Strategies for optimizing the isolation and expansion of sensitive patient-derived duodenoids, ileoids and colonoids

Katlynn Bugda Gwilt, Jay R. Thiagarajah

Division of Gastroenterology, Hepatology and Nutrition, Boston Children's Hospital, Harvard Medical School, Boston, MA, United States

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Abstract

These protocols are an optimization of previous protocols and the original work described in Sato et al., 2009 which provided a foundation for the development of primary duodenoids, ileoids and colonoids from human samples. Here, we optimized methods specifically for use with samples from pediatric patients with genetic intestinal epithelial disorders. These protocols apply specifically to enteroids generated from endoscopic biopsies from patients with Congenital Diarrheas and Enteropathies (CoDE) or Very-Early-Onset Inflammatory Bowel Disease (VEO-IBD) or age-matched patients without any intestinal disease and include samples from the duodenum (duodenoids), ileum (ileoids), and colon (colonoids). The duodenoids, ileoids and colonoids from these patients exhibit a range of cellular phenotypes that potentially impact enteroid growth and maintenance including increased apoptosis, defects in proliferation, polarity and vesicular trafficking. These characteristics make it potentially challenging for long-term culture and expansion, limiting the availability of cells for functional experiments. Using a modified culture media, an expanded initial culture time after cell isolation from patient biopsies, and gentle passaging techniques, we were able to successfully culture and expand several patient lines over multiple passages and maintain the cultures for >5 years. The media conditions and protocols described here allow for reproducible phenotypes as well as scaling for larger functional studies on patient lines. These protocols also provide a useful starting point for further optimization for generating and culturing enteroids from patients with novel disease pathophysiology.

Materials

Key resources table		
Reagent	**Supplier**	**Catalog number**
TrypLE™ Express Enzyme (1X), phenol red	Thermo Fisher Scientific	Cat#12605010
Advanced DMEM/F12	Gibco	Cat#12634-028
L-WRN Conditioned Media	ATCC (Cite Clevers)	CRL-3276
Wnt Conditioned Media	ATCC	CRL-2647
R-spondin-1 conditioned Media	n/a	Hans Clevers
Noggin Conditioned Media	n/a	Hans Clevers
Recombinant Noggin	Peperotech	
Glutamax	Gibco	Cat#35050-061
HEPES	Gibco	Cat#15630-080
Primocin	Invivogen	Cat#Ant-pm-2
Normocin	Invivogen	Cat#Ant-nr-2
B27	Gibco	Cat#12587010
N2	Gibco	Cat#17502-048
Nicotinamide	Sigma-Aldrich	Cat#N0636
N-acetyl-cysteine	Sigma-Aldrich	Cat#A8199
A83-01	Sigma-Aldrich	Cat#SML0788
SB202190	Sigma-Aldrich	Cat#S7067
EGF	Peprotech	Cat#315-09
Gastrin	Sigma-Aldrich	Cat#G9145
Y27632	Sigma-Aldrich	Cat#Y0503
Prostaglandin E2	Sigma-Aldrich	Cat#P5640
CHIR99021	Sigma-Aldrich	Cat#SML1046
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free, 10 mL	Corning	Cat#356231
Cultrex	R&D Technologies	BME001-10
Cultrex Organoid Harvesting Solution, 100 mL	R&D Technologies	3700-100-01
Corning® Cell Recovery Solution, 100 mL	Corning	Cat#354253
Collagen IV from human placenta	Sigma-Aldrich	Cat#C5533
Collagenase, Type 1	Stem Cell	Catalog # 07416
6.5 mm Transwell with 0.4 µm pore polyester membrane insert, TC-treated, sterile, 48/cs	Corning	Cat#CLS3470-48EA
24 well plate	Costar	Cat#3526

Citations

Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., van Es, J. H., Abo, A., Kujala, P., Peters, P. J., & Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*, *459*(7244), 262–265. https://doi.org/10.1038/nature07935

Preparation of Human Organoid Media Components:

Before beginning: dissolve all reagents to the appropriate concentration according to manufacturer's protocols. A * indicates that there were no modifications to the reagent.

[Base Media]

Reagent	Final concentration	Amount
Advanced DMEM/F12*	~80% (*v/v*)	500 ml
HI-FBS (as supplied)*	~20% (*v/v*)	100 ml
Pen/Strep (100x)*	~1% (*v/v*)	5 ml
Total		605ml

Note: Pen/Strep can also be replaced with Anti/Anti (Gibco 15240062) with no effect on organoid cultures

[Colonoid Expansion Media]

Reagent	Final concentration	Amount
L-WRN Conditioned Media	65% (*v/v*)	65ml
R-Spondin-1 Conditioned Media	10% (*v/v*)	10ml
Base Media	25% (*v/v*)	25ml
GlutaMax (100x)*	1x	1ml
HEPES (1M)*	(10mM)	1ml
Primocin (500x)*	n/a	200uL
Normocin (500x)*	n/a	200uL
B27 (50x)*	1X	2ml
N2 (100x)*	1X	1ml
Nicotinamide (1M)	10mM	1ml
N-Acetyl-Cysteine^{+} (500mM)	500µM	200ul
A8301 (500µM)		100ul
SB202190 (5mg/505ul)		33.2ul
EGF (500ug/ml)	50ng/mL	20ul
Gastrin (500µM)	10nM	10ul
Prostaglandin E2 (5mg/ml)	100nM	1ul
Total	n/a	
Noggin Conditioned Media		10ml
Recombinant Wnt3a		10ul

**If working with a challenging to grow colonoid line, adding 10ml Noggin conditioned media and/or Recombinant Wnt3a will help a culture establish and help rescue a dying culture. Add directly to the culture media with no modifications to other volumes

[Colonoid Plating Media]

Reagent	Final concentration	Amount
Colonoid expansion media	working	100ml
Y-27632 (10mM)	10µM	10µl
CHIR (5mM)	250nM	1ul
Total		**100.11ml**

[Duodenoid Expansion Media]

Reagent	Final concentration	Amount
L-WRN Conditioned Media	50% (*v/v*)	50ml
R-spondin-1 conditioned Media		
Base Media	35% (*v/v)*	35ml
GlutaMax (100x)*	1x	1ml
HEPES (1M)*	(10mM)	1ml
Primocin (500x)*	n/a	200uL
Normocin (500x)*	n/a	200uL
B27* (50x)	1X	2ml
N2* (100x)	1X	1ml
Nicotinamide (1M)	10mM	1ml
A8301 (500µM)		100ul
SB202190 (5mg/505ul)		33.2ul
N-Acetyl-Cysteine (500mM)	500µM	200ul
EGF (500ug/ml)	50ng/mL	20ul
Gastrin^{+} (500µM)	10nM	10ul
Total	n/a	
Noggin Conditioned Media		10ml
Recombinant Wnt3a		10ul

**If working with a challenging to grow colonoid line, adding 10ml Noggin conditioned media and/or Recombinant Wnt3a will help a culture establish and help rescue a dying culture. Add directly to the culture media with no modifications to other volumes

[Duodenoid Plating Media]

Reagent	Final concentration	Amount
Duodenoid expansion media	working	100ml
Y-27632 (10mM)	10µM	10µl
CHIR (5mM)	250nM	1ul
Total		**100.11ml**

[Differentiation Media]

Reagent	Final concentration	Amount
Noggin Conditioned Media	10%	65ml
Base Media	90% (*v/v)*	30ml
GlutaMax* (100x)	1x	1ml
HEPES* (1M)	(10mM)	1ml
Primocin* (500x)	n/a	200uL
Normocin* (500x)	n/a	200uL
B27* (50x)	1X	2ml
EGF (500ug/ml)	25ng/mL	10ul
Gastrin (500µM)	10nM	10ul
Prostaglandin E2 (5mg/ml)	100nM	1ul
Total	n/a	

Processing Fresh or Frozen Intestinal Biopsies

Note: Frozen biopsies will take longer to form organoids at first. We routinely leave cells isolated from frozen biopsies in Matrigel for 14 days to allow for the outgrowth of the organoids.

Before you begin: prepare Collagenase to a concentration of 2mg/ml in HBSS. Store aliquots of Collagenase at -20°C.

1. In a sterile tissue culture hood, thaw 1mL of Collagenase Type I (2mg/mL in HBSS), move to 15mL conical tube

2. Prepare a second 15mL conical tube with 3-4mL Base Media

3. If thawing a biopsy, thaw as you would a cell line: in a 37°C water bath until small piece of ice remaining. If working with a fresh biopsy proceed to step 4.

4. Spray with ethanol and bring back to a sterile hood. Using a P1000 remove biopsy from thawed vial or buffer and rinse in the conical tube of Base Media from step 2.t

Note: it is important to rinse the biopsy of DMSO or other buffer reagents that may prevent organoid formation

5. Remove biopsy from Base Media and place into tube of Collagenase Type 1

6. Break up and pipette biopsy piece until fragments easily pass through P1000 tip

7. Incubate in 37°C water bath for 40 minutes

8. Remove tube from water bath and bring into sterile tissue culture hood. Pipette up and down using a P1000 approximately 25x to break up tissue and dissociate crypts

Note: There may be residual fat and tissue left after this step

9. Dilute Collagenase with 5 mL Base Media, inverting the tube gently 3 times dilute collagenase.

10. Centrifuge at 300 x g for 5 minutes at 4°C

11. Aspirate the supernatant from the pellet.

12. Resuspend pellet in Matrigel, plate 50uL droplets into each well of 24-well plate. For small biopsies ~150-200uL Matrigel, for larger pieces add ~300uL.

13. Incubate in 37C/5% CO_2 incubator for at least 15 minutes to polymerize.

14. Add 500uL plating media to each well

15. Replenish the media with plating media every other day for 7 days.

16. Assess organoid formation using brightfield microscopy. If organoids have started to form by day 7, proceed with passaging. If organoids have not formed in the first 7 days, **DO NOT PASSAGE.** Continue feeding with plating media for another 7-10 days.

17. After passaging proceed to normal feeding and passaging protocols.

Standard Culture Splitting/Passaging Protocol

*Note: We **do not** recommend routine use of trypsin when passaging patient organoids. We recommend use of a P200 tip to fragment organoids. This passaging protocol relies on the sheer force of the organoid through the P200 tip, which provides a gentler passaging of healthy and diseased human lines.*

1. Aspirate media with pipet tip

2. Add 0.5ml – 1ml harvesting media
    a. (Either Corning Cell Recovery Solution, Organoid Harvesting Solution (Cultrex 3700-100-01) or 2mM EDTA in PBS)

3. Using mini cell scraper (Weltex 420100) or P1000 pipet tip gently scrape Matrigel
    a. Do not mash up at this point be incredibly gentle with organoids

4. Transfer organoids from the plate to a 15ml conical on ice VERY GENTLY AT THIS POINT. Do not worry about resuspending the matrigel, gentle releases from the matrigel are best

5. Invert tube 3 times and put on ice for 1 hour.

6. After 1 hour, centrifuge at 300xG for 5 minutes and put back on ice

7. After incubation period in take 1 tube at a time in the hood

8. Aspirate the cell recovery and resuspend in 1ml of Base media

9. Using **P200 pipet** and **pipette tip**, triturate at **medium intensity**. Triturate approximately 30 times. Organoids should be fluffy floating in the media along the edges

10. Add 2ml Base Media and mix 5 times gently with P1000

11. Invert 3x and put on ice.

12. Centrifuge at 300xg for 10 minutes at 4°C

13. Aspirate base media from pellet and put on ice

14. Add 35 – 50 µl/well of matrigel to pellet and resuspend gently
    a. Plate in prewarmed plate, all at once, not one at a time using a P1000
        i. For example: if you add 500ul of Matrigel, mix gently, pull up 500ul and then manually pull the rest with the pipet, then plate 10 drops using the P1000
    b. No bubbles
    c. Allow to polymerize for ~5-10 minutes

15. Add prewarmed media @ 500µl/well

Rescue organoid Splitting/Passaging Protocol

Note: use this protocol when organoid growth appears to be slowing down. We do not recommend routine use of trypsin when passaging patient organoids.

1. Mycoplasma test the organoid line
    a. I recommend using a simple kit like MycoStrip (Invivogen: rep-mys-10)
    b. Remove primocin and normocin from one well of culture for 72 hours prior to testing
    c. If mycoplasma positive, or other contamination pops up, go back to a stock vial.
    d. If this is the last vial/plate of a sensitive patient biopsy treat with fungicure and normocure for 21 days. Take care to change media frequently to remove dead debris. If the matrigel develops a significant amount of debris, I will wash the organoids on Tuesday/Thursday and simply replate in fresh matrigel.

If myco/contamination negative, proceed to step 2.

2. Aspirate media from each well of the plate.

3. Rinse with room temp. PBS or cell recovery solution.

4. Aspirate as much of the buffer as you can

5. Add 500ul Trypsin+EDTA to each well. Gently scrape the matrigel dome with a P1000 to dislodge the matrigel and facilitate trypsinization

6. Place plate in 37°C incubator for 45 seconds.

7. Gently mix the matrigel-organoid-trypsin mixture with a P1000, and place plate back in 37°C incubator for 45 seconds.

8. Move the organoids from the plate to a 15ml conical tube. Add 10ml of ice-cold base media to the tube

9. Centrifuge at 300 x g for 5 minutes

10. Aspirate the base media leaving organoids behind.

11. Perform a second wash of the pellet with 5ml of fresh base media.

12. Centrifuge at 300 x g for 5 minutes

13. Remove as much base media as possible from the pellet.

14. Scale down your matrigel from your starting well count I typically scale down by 50-80%.
    a. If there are sparse organoids in 6 wells, I would replate only 2 wells of organoids
    b. If there are medium density, but struggling organoids in 6 wells, I would replate three wells of organoids

15. Using P200, add the appropriate volume of matrigel to the organoid pellet. Mix thoroughly 5-10 times to facilitate the final fragmentation of organoids. Take care to not introduce bubbles.

16. Plate 30-45µl droplets in the center of each well. **Note**: the smaller the matrigel dome, the better chance of recovery. Add 500ul plating media.

17. Keep the trypsinized organoids in plating media for 5 days.

18. Passage between day 5-10 post plating if there are signs of growth.