

Human Crypt Isolation and Propagation

Modified from Clevers and Stappenbeck protocols

Updated 10/2015

Description:

This protocol is for the isolation and propagation of human crypts from primary intestinal tissue.

References:

1. Long term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Sato, *et al. Gastroenterology* (2011).
2. Wnt5a Potentiates TGF-B Signaling to Promote Colonic Crypt Regeneration After Tissue Injury. Miyoshi, *et al. Science* (2012).

Equipment:

- Inverted light microscope
- Tissue culture hood
- Table top centrifuge
- CO₂ incubator/37°C
- Dissecting tools
- Ice bucket

Materials:

- Human intestinal tissue
- PBS without Mg⁺⁺ and Ca⁺⁺
 - (Thermofisher, 10010-023)
- Basement Membrane matrigel
 - (Corning 354234)
- HEPES
 - (Thermofisher, 15630-080)
- L-glutamine
 - (Thermofisher, 25030-081)
- Thiazovivin
 - (Stemgent, 04-0017)
- Advanced DMEM/F12
 - (Thermofisher, 12634-010)
- N2 Supplement
 - (Thermofisher, 17502-048)
- B27 supplement
 - (Thermofisher, 0080085-SA)
- Pen/Strep
 - (Thermofisher, 15140-122)
- N-acetylcysteine
 - (Sigma, A9165-5G)
- EGF
 - R&D Systems, 236-EG-200)
- Nicotinamide
 - (Sigma, N0636)
- LWRN Conditioned Media
- 6-well culture plate
- 4 or 24 well culture plate
- 15cm culture dish
- FBS (Optional)
- 26 or 27 gauge needle and 1mL syringe

Intestinal tissue is usually obtained from warm autopsy donors. Transplant teams remove donor organs, and then we are able to harvest GI tissues.

Intestinal tissue is obtained as 5-10cm pieces of whole-organ, and can be collected from any region of the gut.

5-10cm pieces of intestine are placed in a 50mL conical tube, filled with ice cold PBS w/ pen/strep/gentamycin, and transported back to the lab on ice.

Upon returning to the lab, tissue is cut longitudinally into halves or quarters.

Tissue is placed in a 50mL conical tube, and is filled with fresh ice cold PBS/antibiotics. Tube is shaken vigorously for 10 seconds by hand to remove luminal contents. This is repeated 3-5 more times until most luminal contents have been removed, and the PBS is relatively clear from luminal debris.

Intestinal tissue is then placed in fresh ion-free PBS containing pen/strep and gentamycin and placed at 4C for at least 12 hours and can be processed up to 24 hours after being harvested from donor patient.

I. Crypt Isolation for processing the following day after collection

1. Remove the intestinal tissue from the PBS/antibiotic solution and lay tissue 'face up' (apical surface exposed) in a 15cm petri dish.
2. Dislodge mucosal layer. We have used two techniques that work well.
 - a. Use a scalpel and scrape the mucosal layer and epithelium off the tissue and onto the dish. You can use a decent amount of force here when scraping the tissue.
 - b. Use the scalpel to fillet the mucosa away from the musculature - place scalpel at angle such that the mucosal layer is gently cut away as a thin strip.
3. Fill 3 or 4 wells of a 6-well plate with 3mL of Advanced DMEM/f12 (adding 20% FBS is optional) and TZV (always at 1:4000)
4. Use a cut P1000 tip and transfer mucosa from the petri dish (step 2) to the first well.
5. Pipette up and down vigorously a few times to break up tissue. Check progress of crypt release and then continue vigorously pipetting as necessary until sufficient release of crypts. You can transfer smaller amounts of tissue into new wells to separate the sample out and try different amounts of pipetting to release the crypts.
6. After visually confirming sufficient released the crypts under a stereomicroscope, there will also be other debris from the villi in the well. Here, we do the following:
 - a. If crypts are obvious then you can pick them out individually with a P20 or P200 pipette and transfer the crypts plus media to a 1.5mL microcentrifuge tube.
 - b. Alternatively you can filter the tissue into a new well of the 6-well plate through a 100um cell strainer.
 - c. Using a) and/or b) we try to isolate between 100-200 crypts.
7. After sufficient crypts have been transferred to 1.5mL tube, spin tube @ 300-500rpm for 2-4 minutes. Aspirate media. Use a P200 under the dissecting scope to remove most of the media and place the tube on ice.

8. Fill the tube with an appropriate amount of basement membrane Matrigel. The volume depends on the amount of crypts obtained. Typically we plate about 30-40 crypts in a 50uL droplet per well of a 24 well plate i.e. if you isolated 160 crypts you will have enough for 4 wells and will need 200uL of Matrigel in the microcentrifuge tube.
9. Gently pipette the matrigel/crypt mixture to disperse the pellet and distribute crypts evenly in matrigel, then place a 50uL droplet into each well of the 24-well plate.
10. Let Matrigel solidify in incubator for 10 minutes, and then overlay with 500uL growth media (see below) containing TZV
11. Replace media on day 2, keep TZV in the media.
12. Replace media on day 3, remove TZV
 - a. After day 3 replace every 2 days (MWF feeding schedule works fine)

II. Growth Media for human enteroids:

1. Make up 2x basal media (can make **250mL** at a time)

Component	Amount	Final concentration
Advanced DMEM/F12	250mL	NA
Glutamax (100X-200mM)	5mL	2X
HEPES(100X, 1M)	5mL	2X
N2 supplement (100X)	5mL	2X
B27 (50x)	10mL	2X
Pen/Strep (100X)	5mL	2x
N-acetylcysteine (500mM)	1mL	2mM
Nicotinamide (1M)	5mL	20mM

2. To make up **growth media** add LWRN conditioned media in a 1:1 mixture with Human 2X basal media and add EGF to a final concentration of 100ng/ml (this is referred to as LWRN Complete media).
3. The first 2 days of culturing we feed the enteroids with the growth media containing Thiazovivin (TZV 10mM) at a 1:4000 dilution or Y-compound (also 10mM) at 1:1000 . After that they are fed every other day with the growth media without TZV or Y-compound.

III. Splitting human enteroids:

1. Use a 1000ul micropipette tip to dislodge the matrigel in each well from the plate and collect matrigel/media solution in a microcentrifuge tube. You will want to pool 6-8 wells of enteroids per microcentrifuge tube for splitting (becomes a plates-worth of wells after split).
2. Split enteroids by drawing the mixture through a 26 or 27 gauge needle and then expel them.
3. Spin 2,500xg 10-15 seconds. Layers of liquid media, matrigel, and enteroids will form.
4. Aspirate off media and matrigel, leaving only the enteroid pellet (small amounts of matrigel is OK).
5. Put microcentrifuge tubes with enteroid pellet on ice and allow them to cool before adding matrigel.
6. Pipette new matrigel up and down using a new 1000uL micropipette tip a few times to cool and coat the pipette tip before using.
7. Transfer new matrigel to tubes containing enteroid pellet(s) at 50uL per planned well. Flux the matrigel and pellet a few times to distribute the pellet into solution.

