TECHNICAL MANUAL

Generation of Human Intestinal Organoids Using STEMdiff™ Intestinal Organoid Kit

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1.0 Introduction

Human pluripotent stem cells (hPSCs) are an important tool for regenerative medicine, disease modeling, and compound screening in vitro. Recent studies have shown that hPSCs can be differentiated into tissue-specific cell types by mimicking the signaling pathways in human development. Timing, duration, and concentration of growth factors at specific stages of differentiation determine cell fate decisions and lineage commitment. Recent work by Jason Spence, James Wells, and colleagues have used these principles to develop a multi-stage, in vitro differentiation protocol for the generation of human small intestinal organoids.[1] We developed STEMdiff™ Intestinal Organoid Kit by optimizing these protocols to increase efficiency and consistency across multiple human embryonic stem (ES) and induced pluripotent stem (iPS) cell lines. STEMdiff™ Intestinal Organoid Kit is a serum-free medium that supports differentiation of hPSCs through three distinct stages: definitive endoderm, mid-/hindgut, and small intestine. Cells differentiated using this kit robustly form organoids composed of polarized intestinal epithelia patterned into villus-like structures, and a surrounding, niche factor-producing mesenchyme. The epithelial and mesenchymal components express key markers of these respective intestinal compartments. The organoids generated using this kit can either be cryopreserved or maintained long term by passaging every 7 - 10 days using STEMdiff™ Intestinal Organoid Growth Medium.

2.0 Materials, Reagents, and Equipment

2.1 STEMdiff™ Intestinal Organoid Kit and Growth Medium

The components listed below are available as part of STEMdiff™ Intestinal Organoid Kit or STEMdiff™ Intestinal Organoid Growth Medium and are not available for individual sale.

Refer to the Product Information Sheet (PIS) for STEMdiff™ Intestinal Organoid Kit for component storage and stability information; the PIS is also available at www.stemcell.com, or contact us to request a copy.

PRODUCT	CATALOG #	COMPONENT NAME	COMPONENT #	SIZE
STEMdiff™ Intestinal Organoid Kit	05140	STEMdiff™ Endoderm Basal Medium	05111	100 mL
		STEMdiff™ Definitive Endoderm Supplement CJ (100X)	05113	1.1 mL
		STEMdiff™ Gastrointestinal Supplement PK	05141	0.64 mL
		STEMdiff™ Gastrointestinal Supplement UB	05142	0.64 mL
		STEMdiff™ Intestinal Organoid Basal Medium	05143	100 mL
		STEMdiff™ Intestinal Organoid Supplement*	05144	2 mL
STEMdiff™ Intestinal Organoid Growth Medium	05145	STEMdiff™ Intestinal Organoid Basal Medium	05143	100 mL
		STEMdiff™ Intestinal Organoid Supplement*	05144	2 mL

*This product contains material derived from human plasma. Donors have been tested and found negative for HIV-1 and -2, hepatitis B, and hepatitis C prior to donation. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.

2.2 Additional Required Materials and Reagents

PRODUCT	CATALOG #
mTeSR™1	85850
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free	Corning 356231
Gentle Cell Dissociation Reagent	07174
Tissue culture-treated flat-bottom plate	e.g. 38021 (24 wells)
Falcon® 96-Well Flat-Bottom Microplate, Tissue Culture-Treated	38022
Nunclon® Delta surface treated 24-well, flat-bottom tissue culture plate	Sigma D7039
L-Glutamine	07100
Anti-Adherence Rinsing Solution	07010
DMEM/F-12 with 15 mM HEPES	36254
Cell scraper/lifter	38065 OR 38067
D-PBS (Without Ca++ and Mg++)	37350
Falcon® Conical Tubes, 50 mL	38010
Falcon® Conical Tubes, 15 mL	38009
CryoStor® CS10	07930

For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

2.3 Equipment

- Biosafety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor
- Pipette-aid with appropriate serological pipettes (e.g. Catalog #38002)
- Hemocytometer
- Micropipettor with appropriate tips
- Inverted microscope
- Isopropanol freezing container
- -150°C freezer or liquid nitrogen (LN_2) vapor tank
- -80°C freezer
- -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Coating Cultureware with Corning® Matrigel®

Cultureware must be coated with Corning® Matrigel® hESC-Qualified Matrix (Corning Catalog #354277) when plating hPSCs. Matrigel® should be aliquoted and frozen. Consult the Certificate of Analysis supplied with Matrigel® for the recommended aliquot size ("Dilution Factor") to make up 25 mL of diluted matrix. Ensure to keep Matrigel® on ice when thawing and handling to prevent it from gelling.

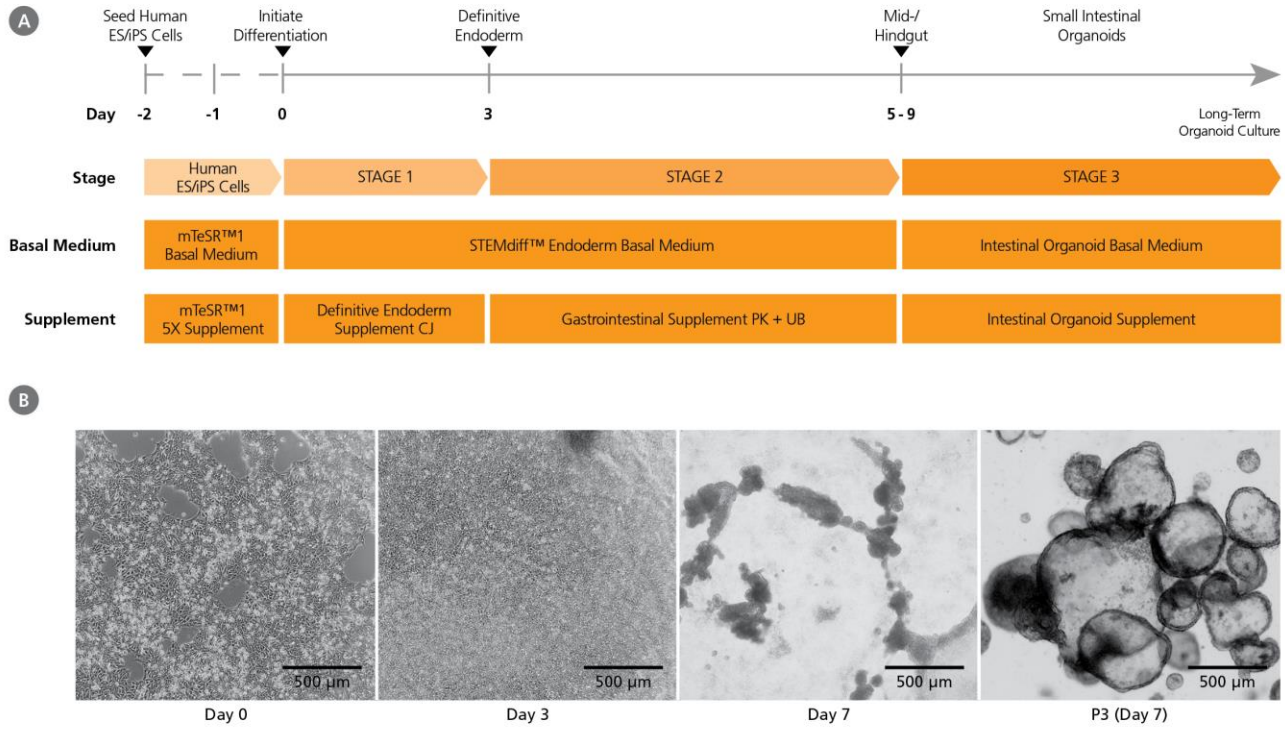
1. Thaw one aliquot of Matrigel® on ice.
2. Dispense 25 mL of cold DMEM/F-12 with 15 mM HEPES into a 50 mL conical tube and keep on ice.
3. Add thawed Matrigel® to the cold DMEM/F-12 and mix well. The vial may be washed with cold medium if desired.
4. Use the diluted Matrigel® solution immediately to coat tissue culture-treated cultureware. See Table 1 for recommended coating volumes.
5. Swirl the cultureware to spread the Matrigel® solution evenly across the surface.
6. Incubate at room temperature (15 - 25°C) for at least 1 hour before use. Do not let the Matrigel® solution evaporate.

   Note: If not used immediately, the cultureware must be sealed to prevent evaporation of the Matrigel® solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 7 days after coating. Allow stored coated cultureware to come to room temperature for 30 minutes before plating cells.

Table 1. Recommended Matrix Volume for Coating Cultureware

TISSUE CULTURE-TREATED CULTUREWARE	VOLUME OF DILUTED MATRIX
24-well plate	250 µL/well
12-well plate	500 µL/well
6-well plate	1 mL/well
100 mm dish	6 mL/dish
T-25 cm^2 flask	3 mL/flask
T-75 cm^2 flask	8 mL/flask

4.0 Protocol Diagram



(A) Human PSC cultures progress through a three-stage differentiation process using stage-specific, specialized media to generate human intestinal organoids.

(B) Representative images from each stage of differentiation are shown. Endoderm differentiation is initiated on Day 0. By Day 3 of the protocol, cultures display characteristics typical of definitive endoderm, and mid-/hindgut differentiation is initiated. During mid-/hindgut differentiation, cells form spheroids that are released from the cell monolayer into the culture medium (Day 5 - 9). These spheroids are collected, embedded in Corning® Matrigel®, and cultured in STEMdiff™ Intestinal Organoid Growth Medium to mature into intestinal organoids that can be repeatedly passaged to maintain cultures.

5.0 Passaging Cells for Differentiation

The following protocols are for passaging human ES or iPS cells cultured in mTeSR™1 from a 6-well plate to a 24-well plate. It is critical that the cells are of high quality (less than 5% differentiation); for complete instructions on maintaining high-quality human ES and iPS cells for differentiation, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com or contact us to request a copy.

Passage cells using the clump passaging method described below (section 5.1). Use sterile technique when performing the protocol. Volumes indicated are for passaging ES or iPS cells from one well of a 6-well plate to one well of a 24-well plate. If using alternative cultureware, adjust volumes accordingly.

Note: Human ES and iPS cells are ready for passaging when cultures are approximately 70% confluent.

5.1 Clump Passaging

1. Coat a 24-well tissue culture-treated plate with Corning® Matrigel® hESC-Qualified Matrix (see section 3.0).
2. Aliquot sufficient mTeSR™1 and warm to room temperature (15 - 25°C).

   Note: Do not warm mTeSR™1 in a 37°C water bath.
3. Use a microscope (4X magnification) to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.
4. Remove regions of differentiation by scraping with a pipette tip or by aspiration. Avoid having the culture plate out of the incubator for more than 15 minutes at a time.

   Note: Removal of differentiated cells will result in better differentiation efficiency.
5. Aspirate medium from the well and add 1 mL of Gentle Cell Dissociation Reagent.
6. Incubate at room temperature (15 - 25°C) for 8 minutes to generate cell clumps.

   Note: Incubation times may vary when using different cell lines or other non-enzymatic cell passaging reagents (e.g. ReLeSR™); dissociation should be monitored under the microscope until the optimal time is determined.
7. Aspirate Gentle Cell Dissociation Reagent and add 1 mL of mTeSR™1. Gently detach the colonies by scraping with a cell scraper/lifter.

   Note: Take care to minimize the breakup of colonies.
8. Transfer the detached cell aggregates to a 50 mL conical tube.

   Optional: Rinse the well with an additional 1 mL of mTeSR™1 to collect remaining cell colonies.

   Note: Centrifugation of cell aggregates is not required.
9. Carefully pipette the cell clump mixture up and down to break up the colonies using either a 1 mL pipettor or a 2 mL serological pipette. A uniform suspension of cell clumps approximately 50 - 200 µm in size is optimal. Avoid creating a single-cell suspension.
10. Gently shake the tube to ensure cell aggregates are evenly distributed. Transfer 5 µL of clump suspension into one well of a flat-bottom 96-well plate containing 50 µL D-PBS (Without Ca++ and Mg++). Count total number of clumps (50 - 200 µm in diameter) in the well.

    Note: If most cell aggregates are > 200 µm in diameter, repeat steps 9 - 10.
11. Calculate the volume (in µL) of clump suspension required to seed 6,000 clumps as follows:

$$Volume\ of\ clump\ suspension\ (\mu L) = 6000\ cell\ clumps\ \div \frac{Number\ of\ clumps\ in\ 5\ \mu L}{5\ \mu L}$$

12. Gently tilt the coated 24-well plate (prepared in step 1) onto one side and allow the excess Matrigel® solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
13. Gently shake the tube to ensure cell aggregates are evenly distributed. Immediately add the appropriate volume for 6,000 clumps (calculated in step 11) to a 24-well plate (prepared in step 1) containing 0.5 mL mTeSR™1 per well. Incubate at 37°C with 5% CO_2 and 95% humidity. Ensure cells are evenly distributed within each well by rocking plate in a back-and-forth and side-to-side motion a few times while the plate is in the incubator. Do not disturb the plate for 24 hours.

    Note: An initial experiment is recommended to determine the optimal clump seeding density for the cell line being used. Seed a range of clump densities (e.g. 4,000, 5,000, and 6,000 clumps per well), and initiate differentiation of each density on the same day.
14. Proceed to section 6.0 for differentiation.

6.0 Differentiation of hPSCs in Monolayer Culture

Prior to initiating differentiation, assess the confluency of cells under a microscope after 24 hours of incubation (prepared in section 5.1). Cells should have < 5% differentiation and should be 85 - 90% confluent. If cells have not yet reached this level of confluency, replace medium with 0.5 - 1 mL of fresh mTeSR™1 (without Y-27632) per well and incubate at 37°C for an additional 24 hours. Initial seeding densities for slow-proliferating cell lines may need to be optimized.

6.1 Differentiation to Definitive Endoderm (DE)

6.1.1 Preparation of DE Medium

On **Day 0**, prepare the volume of DE Medium (STEMdiff™ Endoderm Basal Medium + STEMdiff™ Definitive Endoderm Supplement CJ) required for Day 0, Day 1, and Day 2 (1.7 mL/well). The following example is for preparing 2 mL of DE Medium. For other volumes, adjust accordingly.

1. Thaw Supplement CJ on ice. Mix thoroughly.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately. Do not re-freeze.

2. Thaw entire bottle of Endoderm Basal Medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.

   Note: If not used immediately, store at 2 - 8°C for up to 2 months. Alternatively, aliquot and store at -20°C. Do not exceed the expiry date as indicated on the label. After thawing aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

3. Add 20 µL of Supplement CJ to 1.98 mL of cold (2 - 8°C) Endoderm Basal Medium. Mix thoroughly. Store at 2 - 8°C.

6.1.2 Differentiation to DE

1. **Day 0**: Warm (37°C) the volume of DE Medium required for Day 0 (0.7 mL/well). Store remaining DE medium at 2 - 8°C.
2. Aspirate mTeSR™1 from hPSCs (prepared in section 5.0). Add 0.7 mL DE Medium per well dropwise down the side of the well of a tilted plate.
3. Incubate at 37°C with 5% CO_2 and 95% humidity for a maximum of 24 hours.
4. **Day 1**: Warm (37°C) the volume of DE Medium required for Day 1 use (0.5 mL/well). Store remaining DE medium at 2 - 8°C.
5. Aspirate medium from cells. Add 0.5 mL DE Medium per well dropwise down the side of the well of a tilted plate.
6. Incubate at 37°C with 5% CO_2 and 95% humidity for 24 hours.
7. **Day 2**: Warm (37°C) the remaining DE Medium.
8. Aspirate medium from the cells. Add 0.5 mL DE Medium per well dropwise down the side of the well of a tilted plate.
9. Incubate at 37°C with 5% CO_2 and 95% humidity for 24 hours.
10. **Day 3**: Cells are ready to be assayed for the formation of definitive endoderm (section 9.1). Replicate wells can be carried forward for differentiation into mid-/hindgut (section 6.2).

    Note: Cells will appear to undergo a significant amount of cell death during definitive endoderm induction. Minimize the time the cells spend outside of 37°C incubation as much as possible. After 24 hours of

definitive endoderm induction, cells are very sensitive and cultures require careful medium changes. After 72 hours of incubation, confluent monolayers of tightly packed endodermal cells will form.

6.2 Differentiation to Mid-/Hindgut (MH)

6.2.1 Preparation of MH Medium

On **Day 3**, prepare the volume of MH Medium (STEMdiff™ Endoderm Basal Medium + STEMdiff™ Gastrointestinal Supplement PK + STEMdiff™ Gastrointestinal Supplement UB) required for Day 3 - 8 (3 mL/well). The following example is for preparing 3 mL of MH Medium. For other volumes, adjust accordingly.

1. Thaw Supplements PK and UB on ice. Keep on ice until mixed with basal medium (step 2). Mix thoroughly.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplements. After thawing aliquots, use immediately. Do not re-freeze.

2. Add 30 µL of Supplement PK and 30 µL of Supplement UB to 2.94 mL of cold (2 - 8°C) Endoderm Basal Medium. Mix thoroughly. Store at 2 - 8°C.

6.2.2 Differentiation to MH

1. **Day 3**: Warm (15 - 25°C) a sufficient volume of MH Medium (0.5 mL/well). Store remaining MH Medium at 2 - 8°C.
2. Aspirate medium from cells (prepared in section 6.1.2) and replace with 0.5 mL of MH Medium.
3. Incubate at 37°C with 5% CO_2 and 95% humidity for 24 hours.
4. **Day 4 - 9**: Perform a full-medium change and assess spheroids every 24 hours as described below.

   Note: Ensure cultures are returned to the incubator within 30 minutes of being removed.

   a. Observe monolayer under a microscope. 3D structures may become visible as early as Day 4 of differentiation. Free-floating mid-/hindgut spheroids will appear at Day 5 - 9 of differentiation.
   b. Using a 1 mL pipettor, remove 0.5 mL medium from cells and transfer to a sterile 24-well clear flat-bottom plate to assess the number and concentration of lifted mid-/hindgut spheroids from the cell monolayer.
   c. Add 0.5 mL of fresh MH Medium to the cells. Incubate at 37°C with 5% CO2 and 95% humidity.

   Note: Although spheroids released on Day 5 - 9 are all capable of giving rise to small intestinal organoids, the amount of time that cells are exposed to mid-/hindgut medium will control the regional identity of the developing small intestinal organoids, e.g. duodenum (shorter exposure) or ileum (longer exposure).[2] The timing of the highest mid-/hindgut spheroid yields may vary with different hPSC lines. For reproducible experimental results, consistently harvest and initiate human intestinal organoid cultures using mid-/hindgut spheroids generated on the same day of differentiation. Day 8 is often when the most budding is observed.

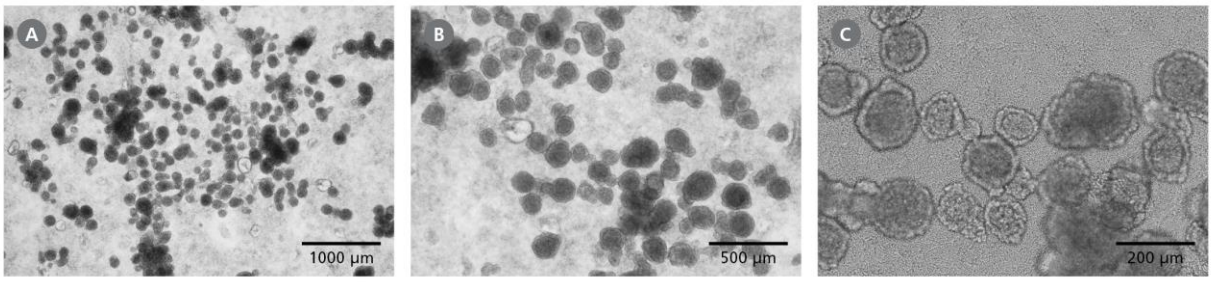


Figure 1. Mid-/Hindgut Cultures with Successful Spheroid Formation and Release

Representative images of Day 6 differentiation cultures demonstrate robust formation of mid-/hindgut spheroids, which bud off the cell monolayer into the culture medium. Image **(C)** shows typical spheroid morphologies of an outer polarized epithelium surrounding an inner cell mass. Magnification: 2X, 4X, and 10X (left to right).

5. **Day of spheroid embedding**: Using a pipettor, add spheroid suspension from each well to one well of a new 24-well plate for counting. Add an appropriate volume, corresponding to ~50 free-floating spheroids (based on prior spheroid counts), to a 15 mL conical tube. Proceed to section 7.0 for culture of intestinal organoids.

   Note: A mid-/hindgut spheroid is a cell aggregate measuring ≥ 75 µm in diameter with the potential to give rise to one human intestinal organoid. Multiple fused spheroids should be counted as one unit, which will generate one human intestinal organoid.

   Note: Remaining monolayer cultures can be assayed for the formation of mid-/hindgut (section 9.2) or further differentiated for additional embedding of spheroids on subsequent days.

7.0 Culture of Human Intestinal Organoids

7.1 Initiation of Human Intestinal Organoid Cultures

7.1.1 Preparation of STEMdiff™ Intestinal Organoid Growth Medium (OGM)

Prepare the volume of STEMdiff™ Intestinal OGM (STEMdiff™ Intestinal Organoid Basal Medium + STEMdiff™ Intestinal Organoid Supplement + L-Glutamine) required for 4 feeds (2 mL/well). The following example is for preparing 2 mL of STEMdiff™ Intestinal OGM. For other volumes, adjust accordingly.

1. Thaw Supplement on ice. Keep on ice until mixed with basal medium (step 2). Mix thoroughly.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplements. After thawing aliquots, use immediately. Do not re-freeze.

2. Add 40 µL of Intestinal Organoid Supplement and 20 µL of L-Glutamine to 1.94 mL of cold (2 - 8°C) Intestinal Organoid Basal Medium. Mix thoroughly. Warm to room temperature (15 - 25°C) before use. Store at 2 - 8°C for up to 2 weeks.

7.1.2 Embedding Spheroids in Matrigel® Domes

1. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free (Corning Catalog #356231) should be aliquoted (e.g. 200 µL per aliquot for 4 domes) and frozen.
2. Thaw an aliquot of Matrigel® (Corning Catalog #356231) on ice (50 µL of Matrigel® will be required per dome). Place a box of sterile 100 µL pipette tips at -20°C.
3. Once all spheroids (collected in section 6.2.2) have settled to the bottom of the conical tube, carefully aspirate and discard supernatant.
4. Add 1 mL of DMEM/F-12 with 15 mM HEPES to the spheroids. Centrifuge at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
5. Using a 1 mL pipettor, carefully remove and discard supernatant.

   Note: It is critical to remove as much supernatant as possible.

6. Remove the 100 µL pipette tips from the freezer and place in the biosafety cabinet.
7. Using a pipettor with a cold 100 µL pipette tip, add 50 µL of cold (2 - 8°C) Matrigel® to the tube. Gently distribute spheroids into the Matrigel® by pipetting up and down ~ 5 times.

   Note: Do not completely empty the pipette tip, as this may introduce bubbles.

8. Using the same pipette tip, gently transfer embedded spheroids into the center of one well of a Nunclon® Delta surface treated 24-well tissue culture dish as follows:
   a. Hold the pipettor vertically over the center of the well. Bring the pipette tip near to but not in contact with the floor of the well.
   b. Slightly depress the plunger until a droplet is visible on the end of the pipette tip.
   c. Slowly lower the pipettor until the droplet touches the floor of the well.
   d. Gently dispense (only to the first stop) the remaining volume while lifting the pipettor vertically and away from the well.
   e. Place the lid on the dish.

   Note: Work quickly to prevent gelling of Matrigel®; however, dispensing Matrigel® too quickly into the culture dish will flatten the dome.

9. Incubate cultures at room temperature for 20 minutes to allow Matrigel® to solidify.

   Note: Alternatively, incubate cultures at room temperature for 5 minutes to ensure the domes harden, then carefully transfer plates into a 37°C incubator for an additional 10 minutes for domes to fully solidify.

10. Warm (15 - 25°C) a sufficient volume of STEMdiff™ Intestinal OGM (0.5 mL/well). Store remaining medium at 2 - 8°C.
11. Add 0.5 mL of STEMdiff™ Intestinal OGM carefully to the side of the well (avoid disturbing the dome). Place the lid on the dish. Incubate at 37°C with 5% CO_2 and 95% humidity.
12. Perform a full-medium change every 3 - 4 days by removing medium then adding fresh medium as described in steps 10 - 11. Incubate at 37°C with 5% CO_2 and 95% humidity.
13. After 7 - 10 days of incubation, proceed to section 7.2 for passaging.

7.2 Passaging Human Intestinal Organoids

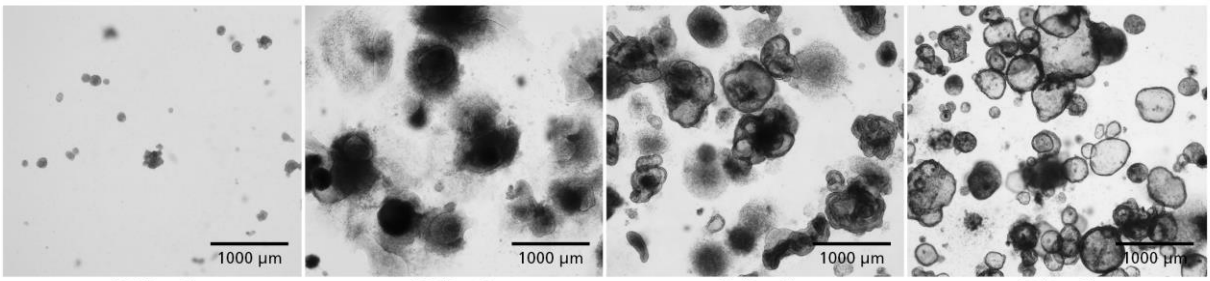


Figure 2. Generation of Human Intestinal Organoids Using STEMdiff™ Intestinal Organoid Kit
Embedded mid-/hindgut spheroids cultured in STEMdiff™ Intestinal OGM mature into intestinal organoids by passaging every 7 - 10 days. Passage number, as well as days post embedding in the passage (parentheses), are indicated below each image. Magnification: 2X.

1. Prepare a volume of STEMdiff™ Intestinal OGM (section 7.1.1) sufficient for 4 feeds (2 mL/well).
2. Thaw an aliquot of Matrigel® (Corning Catalog #356231) on ice (50 µL of Matrigel® will be required per dome). Place a box of sterile 100 µL pipette tips at -20°C. Place DMEM/F-12 with 15 mM HEPES on ice.
3. Pre-wet 15 mL conical tubes (1 per dome) as follows:
   a. Add 1 - 2 mL of Anti-Adherence Rinsing Solution to the tube. Swirl to coat the tube. Remove solution from the tube.
   b. Add 5 mL of D-PBS (Without Ca++ and Mg++) to the tube. Swirl to rinse the tube.
   c. Aspirate as much buffer from the tube as possible.
   d. Cap all coated tubes tightly and store at room temperature (15 - 25°C) until required.
4. Aspirate medium from the domes. Using a 1 mL pipettor, add 1 mL of cold DMEM/F-12 to the dome. Pipette directly onto the dome 5 - 6 times until it detaches from the plate.
5. Using the same 1 mL pipettor, gently pipette the released (or broken) Matrigel® dome up and down, then transfer the suspension to a pre-wetted 15 mL conical tube.
6. Add an additional 1 mL of cold DMEM/F-12 to the well and pipette up and down to harvest any remaining organoids. Transfer to the 15 mL conical tube.

   Note: Confirm successful harvest of organoids by visual inspection of the well under the microscope. If there are residual organoids in the well, repeat step 6.

7. Using a 1 mL pipettor, pipette the suspension up and down to break up organoids until a homogeneous fragment suspension with the desired fragment size (e.g. 100 - 500 µm) has been generated, approximately 5 - 10 times.

   Note: If organoids are large, a 200 µL pipettor may aid in further breaking them up. Fragments should fit through a 200 µL pipette tip. Avoid breaking up fragments into single cells with extended pipetting.

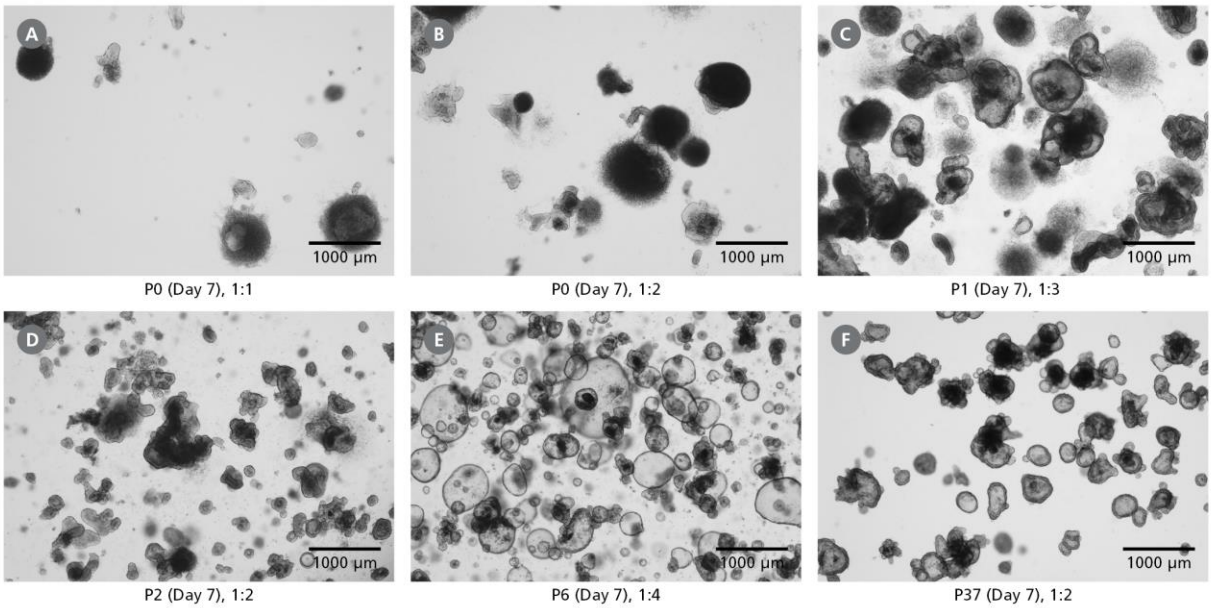


Figure 3. Passaging Organoid Cultures
Representative organoid images on the day of passage. Passage number, days post-embedding in the passage (parentheses), and recommended split ratio are indicated below each image. Magnification: 2X.

8. Determine the desired organoid density based on fragment counts or the split ratio. Split the extra organoid suspension into another pre-wetted 15 mL conical tube or discard.

   Note: Each organoid fragment can give rise to a new intestinal organoid. A density of 40 - 80 intestinal organoids per dome is optimal.

9. Shake the tube. Incubate on ice until organoid fragments have settled by gravity to the bottom of the tube (approximately 5 minutes). Immediately after all fragments have settled to the bottom, proceed to step 10.

   Note: Larger volumes may require a longer incubation time, to ensure that all fragments have settled to the bottom of the tube.

10. Using a pipettor, carefully remove as much supernatant as possible, leaving < 200 µL of DMEM/F-12 containing organoid fragments at the bottom of the tube. Discard supernatant (contains single cells).

    Note: Large organoid fragments should have settled to the bottom of the tube. In early passages, a cloudy phase may be visible (containing Matrigel® and single cells) above the organoid fragment pellet. Use a pipettor to carefully remove this cloudy phase without removing any organoid fragments.

11. Add 2 mL of fresh, cold DMEM/F-12 by directly pipetting onto the pellet.

12. Centrifuge at 200 x *g* for 5 minutes at room temperature (15 - 25°C). Carefully remove and discard supernatant.

    Note: It is critical to remove as much supernatant as possible, without removing any organoid fragments. If this is difficult to achieve due to a loose fragment pellet, remove as much supernatant as possible without disturbing the loose pellet, then repeat steps 11 - 12 up to two times.

13. Remove the 100 µL pipette tips from the freezer and place in the biosafety cabinet.
14. Keep the Matrigel® on ice and within reach, to ensure that it remains cold throughout the protocol.
15. Using a pipettor with a cold 100 µL pipette tip, add 50 µL of cold (2 - 8°C) Matrigel® to the organoid pellet. Gently distribute organoid fragments into the Matrigel® by pipetting up and down several times.

    Note: Do not completely empty the pipette tip, as this may introduce bubbles.
16. Using the same pipette tip, gently transfer embedded spheroids into the center of one well of a Nunclon® Delta surface treated 24-well tissue culture dish as follows:
    a. Hold the pipettor vertically over the center of the well. Bring the pipette tip near to but not in contact with the floor of the well.
    b. Slightly depress the plunger until a droplet is visible on the end of the pipette tip.
    c. Slowly lower the pipettor until the droplet touches the floor of the well.
    d. Gently dispense (only to the first stop) the remaining volume while lifting the pipettor vertically away from the well.
    e. Place the lid on the dish.

    Note: Work quickly to prevent gelling of Matrigel®; however, dispensing Matrigel® too quickly into the culture dish will flatten the dome.
17. Incubate at room temperature for 20 minutes to allow Matrigel® to solidify.

    Note: Alternatively, incubate cultures at room temperature for 5 minutes to ensure the domes harden, then carefully transfer plates into a 37°C incubator for an additional 10 minutes for domes to fully solidify.
18. Warm (15 - 25°C) a sufficient volume of STEMdiff™ Intestinal OGM (0.5 mL/well). Store remaining medium at 2 - 8°C.
19. Add 0.5 mL of STEMdiff™ Intestinal OGM carefully to the side of the well (avoid disturbing the dome). Place the lid on the plate and incubate at 37°C with 5% CO_2 and 95% humidity.
20. Perform a full-medium change bi-weekly (every 3 - 4 days) by removing medium and following steps 18 - 19.

    Note: Cultures with organoid densities higher than 40 - 80 intestinal organoids per dome may require medium changes every other day.
21. Passage organoid cultures every 7 - 10 days, depending on organoid density, size, and morphology.

8.0 Cryopreservation of PSC-Derived Human Intestinal Organoids Using CryoStor® CS10

Human PSC-derived intestinal organoids can be cryopreserved as early as passage 3 once they are fully established and display mature, budding or cystic morphologies. To achieve best results, organoids should be cultured for at least 7 days but not longer than 10 days in STEMdiff™ Intestinal OGM before cryopreservation. High-quality organoids are required for optimal freezing and recovery. If organoids have been thawed, they should be passaged at least twice before re-freezing.

8.1.1 Cryopreserving Organoids

Note: It is critical to pre-wet any pipette tip or tube with Anti-Adherence Rinsing Solution to minimize loss of organoid fragments due to adherence to plasticware. Up to 7 organoid-containing domes can be combined into a single 15 mL conical tube. Volumes indicated are for harvesting organoids from one well of a 24-well plate. If pooling multiple wells, adjust volumes accordingly.

1. Aspirate medium from dome cultures. Using a 1 mL pipettor, add 1 mL of cold DMEM/F-12 to the dome containing organoids. Pipette directly onto the dome 5 - 6 times until it detaches from the plate.
2. Using the same 1 mL pipettor, gently pipette the released (or broken) Matrigel® dome up and down, then transfer the suspension to a pre-wetted 15 mL conical tube.
3. Add an additional 1 mL of cold DMEM/F-12 to the well and pipette up and down to harvest any remaining organoids. Transfer to the 15 mL conical tube.

   Note: Confirm successful harvest of organoids by visual inspection of the organoid culture well under the microscope. If there are residual organoids in the well, repeat step 3.
4. Using a 1 mL pipettor, pipette the suspension up and down to break up organoids until a homogeneous fragment suspension with the desired fragment size (e.g. 200 - 500 µm) has been generated, approximately 5 - 10 times.

   Note: If organoids are large, a 200 µL pipettor may aid in further breaking them up. Fragments should fit through a 200 µL pipette tip. Avoid breaking up fragments into single cells with extended pipetting.
5. Shake the tube. Incubate on ice until organoid fragments have settled by gravity to the bottom of the tube (approximately 5 minutes). Immediately after all fragments have settled to the bottom, proceed to step 6.

   Note: A longer incubation time may be required to ensure that all organoid fragments have settled to the bottom of the tube.
6. Using a pipettor, carefully remove as much supernatant as possible, leaving < 200 µL of DMEM/F-12 containing organoid fragments at the bottom of the tube. Discard supernatant (contains single cells).

   Note: Large organoid fragments should have settled to the bottom of the tube. In early passages, a cloudy phase may be visible (containing Matrigel® and single cells) above the organoid fragment pellet. Use a pipettor to carefully remove this cloudy phase without removing any organoid fragments.
7. Add 2 mL of fresh, cold DMEM/F-12 by directly pipetting onto the pellet.
8. Repeat steps 5 - 7, then proceed to step 9.
9. Add 1 mL of fresh, cold DMEM/F-12 by directly pipetting onto the pellet. Shake the tube until an evenly distributed organoid fragment suspension has been generated.

   Note: Avoid breaking up organoids further by pipetting up and down.
10. Immediately transfer 5 µL of organoid fragment suspension into one well of a flat-bottom 96-well plate containing 50 µL D-PBS (Without Ca++ and Mg++). Count the total number of organoid fragments in the well. Keep the 15 mL tube containing the organoid suspension on ice.

11. Calculate the total number of organoid fragments in the suspension. We recommend cryopreserving a variety of fragment densities (e.g. 2000 - 8000 fragments per vial) for optimal long-term storage and recovery. For example, to calculate the volume of fragment suspension required for cryopreserving 2000 organoid fragments in one cryovial:

$$Volume\ of\ fragment\ suspension\ (\mu L) = 2000\ fragments \div \frac{Number\ of\ fragments\ in\ 5\ \mu L}{5\ \mu L}$$

12. Add the appropriate volume of fragment suspension to a new pre-wetted 15 mL conical tube.

    Note: Depending on the total fragment quantity, a multiple of the calculated fragment suspension volume can be added to a single 15 mL conical tube; this will be used for the same multiple number of cryovials (see step 14).

13. Centrifuge at 200 x *g* for 5 minutes at room temperature (15 - 25°C). Carefully remove and discard supernatant.

    Note: It is critical to remove as much supernatant as possible, without removing any fragments. If this is difficult to achieve due to a loose fragment pellet, remove as much supernatant as possible without disturbing the loose pellet, then add 1 mL of fresh, cold DMEM/F-12 by directly pipetting onto the pellet. Repeat step 13.

14. Add cold CryoStor® CS10 to the pellet; use 1 mL for each cryovial to be prepared. Gently distribute fragments by pipetting up and down several times.

15. Using a 1 mL pipettor, transfer 1 mL of fragment suspension into a pre-labeled cryovial. Place the cryovial into a freezing container.

    Note: If preparing multiple cryovials from one tube, constant shaking of the tube is required to achieve an even distribution of fragments across all cryovials.

16. Transfer freezing container into a -80°C freezer for at least 24 hours before moving the cryovials into long-term liquid nitrogen storage (-135°C).

8.1.2 Thawing Organoids

Note: It is critical to pre-wet any pipette tip or tube with Anti-Adherence Rinsing Solution to minimize loss of organoid fragments due to adherence to plasticware.

1. Remove the vial containing organoid fragments from the liquid nitrogen and place the vial immediately into a 37°C water bath.
2. Observe thawing process closely and retrieve cryovial from the water bath before fragments are completely thawed.

   Note: The thawing process should take no longer than 1 minute.

3. Using a 2 mL serological pipette, carefully wash the fragments with 2 mL of room temperature DMEM/F-12 to completely thaw the organoid fragment suspension.
4. Transfer the washed fragments to a 15 mL conical tube. Centrifuge at 200 x *g* for 5 minutes.
5. Carefully aspirate and discard the supernatant. Resuspend the fragments in 2 mL of DMEM/F-12.

   Note: Avoid breaking up fragments into single cells with extended pipetting.

6. Aliquot the fragment suspension into a new 15 mL conical tube so that each tube contains the desired number of organoid fragments for embedding. We recommend embedding 250 - 1000 fragments.

   Note: A higher density of fragments will require more frequent feeds and earlier passaging than lower density cultures.

7. Centrifuge at 200 x *g* for 5 minutes. Carefully aspirate and discard as much supernatant as possible.
8. Refer to section 7.2 steps 1 - 3 and 13 - 20 for embedding organoid fragments and maintenance.

9. Assess organoids every day after embedding to determine if passaging is required. Organoids during the thawed passage will appear smaller than organoids that have not been cryopreserved. Darkening of organoids will indicate they are ready for passaging; typically, this will occur 4 - 7 days after embedding.
10. After passaging the previously cryopreserved organoids, the passaging schedule will return to every 7 - 10 days, depending on organoid density, size, and morphology.

9.0 Characterization of Differentiation Cultures

9.1 Characterization of DE Cells

Purity of definitive endoderm cells can be measured by flow cytometry after labeling with the following antibodies:

- Anti-FOXA2 antibody

  e.g. Human HNF-3 beta/FoxA2 Alexa Fluor® 488-conjugated Antibody (R&D Systems Catalog #IC2400G)
- Anti-SOX17 antibody

  e.g. Human SOX17 APC-Conjugated Antibody (R&D Systems Catalog #IC1924A)
- Anti-CD184 (CXCR4) antibody

  e.g. PE Anti-Human CD184 (CXCR4) Antibody, Clone 12G5 (STEMCELL Catalog #60089)
- Anti-CD117 (C-KIT) antibody

  e.g. APC Anti-Human CD117 (c-kit) Antibody, Clone 104D2 (STEMCELL Catalog #60087)

Cells may also be assessed by immunocytochemistry after labeling with the following antibodies:

- Anti-FOXA2 antibody

  e.g. Mouse Anti-Human FoxA2 Antibody, clone N17-280 (BD Biosciences Catalog #561580)
- Anti-SOX17 antibody

  e.g. Goat Anti-Human SOX17 Antibody (R&D Systems Catalog #AF1924)

Results may vary depending on cell line used.

9.2 Characterization of MH Cells

Purity of mid-/hindgut cells can be measured by flow cytometry after labeling with the following antibodies:

- Anti-CDX2 antibody

  e.g. Alexa Fluor® 647 Mouse Anti-CDX-2, clone M39-711 (BD Biosciences Catalog #560395)
- Anti-SOX2 antibody

  e.g. PE Mouse Anti-Sox2, clone 245610 (BD Biosciences Catalog #560291)

Cells may also be assessed by immunocytochemistry after labeling with the following antibodies:

- Anti-CDX2 antibody

  e.g. Mouse CDX2 antibody, clone CDX2-88 (Biocare Medical Catalog #CM226A) or Rabbit CDX2 antibody, clone EPR2764Y (Thermo Fisher Catalog #MA5-14494)
- Anti-E-Cadherin antibody

  e.g. Purified Mouse Anti-E-Cadherin, clone 36/E-Cadherin (BD Biosciences Catalog #610182)
- Anti-Vimentin antibody

  e.g. Anti-Vimentin Antibody - Cytoskeleton Marker, clone EPR3776 (abcam Catalog #ab92547)

Results may vary depending on cell line used.

9.3 Characterization of Human Intestinal Organoids

Purity of Human Intestinal Organoid cultures can be assessed by immunocytochemistry after labeling with the following antibodies:

- Anti-CDX2 antibody

  e.g. Mouse CDX2 antibody, clone CDX2-88 (Biocare Medical Catalog #CM226A) or Rabbit CDX2 antibody, clone EPR2764Y (Thermo Fisher Catalog #MA5-14494)
- Anti-E-Cadherin antibody

  e.g. Purified Mouse Anti-E-Cadherin, clone 36/E-Cadherin (BD Biosciences Catalog #610182)
- Anti- EpCAM/TROP-1 antibody

  e.g. Human EpCAM/TROP-1 Antibody (R&D Systems Catalog #AF960)
- Anti-Mucin 2 antibody

  e.g. Purified Mouse Anti-Human MUC2, clone CCP58 (BD Biosciences Catalog #555926)
- Anti-Cytokeratin 20 (KRT20) antibody

  e.g. Monoclonal Mouse Anti-Human Cytokeratin 20, clone Ks20.8, Agilent Technologies (Catalog #M701929-2)
- Anti-Vimentin antibody

  e.g. Anti-Vimentin Antibody - Cytoskeleton Marker, clone EPR3776 (abcam Catalog #ab92547)
- Anti-Desmin antibody

  e.g. Anti-Desmin Antibody, clone Y66 – Cytoskeleton Marker (abcam Catalog #ab32362)
- Anti-Villin antibody

  e.g. Purified Mouse Anti-Human Villin, clone 12/Villin (BD Biosciences Catalog #610358)
- Anti-KLF5 antibody

  e.g. Anti-KLF5 Antibody (abcam Catalog #137676)
- Anti-SOX9 antibody

  e.g. Anti-Sox9 Antibody (Millipore Sigma Catalog #AB5535)
- Anti-Chromogranin A (CHGA) antibody

  e.g. Purified Mouse Anti-Human Chromogranin A, clone S21-537 (BD Biosciences Catalog #564562)

Results may vary depending on cell line used.

10.0 Troubleshooting

PROBLEM	POSSIBLE CAUSE	SOLUTION
Low efficiency of definitive endoderm induction	Low-quality hPSC starting culture	Ensure high quality of undifferentiated hPSCs starting culture by removing all hPSC colonies that have spontaneously differentiated from maintenance cultures (section 5.1 steps 3 - 4)
Cells lift off during definitive endoderm induction	• Cell density on Day 0 too high • Time between medium changes > 24 hours	• Start definitive endoderm differentiation earlier with an optimal cell density of 85 - 90% • Perform medium changes every ≤ 24 hours for the first 3 days of definitive endoderm induction
No spheroid formation during mid-/hindgut differentiation	Cell density on Day 3 too low	Seed 3 different cell clump densities (4000, 5000, and 6000) and start definitive endoderm induction of all three seeding densities on the same day, once one density has reached 85 - 90% confluence. Continue with mid-/hindgut induction of endoderm monolayers (Day 3), which show the most compact and overcrowded cell densities. Discontinue with all wells that don't have a confluent monolayer at the end of definitive endoderm induction (Day 3).
Uneven spheroid formation across the well during mid-/ hindgut induction	ES/iPS cells were not plated evenly	Ensure cell clumps are evenly distributed among each well by rocking plate in a back-and-forth and side-to-side motion a few times while plate is in the incubator (section 5.1 step 12)
Matrigel® dome collapses after plating onto cell culture dish	• Non-suitable cell culture plastic for Matrigel® dome cultures • Low protein content of Matrigel® lot • Diluted Matrigel® due to residual medium	• A Nunclon® Delta surface treated 24-well tissue culture dish is recommended for organoid dome cultures • Use Matrigel® lots with a minimum protein content of 8 mg/mL • Remove as much residual medium as possible from pelleted spheroids (section 7.1.2 step 5)
Spheroids sink to the bottom of the plastic and attach during embedding in Matrigel®	Diluted Matrigel® due to residual medium	Remove as much residual medium as possible from pelleted spheroids (section 7.1.2 step 5)
Spheroids clump together in Matrigel® domes	Matrigel®/spheroid suspension was not mixed sufficiently	Mix Matrigel®/spheroid suspension thoroughly by pipetting up and down several times, before formation of Matrigel® domes (section 7.1.2 step 7)
Organoids do not recover after passaging, or they grow slowly	• Organoids were broken up too much • Fragment seeding density was too low	• Generate larger organoid aggregates during mechanical breakup by pipetting up and down; optimal size is 100 - 500 µm per fragment (section 7.2 step 7) • Seed a greater number of fragments
Low quantities of differentiated intestinal cell types	Organoids were harvested too early	Keep organoids in culture for 2 weeks without passaging. Perform a full-medium change every 3 - 4 days using 0.5 mL of fresh STEMdiff™ Intestinal OGM. Extended organoid culture will enrich for differentiated intestinal cell lineages.

11.0 References

1. Spence JR et al. (2011) Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. Nature 470(7332): 105–9.
2. Tsai YH et al. (2017). In vitro patterning of pluripotent stem cell-derived intestine recapitulates in vivo human development. Development 144(6): 1045–55.

TECHNICAL MANUAL

Generation of Human Intestinal Organoids Using STEMdiff™ Intestinal Organoid Kit

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