# Working with patient-derived enteroids and colonoids

*Last updated: 11/16/2023 (TK)*

Reagents:

- Matrigel (Corning #354234): keep on ice! After thawing the 10 mL bottle (on ice, will take a while - best to do overnight), dispense into 500 μL aliquots (pre-chill the tubes on ice) and store at -20°C
- Enteroid expansion medium (EEM): see section (5) for details
    - A commercially available alternative is Human IntestiCult Organoid Growth Medium (StemCell # 06010).
- Cryopreservation medium: 90% FBS + 10% DMSO
- PBS or HBSS, ice-cold is best, but not necessary
- Trypsin-EDTA, 0.05% (Gibco #25300-054)
    - We have also used 0.25% Trypsin-EDTA (Gibco #25200-056) and TrypLE Express (Gibco 12605-010), with comparable results
- DNAse I (Roche # 10104159001): reconstitute 100 mg in 4 mL sterile PBS, yielding a 50U/mL solution (100x). Aliquot 100 uL/tube and store at -20°C
- Soybean trypsin inhibitor (STI, Sigma-Aldrich #T9128, 250 mg/mL in PBS) or FBS, for neutralizing trypsin
- 24-well tissue culture-treated plate (a pre-warmed plate is best, but not necessary)

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(1) Recovery from cryopreservation:

Unless noted otherwise, recover one vial into one well of a 24-well plate

1. Quickly thaw the vial in a 37°C water bath.
    - (If using a bead bath, place a small beaker of pre-warmed water into the beads – this shortens the thaw time)
2. Transfer the suspension into an Eppendorf tube
3. Spin 30 s – 700 g, remove supernatant by pipetting
4. Wash with 1 mL PBS, spin 30s - 700g, remove supernatant by pipetting
5. Place the tube on ice to cool the plastic
6. Resuspend the pellet in 50 μL Matrigel per dome, plate
7. Let the dome(s) solidify at 37°C for 1 h (incubator), then add 500 μL/well of pre-warmed EEM
8. Change media every other day (ex. Monday, Wednesday, Friday), monitor enteroid formation and growth
9. Enteroids will be ready for passage on day 5-7

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(2) Passaging – single cell:

This protocol is for passaging of enteroids from one well, if you have more - scale the volumes up accordingly

Optimal seeding density may vary from line to line, we recommend starting by seeding 10000 – 15000 live cells per 50 μL dome

1. Remove spent medium
2. Dislodge the Matrigel dome with 500 μL PBS by pipetting up and down through a P1000 tip, transfer to an Eppendorf tube
3. Spin 30 s – 700 g, remove supernatant by pipetting
4. Add 1 mL PBS and mix
5. Spin 30s - 700g, remove supernatant by pipetting
6. Resuspend the pellet in 300 μL 0.05% Trypsin-EDTA, supplemented with 3 μL DNAse I
7. Incubate at 37°C for 5-10 m
    - 10 m may reduce viability, but will get better single cell separation
    - We recommend incubating in a thermomixer with shaking (for example, Eppendorf model 5385000024 at 800 RPM). If unavailable, can keep in the incubator and flick the tube every 1-2 m.
8. Neutralize the trypsin with either 300 μL STI or 100 μL FBS
9. Spin 30 s – 700 g, remove supernatant by pipetting
10. Reconstitute in 100 μL EEM and count the cells
11. Place the tube on ice to cool the plastic
12. Seed 15000 live cells per 50 μL dome of 80% Matrigel:
    - Bring your cell suspension to $15x10^5$ live cells/mL (1500 cells/μL)
    - For 1 dome, mix 10 μL of this suspension + 40 μL Matrigel
13. Seed 50 μL domes
14. Let the domes solidify at 37°C for 1h (incubator), then add 500 μL/well of pre-warmed EEM
15. Change media every other day (ex. Monday, Wednesday, Friday), monitor enteroid formation and growth
16. Enteroids will be ready for harvest on day 14

# Working with patient-derived enteroids and colonoids

(3) Passaging – mechanical splitting:

This protocol is for passaging of enteroids from one well, if you have more - scale the volumes up accordingly

One well is typically split into 3-5 wells

1. Remove spent medium
2. Dislodge the Matrigel dome with 500 μL PBS by pipetting up and down through a P1000 tip, transfer to an Eppendorf tube
3. Spin 30 s – 700 g, remove supernatant by pipetting
4. Resuspend in 250-500 μL cold PBS (depending on the pellet size) and mechanically break up the enteroids by passing the suspension 10-15 times through a P200 tip on top of a P1000 tip
5. Add 1 mL PBS and mix
6. Spin 30 s – 700 g, remove supernatant by pipetting
7. Use a small volume of EEM (e.g. 30 or 50 μL when seeding 3 or 5 domes, respectively) to resuspend the pelleted fragments (this will also help to get more even distribution in Matrigel)
8. Place the tube on ice to cool the plastic
9. Mix the fragment suspension with Matrigel to 80% ( e.g. 120 or 200 μL when seeding 3 or 5 domes, respectively)
10. Seed 50 μL domes
11. Let the domes solidify at 37°C for 1 h (incubator), then add 500 μL/well of pre-warmed EEM
12. Change media every other day (ex. Monday, Wednesday, Friday), monitor enteroid formation and growth
13. Enteroids will be ready for harvest on day 7

(4) Cryopreservation:

On average, a well with ~20 enteroids should be cryopreserved in one vial

1. Remove spent medium
2. Dislodge the Matrigel dome with 500 μL ice-cold PBS by pipetting up and down through a P1000 tip, transfer to an Eppendorf tube
3. Spin 30 s – 700 g, remove supernatant by pipetting
4. Resuspend in 250-500 μL cold PBS (depending on the pellet size) and mechanically break up the enteroids by passing the suspension 10-15 times through a P200 tip on top of a P1000 tip
5. Add 1 mL PBS and mix
6. Spin 30 s – 700 g, remove supernatant by pipetting
7. Reconstitute the pellet in 1 mL cryopreservation medium and transfer to a labeled cryovial
8. Place the cryovial in a Mr. Frosty (or an equivalent cell freezing container) and transfer to -80$^{o}$C
9. Keep at -80$^{o}$C overnight and transfer to cryostorage

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(5) EEM recipe and reagent information

| Reagent (stock concentration) | For 50 mL | For 10mL | Final conc. |
|---|---|---|---|
| Basal medium | 23 mL | 4.6 mL | - |
| L-WRN CM* | 25 mL | 5mL | 50% |
| N2 Supplement (100X) | 500 µL | 100 µL | 1x |
| B27 Supplement (50X) | 1 mL | 200 µL | 1x |
| Hs EGF (500ug/ml) | 5 µL | 1 µL | 50 ng/mL |
| Gastrin (100 uM) | 5 µL | 1 µL | 10 nM |
| N-Acetylcysteine (0.5M) | 100 µL | 20 µL | 1 mM |
| Nicotinamide (1M) | 500 µL | 100 µL | 10 mM |
| SB202190 (10mM) | 15 µL | 3 µL | 3 µM |
| A83-01 (5mM) | 5 µL | 1 µL | 500 nM |
| Y27632 (50mM) ** | 10 µL | 2 µL | 10 µM |
| Anti-anti (100x) | 500 µL | 100 µL | 1x |

*Conditioned medium with Noggin/R-Spondin/Wnt3a, produced in L-WRN cells in Basal medium supplemented with 20% FBS

**The ROCK inhibitor should only be used at seeding (or recovery from cryopreservation)

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Stock solutions:

**Basal medium**:

- 500 mL Advanced DMEM/F12 (Thermo #12634028)
- 5 mL GlutaMax (Thermo #35050061)
- 5 mL HEPES (Thermo #15630080)
- 5 mL Anti-anti (Thermo #15240062)

**N2 Supplement (100x), serum-free**: Gibco # 17502048.

- Thaw overnight in the fridge or quickly in bead-bath (do not let it sit at 37$^{o}$C once thawed) and make 500 $\mu$L aliquots.
- Store @-20$^{o}$C.

**B-27 Supplement (50x), serum-free**: Gibco # 17504044.

- Thaw overnight in the fridge or quickly in bead-bath (do not let it sit at 37$^{o}$C once thawed) and make 1 mL aliquots.
- Store @-20$^{o}$C.

**EGF**: Peprotech # AF-100-15.

- To make 500 $\mu$g/mL solution, reconstitute 1 mg in 2 mL Basal Medium, dispense into 200 $\mu$L aliquots.
- After thawing, dispense each 200 $\mu$L into 25 $\mu$L aliquots.
- Store @-20$^{o}$C.

**[Leu15]-Gastrin I**: Sigma # G9145.

- To make 100 $\mu$M working stock, resuspend 0.5 mg in 2.4 mL DPBS, make 200 $\mu$L aliquots.
- After thawing, dispense each 200 $\mu$L into 10 $\mu$L.
- Store @-20$^{o}$C.

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**NAC/N-Acetylcysteine**: Sigma # A9165.

- To make 0.5 M stock solution, dissolve 408 mg in 5 mL DPBS, sterile-filter and aliquot 200 $\mu$L tube.
- Store @-20$^{o}$C

**Nicotinamide**: Sigma # N0636.

- To make 1 M stock solution, dissolve 1.12 g in 10 mL DPBS, sterile-filter and aliquot 500 $\mu$L tube.
- Store @-20$^{o}$C

**SB202190**: Selleck Chemicals #S1077.

- To make 10 mM solution, resuspend 5 mg in x 1.5 mL sterile DMSO, aliquot 200 $\mu$L/tube.
- Once thawed, dispense each 200 $\mu$L into 10x20 $\mu$L aliquots.
- Store @-20$^{o}$C.

**A83-01**: Cayman # 9001799.

- To make 5 mM stock solution, dissolve 5 mg in 2.37 mL sterile DMSO, 250 $\mu$L aliquots.
- Once thawed, dispense each 250 $\mu$L into 20x25 $\mu$L aliquots.
- Store @-20$^{o}$C

**Y27632**: LC labs # Y-5301.

- To make 50 mM solution, resuspend 10 mg in 625 $\mu$L sterile DMSO and make 50 $\mu$L aliquots.
- Once thawed, dispense each 50 $\mu$L into 5x10 $\mu$L aliquots.
- Store @-20$^{o}$C.