

*** Keep all the solutions and cells on ice as much as possible ***

Matrigel – thaw in refrigerator or ice ahead of time

1. Perform mucosectomy on isolated segment of intestine.
2. Use 1–2 grams mucosa (lower end if dry weight, upper end if wet weight).
3. Cut mucosa into 1 x 1 cm pieces.
4. Wash mucosa by adding it to a 50 mL tube with 30 mL ice-cold PBS, vortex for 30 seconds (3 pulses of 10 second duration) and remove supernatant.
5. Repeat Step 4 until supernatant is as clear as possible. Usually about 8-15 times.
6. Add 30 ml ice-cold PBS containing EDTA (final 3 mM) and DTT (final 1 mM).
7. Rock sample for 30 minutes at 4 °C (in cold room).
8. Make every effort to remove all supernatant.
9. Add PBS to a total volume of ~25–30 mL and vortex gently for 30 Sec. in 3 sec pulses. Transfer supernatant to a 15 mL tube after tissue settles down. Repeat 6 times until most of crypts are released, and make sure the volume in each 15 mL tube is the same.
10. Spin down the crypt fractions at 140 rcf and 4 °C for 2 min and remove supernatant (leave 2 mL in tube to re-suspend).
11. Select tubes 2-6 (If all tubes have significantly large pellets, then select tubes 3-6) to re-suspend. Add FBS to each tube to make the solution 10%FBS by volume and mix up and down by transfer pipette.
12. Combine fractions (tubes) 2-6 or 3-6 and filter through 100 um cells strainer.
13. Centrifuge filtrate at 140 rcf and 4 °C for 2 min to form pellet.
14. Remove all supernatant and re-suspended pellet in 5mL of Basic Media.

15. Count crypts: Place 2 μ L from the 5 mL (5000 μ L) crypt suspension on a glass slide and count the number of crypts by inverted microscopy.

Total number of crypts (in 5 mL) = numbers of crypts counted x 2500.

16. Calculate volume of suspension needed to provide desired number of cells:

$$\text{Volume of crypt suspension needed}(\mu\text{L}) = \frac{\text{Crypts needed}}{\text{Crypts in } 1 \mu\text{L}}$$

17. Place desired volume of crypt suspension in microcentrifuge tube and do quick spin to pellet crypts. Usually 3 quick spins of a couple of seconds each will suffice.

18. Quickly remove supernatant and re-suspend in Matrigel to final concentration of 100–250 crypts/25 μ L. Use chilled 200 μ L pipette tips. Avoid introducing bubbles when mixing crypts with Matrigel. Keep Matrigel on ice to avoid premature hardening.

19. Plate on a 48 well plate. Incubate plate at 37 °C / 5% CO₂ for 20 minutes to allow Matrigel to harden then carefully adding 250 μ L of enteroid media per well. Replace fresh enteroids media every other day.

Basic Media

Advanced DMEM/F12

2 mM GlutaMax

10mM Hepes

100 U/ml Penicillin/100 μ g/ml Streptomycin

2X Complete medium

Advanced DMEM/F12

4 mM GlutaMax

20 mM Hepes

200 U/ml Penicillin/100 ug/ml Streptomycin

2x N2 supplement

2x B27 supplement

2 mM N-Acetylcysteine

Enteroids Media

50% Wnt3a condition media

50% 2X Complete medium

50 ng/ml EGF

100 ng/ml Noggin

1 ug/ml R-spondin

10 uM Y27632

500 nM A8301

2.5 uM GSK (Only on P0d0 for the crypts)

Cell Line Description Organism: *Mus musculus* (mouse) **Strain:** C3H/An
Tissue: subcutaneous connective tissue; areolar and adipose; transfected with Wnt-3A expression vector

L-M(TK-) cells (ATCC CCL-1.3) were transfected with a Wnt-3A expression vector and stable clones were selected in medium containing G418.

The Wnt-3A gene encodes a secreted glycoprotein with a variety of signaling effects. Wnt genes control many of the patterning and growth events during embryonic development.

(ATCC CRL-2648).

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium:

10% Fetal bovine serum and 1X Penicillin/Streptomycin.

Incubator

5% CO₂ in air atmosphere at 37°C

Protocol for Wnt-3A conditioned medium

1. Thaw out frozen cell and grow in 1 T25 flask media with 0.4mg/ml of G418 (only in this step) until 100% confluent for 2 ~ 3 days.
2. Cells were split from 1 T25 to 1 T75 until confluent (about 2 days).
3. Split the cells 1:8 of T-75 flasks (10ml) or 1:5 of T150 flask (15ml) and let the cells grow for 4 days (approximately to confluent).
4. Transfer the 1st batch condition medium from flask to filter and store it at 4°C.
5. Add 10 ml fresh medium to each of the T75 flask or 15ml to the T150 flask and incubate for another 3 days.
6. Transfer the 2nd batch of the condition medium from flask to combine the first batch of condition medium filtering with a 0.22 µm filter and discard the cells.
7. The Wnt-3A condition medium is stable at 4° C fridge for one or two month.

