

Human Colon Organoid Culture

Base Culture Media:

- 500 mL Advanced DMEM/F-12 [12634010](#)
- 5 mL 200 mM L-Glutamine (2 mM final) [25030081](#)
- 5 mL HyClone™ Pen / Strep Solution [SV30010](#)
- 1 mL 500 mM N-Acetyl-L-Cysteine (NALC) (1 mM final) [A9165-25G](#)
- 5 mL 1M HEPES (10 mM final) [H3375-25G](#)
- 1 mL B-27 (50×) [17504044](#), 500 µL N2 (100×) [17502048](#) supplements to make 50 mL aliquots

Complete Culture Media:

- Human R-spondin-1* (500-800 ng / mL final) [120-38](#)
- Murine Wnt-3a* (100 ng / mL final); [315-20](#)
- Murine EGF (50 ng / mL final); [PMG8041](#)
- Murine Noggin* (100 ng / mL final); [250-38](#)
- Gastrin I (10 nM final) [SCP0152](#)
- A83-01 (500 nM final) [04-0014](#)
- SB202190 (10 µM final) [CAYM10010399-10](#)
- Nicotinamide (10 mM final) [N0636](#)
- Rho-associated protein kinase inhibitor (Y-27632) – only for thawing organoids (10 µM final), [Y0503](#)

* Growth factors can be replaced by addition of ~20% L-WRN-conditioned media (L-WRN - a cell line that stably expresses mWnt-3A, hR-Spondin-1, hNoggin - [Takahashi, Y. et al.](#))

Additional Materials and Instruments:

- Growth Factor Reduced Matrigel [354230](#)
- PBS, Ca²⁺/Mg²⁺-free [10010056](#)
- Sterile 1.5 mL Eppendorf tubes [30120086](#)
- TrypLE™ Express Enzyme [12604013](#)
- Cell culture plates (12-well plate) [92412T](#)
- Recovery™ Cell Culture Freezing Medium [12648010](#)
- Cryovials [377267](#)
- Mr. Frosty freezing container [479-3200](#)
- Tabletop centrifuge

Organoid Thawing

Day 0

1. Slowly thaw cryovials with organoids at 37 °C in water bath.
2. Transfer organoids into a sterile 1.5 mL Eppendorf and top up with 0.5 mL ice-cold PBS.
3. Spin down briefly (10 sec) using a benchtop centrifuge and carefully remove the supernatant.
4. Wash the organoids with 1 mL ice-cold PBS and centrifuge again.
5. Remove supernatant and resuspend the cells in X μ L of Matrigel (volume depends on the size of the organoid pellet).
6. Plate 3 \times 25 μ L of organoid / Matrigel droplets per well (12-well plate format).
7. Incubate the plate at 37 °C for 5-10 mins to allow the Matrigel to polymerise.
8. Add 1 mL of complete culture media (with Y-27632) and incubate the cells at 37 °C, 5% CO₂.

Day 1

NOTE: Fragmented organoids will close up to form organoids, media change is required to remove Y-27632.

1. Remove media and replace with fresh complete media (without Y-27632).
2. *Optional: Organoids can be passaged to remove cell debris to clean up the culture.*

Organoid Passaging and Maintenance

1. Remove media from the wells and add 1 mL ice-cold PBS.
2. Mechanically break up the Matrigel droplets by P1000 pipetting.
3. Transfer PBS / Matrigel / organoids into a 1.5 mL Eppendorf and spin down in a benchtop centrifuge. Remove supernatant and repeat wash.
4. Remove supernatant and add 250 μ L TrypLE, incubate the cells at room temperature for 3-5 mins.
5. Mechanically break up the organoids by P200 pipetting, add 750 μ L ice-cold PBS and centrifuge.
6. Remove supernatant and wash the cells with 1 mL ice-cold PBS. Centrifuge again and repeat wash.
7. Remove supernatant and resuspend cells in X* μ L Matrigel to seed Y* number of wells.
8. Incubate the plate at 37 °C for 5-10 mins to allow the Matrigel to polymerise.
9. Add 1 mL of growth media and incubate at 37 °C, 5% CO₂.
10. Change media every 2-3 days and passage the organoids every 4-7 days.

**Organoids are split into fragments that may or may not contain stem cells that will develop into new organoids. It is therefore not possible to count the number of viable stem cells before seeding. Thus, the number of wells to seed after passage is determined from the size of the pellet.*

Note: Depending on the growth pattern of the organoids, sometimes it is necessary to re-plate them without splitting to clean up the culture.

Organoid Freezing and Storage:

1. Harvest cells as described above.
2. Resuspend cell pellets in Cell Freezing Medium (0.5 mL / cryovial).
3. Transfer the cryovials to a Mr. Frosty and store at -80 °C for 24 hours.
4. Transfer the cryovials to liquid nitrogen storage.