# SOP #001
# Human Kidney Organoid Differentiation from Pluripotent Stem Cells

## 1. Coating plates for organoid differentiation

Adherent organoid differentiation requires the pre-coating of tissue culture plates with a suitable matrix for the proper attachment of cells. There are several matrix alternatives, and the manufacturer guidelines provide starting concentrations. The following protocol describes the use of GelTrex or Matrigel for coating, two commonly used matrices that work well for the kidney differentiation protocol. These should be kept in ice at all times to prevent polymerization. The use of pre-chilled pipette tips and tubes is recommended.

*Materials*

- Geltrex LDEV-Free, hESC-qualified, reduced growth factor (Thermo Fisher #A4000046702) or Matrigel GFR (Corning #354230)
- DMEM/F12 (Thermo Fisher #11320033)
- Tissue culture plates: 24-well (Sigma #Z707805) or 96-well (Grenier #655090)

*Protocol*

1. If frozen, thaw Geltrex or Matrigel overnight on ice in a 4°C refrigerator.

2. Mix the desired amount of cold DMEM/F12 media with Geltrex (1:100 dilution) or Matrigel (1:60 dilution) in a pre-chilled tube. Slowly pipet up and down several times to make a homogeneous solution, avoiding introducing air bubbles.

3. Add the recommended coating amount to plates as follows:
1 mL per 6 well
300 µl per 24 well
60 µl per 96 well

4. Rock the plate back and forth to evenly coat the bottom. Incubate for at least 1 hour at 37°C. Then seal the plates with Parafilm and store at 4°C overnight. The plates are now ready to use. They may be stored in the refrigerator for up to two weeks after coating. Allow stored plates to equilibrate at room temperature for 30 minutes before using.

## 2. Differentiation of human pluripotent stem cells (hPSC) into kidney organoids

*Materials*

- hPSC (50-80 % confluent) growing feeder-free
- Accutase (StemCell Technologies #07920)
- mTeSR1 (StemCell Technologies #85850)
- PBS pH 7.4 (Thermo Fisher #10010-049)
- Advanced RPMI (Thermo Fisher #12633020) + Glutamax (#35050061)
- 'RB' media: Advanced RPMI + Glutamax + B27 Supplement (#17504-044)
- Rock inhibitor Y27632 (Tocris #1254), 10 mM stock solution in DMSO
- CHIR99021 (we prefer StemGent #04-004), 10 mM stock solution in DMSO
- Noggin (Thermo Fisher #120-10C-100UG), 100 µg/ml stock solution in PBS
- BMP4 (R&D #314-BP-010/CF), 50 µg/ml stock solution in PBS
- Penicillin-Streptomycin (Thermo Fisher #15140122) in all cell culture media
- Coated plates (from Section 1 above)

*Protocol*

**Day -3: Preparing single-cell suspension and plating cells**

hPSC should be 50-80% confluent on the day of plating. It is not recommended to let the cells get over confluent as this will decrease cell viability and may lead to spontaneous differentiation. The optimal cell density for plating will vary depending on the cell line and it is recommended that several densities be tested the first time setting up the experiment.

1. Wash 6-wells containing hPSC briefly with PBS, 2mL/well. Aspirate and add 1mL of Accutase.

2. Incubate at 37°C for 5 minutes-10 minutes. Gently tap the plate to help the cells detach.

3. Dissociate the cells in Accutase by pipetting them up and down 5-10 times with a P1000 in the wells. Collect all the cells and transfer to a 1.5mL or 15 mL tube.

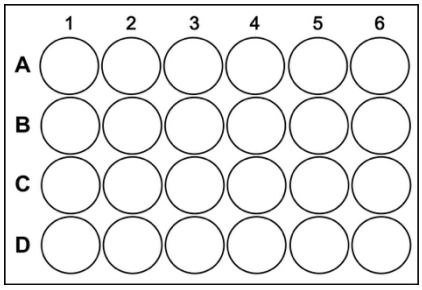
4. Centrifuge at 300 rcf for 4 minutes at room temperature.

5. Carefully aspirate the Accutase and resuspend the cell pellet in 1 mL of mTeSR with 10 µM ROCK inhibitor Y-27632. Y-27632 substantially increases cell survival when plating single cell suspensions of hPSC.

6. Count cells to determine the concentration of the cell suspension using software such as CytoSMART Cell Counter.

7. Prepare a solution of 40,000 cells/mL in 16 mL mTeSR1 + 10 µM ROCK inhibitor (640,000 total cells). Mix well by pipetting up and down 5-10 times to make a homogeneous solution. Avoid adding air bubbles while mixing.

8. Tilt a coated 24-well plate 45° and aspirate the coating media. Add 250 µL mTeSR + ROCK inhibitor to row A. Then, pipet the cell solution prepared in step 7 to the plate as follows:




**A** 250µL (1,000 - 10,000 cells/well)

**B** 500µL (2,000 - 20,000 cells/well)

**C** 750µL (3,000 - 30,000 cells/well)

**D** 1mL (4,000 - 40,000 cells/well)

**It is very important to mix the cell solution well before pipetting to the plate. Cells can settle quickly and not mixing them could lead to inaccurate cell densities. The suggested cell densities are a starting point, but certain hPSC may require lower or higher densities and they should be adjusted as necessary.**

9. Let the plate sit in the culture hood for 5 minutes without disturbing to let the cells settle on the wells.

10. Examine the plate under a microscope. The cells should look evenly spread across the well. If the cells look aggregated, rock the plate up and down several times to disperse them evenly across the well.

**Day -2: Matrix sandwiching**

1. During the morning of day 2, prepare 12 mL of ice-cold mTeSR with 1.5% Geltrex or 2.25% Matrigel.

2. Tilt the 24-well plate in 45° and carefully aspirate the media from the wells. Immediately proceed to the next step, cells will dry if left without media for too long.

3. Slowly add 500 µL of the cold mTeSR + Geltrex or Matrigel solution to each well, drop by drop, being careful of not disturbing the cells.

4. Incubate at 37°C overnight.

**Day -1: Feed the cells with mTeSR**

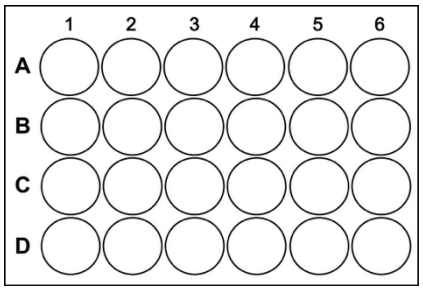
1. Tilt the 24-well plate in 45° and carefully aspirate the media from the wells.

2. Slowly add 500 µL of mTeSR (warm to room temperature) to each well. Spheroid formation should be evident.

**Day 0: CHIR99021 pulse**

1. During the evening of day 4, aspirate the mTeSR1 and replace with 1 mL/24 well of Advanced RPMI + Glutamax supplemented with 12 µM CHIR99021. Some hPSC lines may require addition of 10 ng/mL Noggin or 2 ng/mL BMP4 in addition to the CHIR99021, try these formulations as well along the y-axis of the 24-well plate for each of the cell densities.

2. Incubate for **36 hours** at 37°C.




**1 & 2** 12µM CHIR99021

**3 & 4** 12µM CHIR99021 + 10 ng/mL Noggin

**5 & 6** 12µM CHIR99021 + 2 ng/mL BMP4

**Day 1.5: CHIR99021 removal**

After 36 hours in CHIR99021, change the media to RB (1 mL/24 well). Aspirate and drip on the media gently from this point forward in the protocol, as the structures are delicate and can be easily disrupted. There is no need for Noggin or BMP4 from this point onwards.

**Day 3 and onwards: Feeding with RB media**

Replace the RB media in the plates after two days and every third day after that. Discrete nests of translucent, convoluted tubular structures should emerge by day 18 at the latest in at least one of the conditions (**Figure 1**).

Count each nest of tubules as a separate 'organoid'. Different cell lines have different tendencies to make organoids. If organoids do not emerge, troubleshoot cell number, concentration of CHIR99021 and other factors. You may also refer to *Physiology Assays in Human Kidney Organoids* (PMID 35379001) for additional information on troubleshooting organoid differentiation

Cysts will begin to emerge on day 21 and will continue to grow over the next two weeks (**Figure 1**). These will be present in ~15% of all organoids.

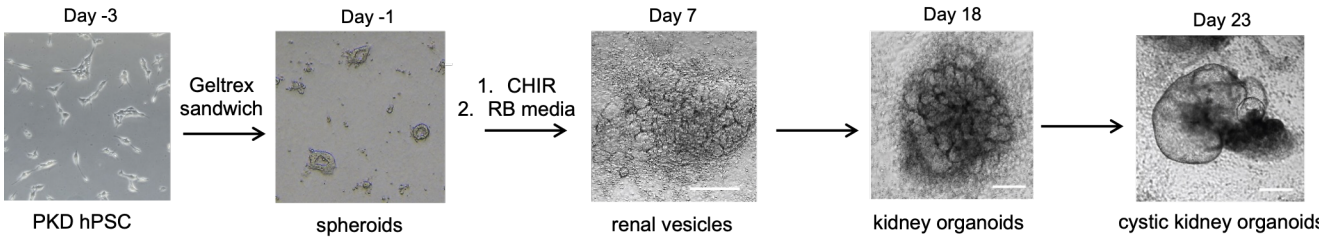



**Figure 1. Adherent kidney organoid differentiation and PKD cyst formation.** Phase contrast images of representative cultures at different days of the protocol.