loading, centrifuge the 50% Matrigel solution at 1,000 rpm for 2 minutes to remove air bubbles generated during the mixing process.
9. Place a LoadingPlate on a flat surface. Dispense 2 - 2.5 mL of the diluted Matrigel solution into each small block without introducing big bubbles, and spread the solution evenly using the pipette tip (**Fig. 5**).
   Note: *It is critical to maintain a sufficient volume of Matrigel in each block of the LoadingPlate; a minimum volume of 2 mL per block is recommended. Matrigel should be distributed uniformly to ensure complete wetting of all pillars. Improper loading of Matrigel onto the pillars during pillar stamping may result in macro-bubble formation on the pillars after spheroid transfer. Do not leave the diluted Matrigel solution on the LoadingPlate for longer than 5 minutes to avoid premature gelation during the stamping process. Because pillar stamping is performed rapidly, it is generally not necessary to place the LoadingPlate containing diluted Matrigel on ice during this step.*

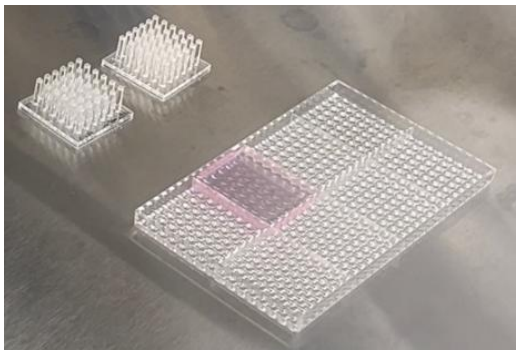


Figure 5. Dispensing 2 - 2.5 mL of diluted Matrigel per block in the LoadingPlate for rapid loading of Matrigel on the pillar plate.

10. Stamp the 36PillarPlate on the LoadingPlate and press gently to load diluted Matrigel evenly on the entire pillar plate. Repeat this Matrigel loading step for another pillar plate (**Fig. 6**)
    Note: *Using 2 - 2.5 mL of the diluted Matrigel solution, it is possible to prepare at least four 36PillarPlates (5 µL Matrigel per pillar or 180 µL per 36PillarPlate) without introducing macro-bubbles on the pillars. For uniform pillar wetting and robust Matrigel loading, gently wiggle the pillar plate during stamping. Add additional Matrigel solution to the LoadingPlate as needed.*

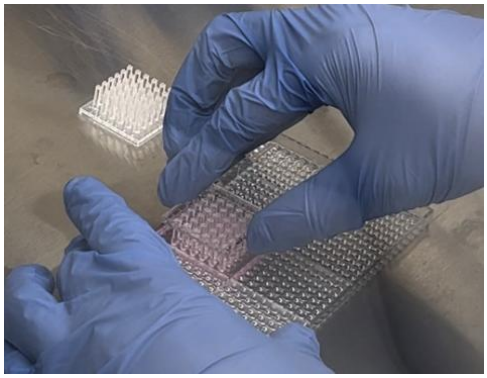


Figure 6. Stamping of the 36PillarPlate on the LoadingPlate to load Matrigel on all the pillars at once.

11. For minimizing water evaporation during complete Matrigel gelation, insert two 36PillarPlates loaded with diluted Matrigel into a 36PetriLid placed on a 100 x 20 mm petri dish containing 5 mL of sterile, distilled water (**Fig. 1**).

12. Incubate the assembly in a humidified 5% CO_2 incubator at 37°C for 20 minutes to allow complete gelation of the diluted Matrigel.
    Note: *It is critical to minimize water evaporation during Matrigel gelation to ensure proper spheroid transfer. Ensure that the pillars are not immersed in water when transferring the assembly to the CO_2 incubator, as excess water on the pillars may interfere with proper gelation.*
13. Align one corner pillar of the 36PillarPlate with the corresponding corner well of the ULA 384-well plate. Carefully sandwich the pillar plate with Matrigel onto the ULA 384-well plate containing EBs. Cover the assembled plates with a 384-well plate lid and quickly invert the sandwiched plates so that the pillar plate faces downward to initiate spheroid transfer (**Fig. 7**).
    Note: *To position EBs at the center of the pillars, ensure that there is no excess hydrogel on the pillars and gently insert the pillar plate into the ULA 384-well plate. Remove excess hydrogel from the pillars by horizontally sliding a 1 mL pipette tip across the pillar surfaces. In addition, carefully transport the inverted and sandwiched plates to a 5% CO_2 incubator without tilting, so as not to disturb vertical spheroid precipitation onto the pillars.*

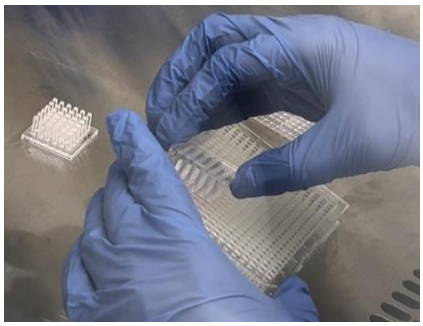
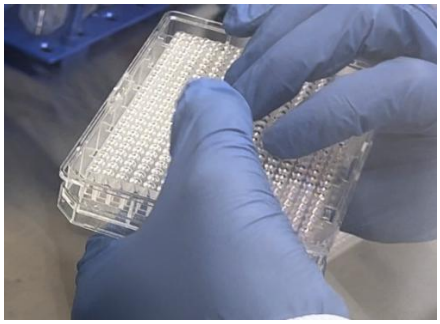
(A)  (B) 

Figure 7. (A) Insertion of the 36PillarPlate into the ULA 384-well plate containing EBs. **(B)** Inversion of the sandwiched plates for EB transfer

14. Incubate the inverted and sandwiched plates in a humidified 5% CO_2 incubator at 37°C for 4 hours to allow spheroid transfer from the ULA 384-well plate onto the pillar plate and attachment of the spheroids to the Matrigel.
15. After incubation, carefully re-invert the sandwiched plates to return the 36PillarPlate to the top position, and then separate the pillar plate containing the transferred EBs attached to the Matrigel.
16. Immediately insert the pillar plate into the 384DeepWellPlate containing 70 µL/well of prewarmed NIM.
    Note: *It is critical to prewarm the growth medium in the 384DeepWellPlate for at least 1 hour and gently tap the plate to dislodge any air bubbles in the wells before sandwiching with the pillar plate. Small micro-bubbles may appear at the edges of the pillars after sandwiching; however, these bubbles typically disappear within 1 - 2 days during routine medium changes.*
17. Inspect the pillar plate under a brightfield microscope to ensure successful EB transfer.
18. Culture the EBs on the pillar plate in a 5% CO_2 incubator at 37°C, replacing the NIM in the 384DeepWellPlate every 2 days till day 12 for neuroectodermal generation.

2.4 Cerebral organoid differentiation

1. On day 13, inspect the pillar plate under the microscope and confirm the morphology of neuroectoderms with neuroepithelial buds.
2. Gently dispense 70 µL of the cerebral differentiation and maturation medium without vitamin A (CDM - vit A) per deep well in a new 384DeepWellPlate to avoid big air bubble formation, cover it with a well plate lid, and place it in a 5% CO_2 incubator at 37°C for at least 1 hour to warm up the medium.

3. Gently tap the 384DeepWellPlate with the CDM - vit A medium on the surface of the biosafety cabinet to remove big air bubbles entrapped in deep wells.
4. To replace the spent medium, separate the 36PillarPlate and sandwich it within the 384DeepWellPlate filled with warm, fresh CDM - vit A medium.
5. Incubate the assembled plates in the 5% CO_2 incubator at 37°C with the CDM - vit A medium change every 2 days until day 40.
6. On day 40, gently dispense 70 µL of the cerebral differentiation and maturation medium with vitamin A (CDM + vit A) per deep well in a new 384DeepWellPlate to avoid big air bubble formation, cover it with a well plate lid, and place it in the CO_2 incubator at 37°C for at least 1 hour to warm up the medium.
7. Gently tap the 384DeepWellPlate with the CDM + vit A medium on the surface of the biosafety cabinet to remove big air bubbles entrapped in deep wells.
8. To replace the spent medium, separate the 36PillarPlate and sandwich it within the 384DeepWellPlate filled with warm, fresh CDM + vit A medium.
9. Incubate the assembled plates in the CO_2 incubator at 37°C with the CDM + vit A medium change every 2 days.

2.5 Assessment of cerebral organoid viability on the pillar plate

The viability of cerebral organoids on the pillar plate can be assessed using a CellTiter-Glo® 3D cell viability assay kit from Promega which enables the quantification of ATP levels in metabolically active cells. The recommended storage condition of the kit is - 20°C up to the expiration date. Prepare 5 mL aliquots of the reagent by following the manufacturer's recommended protocol to prevent multiple cycles of freezing and thawing.

1. Calculate the total volume of the CellTiter-Glo 3D reagent required to assess the viability of cerebral organoids on the pillar plate. The CellTiter-Glo reagent is diluted with the cell culture medium at a 1:1 ratio to prepare the final assay solution.
   Note: *The volume of the reagent needed is 40 µL per well or 1,440 µL for 36 wells per 36PillarPlate.*
2. Thaw aliquots of the CellTiter-Glo 3D reagent at room temperature as required for the number of cerebral organoids on the pillar plate.
   Note: *It is crucial for the reagent to reach room temperature before use in the assay as it is temperature sensitive.*
3. Mix the CellTiter-Glo 3D reagent and the DMEM/F-12 medium at a 1:1 ratio in a 15 mL conical tube.
4. Dispense 40 µL of the diluted reagent in each well of the opaque white 384-well plate using a multichannel pipette.
5. Separate the 36PillarPlate from the 384DeepWellPlate, insert it onto the opaque white 384-well plate containing the diluted CellTiter-Glo reagent, and incubate for 30 - 45 minutes at room temperature on an orbital shaker at 80 rpm in the dark to ensure the complete lysis of the organoids (**Fig**. **8**).
   Note: *The manufacturer's protocol recommends 5 minutes of vigorous shaking for cell lysis and stabilization of signals for 25 minutes. However, for large organoids on the pillar plate, 30 - 45 minutes on an orbital shaker is required to ensure complete cell lysis. For small organoids, the incubation time for cell lysis can be reduced.*

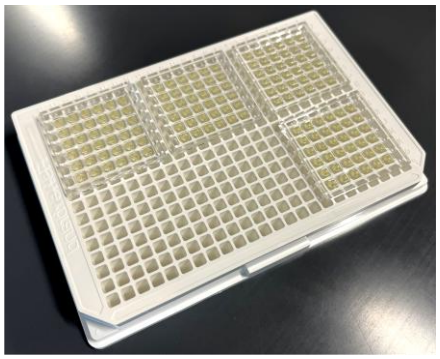


Figure 8. The four 36PillarPlates with cerebral organoids inserted onto the opaque white 384-well plate containing the diluted CellTiter-Glo reagent.

6. Separate the 36PillarPlate from the opaque white 384-well plate. Incubate the opaque white 384-well plate at room temperature for 15 - 20 minutes in the dark to stabilize the luminescence signal.
7. Record luminescence at an emission wavelength of 560 nm (typical gain at 135) from the opaque white 384-well plate using a microtiter well plate reader.

2.6 Immunofluorescence (IF) staining of cerebral organoids

Whole cerebral organoids on the pillar plate can be stained in parallel without detaching the organoids from the pillar plate. Refer to the video clip of the immunofluorescence staining process at youtu.be/tBGvF83wIew.

1. For rinsing, insert a 36PillarPlate with cerebral organoids in the 36PetriLid on a 90 x 15 mm petri dish containing 20 mL of 1x phosphate-buffered saline (PBS) and incubate for 10 minutes at room temperature (**Fig. 9**).
   Note: *Perform organoid rinsing in a static condition to avoid organoid detachment from the pillar plate. If the cells are encapsulated in alginate on the pillar plate, use Tris-buffered saline (TBS) instead of PBS due to alginate degradation in PBS.*

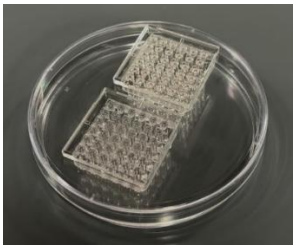


Figure 9. The 36PillarPlate with cerebral organoids inserted in the 36PetriLid on a 90 x 15 mm petri dish containing phosphate-buffered saline (PBS) for rinsing.

2. For fixation, prepare a petri dish with 20 mL of 4% paraformaldehyde solution, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 4% paraformaldehyde, and incubate for 2 hours at room temperature.
   Note: *Bring the 4% paraformaldehyde solution to room temperature for fixation as Matrigel might weaken at low temperature, resulting in spot detachment.*
3. Prepare a petri dish with 20 mL of 0.1% (w/v) sodium borohydride in PBS and insert the 36PillarPlate in the 36PetriLid on the petri dish containing 0.1% (w/v) sodium borohydride. Incubate for 15 minutes at room temperature. Repeat this step once.
   Note: *This step is important to reduce background due to residual aldehyde.*

4. For permeabilization. prepare a petri dish with 20 mL of 0.5% Triton X-100 in PBS, insert the 36PillarPlate in the 36PetriLid on the petri dish containing 0.5% Triton X-100, and incubate for 15 minutes at room temperature with rocking at 3 - 5º tilt angle and speed 5 (**Fig. 10)**. Repeat this step twice to have a total permeabilization time of 45 minutes.

Figure 10. The petri dish with the pillar plates on a low-speed rocker for permeabilization.

5. For blocking, prepare a petri dish with 5% normal donkey serum (NDS) diluted in 1x PBS with 0.25% Triton X-100 (blocking buffer), insert the 36PillarPlate in the 36PetriLid on the petri dish containing 5% NDS in the blocking buffer, and incubate for 2 - 4 hours at room temperature.
   Note: *This step is critical to prevent non-specific binding of primary antibodies. If necessary, you can incubate it overnight at 4°C instead of 2 - 4 hours of incubation at room temperature.*
6. For primary antibody staining, dilute the desired primary antibody solution in the blocking buffer according to the manufacturer's recommendation.
7. Dispense 70 µL/well of the diluted primary antibody solutions in a 384DeepWellPlate, insert the 36PillarPlate in the 384DeepWellPlate containing primary antibodies, and incubate for 24 hours at 4°C on the rocker at 3 - 5º tilt angle and speed 5 (**Fig. 11**).

Figure 11. The 36PillarPlate inserted onto the 384DeepWellPlate with primary antibodies on the low-speed rocker at 4°C for primary antibody staining.

8. Separate the 36PillarPlate from the 384DeepWellPlate, insert it in the 36PetriLid on a petri dish containing 20 mL of the blocking buffer, and rinse the 36PilllarPlate for 20 minutes each at room temperature on the rocker. Repeat this step twice with fresh blocking buffer.
9. For secondary antibody staining, dilute fluorophore-conjugated secondary antibodies 200-fold in the blocking buffer, dispense 70 µL/well of the 200-fold diluted secondary antibody solutions in a 384DeepWellPlate, insert the 36PillarPlate in the 384DeepWellPlate containing secondary antibodies, and incubate for 2 - 4 hours at room temperature on the rocker.
10. Prepare a 384DeepWellPlate with 70 µL/well of 0.5 µg/mL DAPI in 1x PBS, insert the 36PillarPlate in the 384DeepWellPlate containing 0.5 µg/mL DAPI, and incubate for 25 minutes at room temperature with gentle rocking.
11. For rinsing, insert the 36PillarPlate with stained organoids in the 36PetriLid on a petri dish containing 20 mL of 1x PBS for rinsing and incubate for 20 minutes at room temperature on the rocker. Repeat this step once.

12. For organoid clearing, dispense 30 µL/well of Visikol Histo-M or 1x RapiClear 1.52 in a regular 384-well plate for organoid clearing, pre-warm the 384-well plate containing the clearing solution for 20 minutes at 37°C, insert the 36PillarPlate with stained organoids in the 384-well plate, and incubate for 1 hour at room temperature.
    Note: *The tissue clearing step is necessary to avoid light scattering of large organoids or spheroids and enable deep imaging of the large cell samples.*
13. For organoid imaging, separate the 36PillarPlate with stained and cleared organoids from the 384-well plate and attach a thin microscopic glass slide on the pillar plate for imaging (**Fig. 12**).

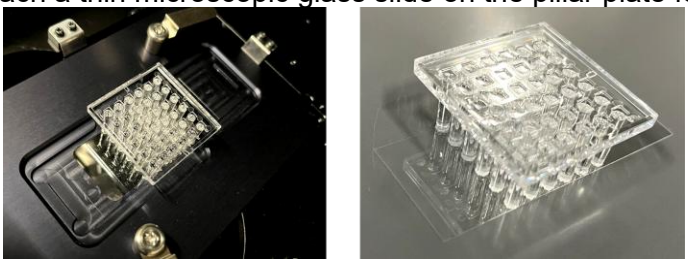


Figure 12. Thin microscopic glass slide attached to the pillar plate with organoids for image acquisition.

14. Mount the 36PillarPlate with the thin microscopic glass slide and obtain fluorescence images using a confocal microscope (**Fig. 13**).

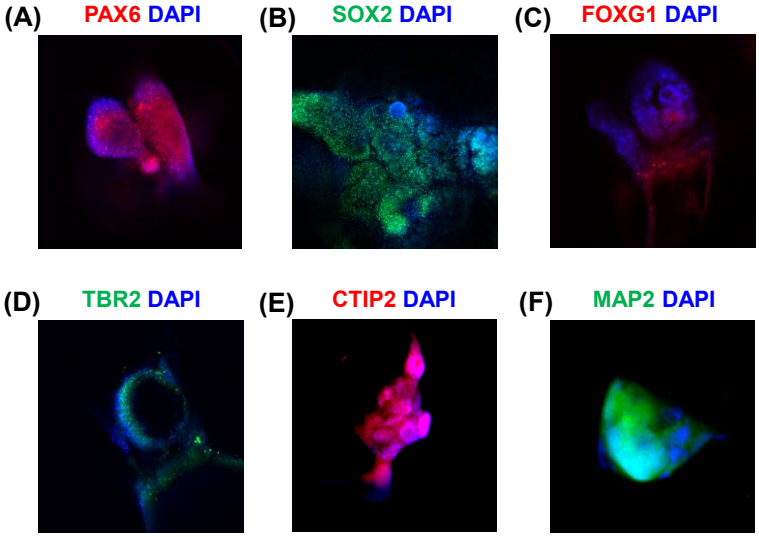


Figure 13. Stained images of cerebral organoids obtained from the 36PillarPlate for measuring neuronal biomarkers, including **(A)** PAX6 neural progenitor cell, **(B)** SOX2 neural stem cell, **(C)** FOXG1 forebrain, **(D)** TBR2 intermediate progenitor cell, **(E)** CTIP2 deep cortical neuron, and **(F)** MAP2 mature neuron.

2.7 RT-qPCR analysis of HBOs on the pillar plate

Cerebral organoids can be harvested from the pillar plate by incubation in cold Cultrex™ organoid harvesting solution for RT-qPCR and cryosectioning. Typically, 10 - 15 organoids can be harvested from the pillar plate for RT-qPCR analysis of biomarkers expressed in cerebral organoids.

2.7.1 Organoid harvest from the pillar plate

1. For cerebral organoid detachment from the pillar plate for RT-qPCR, add 5 mL of cold basal medium to one well of a 6-well plate.

2. Insert the pillar plate with organoids in the well containing 5 mL of cold basal medium, cover the lid, and incubate for 5 minutes.
3. Dispense 5 mL of cold Cultrex organoid harvesting solution to a new well of the 6-well plate.
4. Insert the pillar plate with organoids in the well, cover the lid, and incubate for 45 - 60 minutes at 4°C.
5. After incubating with Cultrex organoid harvesting solution, tap the 6-well plate with the pillar plate inserted for a few times to detach the organoids completely.
   Note: *The 6-well plate with the pillar plate can be gently rocked during the incubation at 4°C to speed up the detachment process.*
6. Check the 6-well plate under the microscope for the number of organoids detached from the pillar plate.
   Note: *The efficiency of organoid detachment depends on the size of the organoids and the amount of extracellular matrices (ECMs) they secrete. If some organoids remain attached to the pillar plate, incubate them with Cultrex organoid harvesting solution for additional 10 - 15 minutes with gentle rocking. Do not use proteolytic enzymes such as Accutase, TrypLE, and Trypsin for organoid harvesting, as these enzymes degrade organoid morphology. If complete harvesting is not necessary, a few organoids can be manually collected from the pillar plate using pipette tips instead of the Cultrex solution or centrifugation.*
7. Rinse the detached organoids with PBS for RNA extraction using RNeasy plus mini kit.
   Note: *Ensure to collect 10 - 15 replicates of organoids from the pillar plate for RNA extraction. The detailed protocol for the use of the RNeasy plus mini kit can be found on the Qiagen website (RNeasy Plus Mini Handbook - QIAGEN).*

2.7.2 RNA isolation from HBOs

1. Add 4 volumes of 96 – 100% ethanol to Buffer RPE to prepare a washing buffer solution.
2. Prepare a lysis buffer by adding either 10 µL of β-mercaptoethanol (β-ME) or 20 µL of 2 M dithiothreitol (DTT) in 1 mL Buffer RLT.
   Note: *β-ME or DTT in Buffer RLT is necessary for purifying RNA from cell lines or tissues containing RNases. Buffer RLT containing β-ME or DTT can be stored at room temperature for up to 1 month.*
3. For cell lysis, collect at least 10 - 15 organoids in a 1.5 mL Eppendorf tube, add 350 µL or 600 µL of Buffer RLT depending on the cell numbers, and vortex for 30 seconds for disruption and homogenization of the cells (**Table 2**).
   Note: *The organoids should be dissolved in the lysis buffer completely in 30 seconds. If not lysed completely, use a pestle to mechanically disrupt organoids. The number of organoids to be collected for RNA extraction depends on the size of organoids.*
4. Add 1 volume of 70% ethanol to the lysate from Step 3 and mix well by pipetting.
   Note: *Do not centrifuge it. Proceed to Step 5 immediately.*
5. Transfer up to 700 µL of the lysate-ethanol sample, including any precipitate, to a RNeasy mini spin column placed in a 2 mL collection tube (provided by the vendor), close the lid, and centrifuge for 15 seconds at ≥ 8000 x g. Discard the flow-through.
6. Add 700 µL Buffer RW1 to the RNeasy spin column, close the lid, and centrifuge for 15 seconds at ≥ 8000 x g. Discard the flow-through.
7. Add 500 µL Buffer RPE to the RNeasy spin column, close the lid, and centrifuge for 15 seconds at ≥ 8000 x g. Discard the flow-through.
8. Add 500 µL Buffer RPE to the RNeasy spin column, close the lid, and centrifuge for 2 minutes at ≥ 8000 x g.
9. Place the RNeasy spin column in a new 1.5 mL collection tube (provided by the vendor) and centrifuge at a maximum speed for 1 minute to dry the membrane.

10. Place the RNeasy spin column in a new 1.5 mL Eppendorf tube (provided by the vendor), add 30 - 50 µL RNase-free water directly to the spin column membrane, close the lid, and centrifuge for 1 minute at ≥ 8000 x g to elute the RNA.
11. If the expected RNA yield is > 30 µg, repeat Step 10 using another 30 - 50 µL of RNase-free water, or using the eluate from Step 10 (if high RNA concentration is required). Reuse the collection tube from Step 10.
12. Measure the concentration of RNA using a nanodrop and prepare 1 µg RNA per 10 µL of distilled water.

Table 2. Volumes of Buffer RLT necessary for sample disruption and homogenization

Sample	Amount (cell #)	Cell culture dish	Buffer RLT (µL)	Disruption and homogenization
Animal cells	< 5 x 10^6 < 1 x 10^7	< 6 cm 6 - 10 cm	350 600	Add Buffer RLT and vortex (≤ 1 x 10^6 cells)

2.7.3 cDNA conversion

1. To synthesize cDNA from 1 µg of RNA, use the high-capacity cDNA reverse transcription kit.
   Note: *The kit consists of 4 components including RT buffer, dNTP mix, RT primer, and MultiScribe reverse transcriptase. The detailed protocol for the use of the kit can be found on the ThermoFisher website (High-Capacity cDNA Reverse Transcription Kit User Guide).*
2. Mix 2 µL of RT buffer (10x), 0.8 µL of dNTP mix, 2 µL of RT primer (10x), 1 µL of MultiScribe reverse transcriptase, and 4.2 µL of distilled water in a PCR tube. The total volume of this mixture will be 10 µL.
3. Add 1 µg RNA in 10 µL of distilled water from Step 12 in the RNA isolation protocol in the PCR tube. The final volume of the mixture will be 20 µL.
4. Place the PCR tube in a thermocycler with the following setting: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C for ∞.

2.7.4 qPCR analysis

1. Perform RT-qPCR using SYBR™ Green Master Mix containing two solutions, synthesized cDNA, and primers for the genes of interest in QuantStudio™ 5 Real-Time PCR System.
   Note: *The detailed protocol for the use of the kit can be found on the ThermoFisher website (PowerTrack SYBR Green Master Mix User Guide).*
2. Mix 10 µL of the SYBR Green reagent, 0.5 µL of yellow solution in the kit, 1 µL of forward primer, 1 µL of reverse primer, 6.5 µL of distilled water, and 1 µL of 5 ng cDNA in a 96-well plate used for running qPCR.
   Note: *cDNA needs to be diluted in distilled water to make a final concentration of 5 ng/µL. In addition, forward and reverse primers need to be diluted in TE buffer (Fisher Scientific, Cat. no. 12-090-015) to make a final concentration of 1 mM.*
3. Run the 96-well plate containing the samples in QuantStudio™ 5 Real-Time PCR System with the following setting: 95°C denaturation for 30 seconds, 58 - 62°C annealing for 45 seconds (depending on primer pair), and 72°C extension for 30 seconds with 35 - 45 cycles.
   Note: *Genes such as glyceraldeyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB) can be used as house-keeping genes.*
4. Calculate the fold-change of relative gene expression using the delta-delta Ct method.