TECHNICAL MANUAL

Generation of Human Gastric Organoids Using STEMdiff™ Gastric Organoid Differentiation 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. McCracken and colleagues have used these principles to develop a multi-stage, in vitro differentiation protocol for the generation of human gastric organoids [1]. We have developed STEMdiff™ Gastric Organoid Differentiation Kit by optimizing these protocols to increase efficiency and consistency across multiple human embryonic stem (ES) and induced pluripotent stem (iPS) cell lines. STEMdiff™ Gastric Organoid Differentiation Kit is a serum-free medium that supports differentiation of hPSCs through four distinct stages: definitive endoderm, posterior foregut, stomach patterning, and gastric organoid generation. Cells differentiated using this kit robustly form organoids composed of gastric epithelia and a surrounding, niche factor-producing mesenchyme. The epithelial and mesenchymal components express key markers of these respective gastric compartments. In addition to the differentiation workflow, we have optimized the medium formulation described by Bartfeld and colleagues [2] for long-term expansion and maintenance of hPSC-derived gastric organoids. Thus, the organoids generated using this kit can be either fully differentiated or maintained long-term and cryopreserved using STEMdiff™ Gastric Organoid Expansion Medium.

2.0 Materials, Reagents, and Equipment

2.1 STEMdiff™ Gastric Organoid Differentiation Kit and Expansion Medium

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

For component storage and stability information, refer to the Product Information Sheet (PIS) for STEMdiff™ Gastric Organoid Differentiation Kit, available at www.stemcell.com or contact us to request a copy.

PRODUCT	CATALOG #	COMPONENT NAME	COMPONENT #	SIZE
STEMdiff™ Gastric Organoid Differentiation Kit	100-0475	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™ Posterior Foregut Supplement	100-0477	0.64 mL
		STEMdiff™ Gastric Organoid Medium*	100-0478	100 mL
		STEMdiff™ Gastric Organoid Expansion Supplement	100-0479	1.1 mL
STEMdiff™ Gastric Organoid Expansion Medium	100-0490	STEMdiff™ Gastric Organoid Medium*	100-0478	100 mL
		STEMdiff™ Gastric Organoid Expansion Supplement	100-0479	1.1 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 OR mTeSR™ Plus	85850 OR 100-0276
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free (≥ 8 mg/mL protein)	Corning 356231
Gentle Cell Dissociation Reagent	07174
24-well tissue culture-treated flat-bottom plate	e.g. 38017 or 38021
Anti-Adherence Rinsing Solution	07010
Retinoic acid	e.g. 72262
DMEM/F-12 with 15 mM HEPES	36254
Microcentrifuge tubes, 1.7 mL	e.g. 38089
Cell scraper/lifter	e.g. 38065 OR 38067
D-PBS (Without Ca++ and Mg++)	37350
Conical tubes, 15 mL and 50 mL	e.g 38009 and 38010
CryoStor® CS10	07930

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

2.3 Equipment

- Biohazard safety 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)
- Pipettor 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 prepare 25 mL of diluted matrix. Always 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. If desired, the vial may be washed with cold medium.
4. Use the diluted Matrigel® solution immediately to coat tissue culture-treated cultureware. Refer to 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 Matrigel® Volume for Coating Cultureware

TISSUE CULTURE-TREATED CULTUREWARE	VOLUME OF DILUTED MATRIGEL®
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 Passaging hPSCs for Differentiation

The following protocols are for passaging human ES or iPS cells cultured in mTeSR™1 or mTeSR™ Plus 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 for mTeSR™1 or mTeSR™ Plus, available at www.stemcell.com or contact us to request a copy.

Passage cells using the clump-passaging method described below. 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.

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

   Note: Do not warm medium 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 for 6 - 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™, Catalog #100-0484); 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 or mTeSR™ Plus. 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 or mTeSR™ Plus 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 10 µL of clump suspension into one well of a flat-bottom 96-well plate containing 90 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 4000 clumps, as follows:

    $$Volume\ of\ clump\ suspension\ (\mu L) = 4000\ cell\ clumps\ \div \frac{Number\ of\ clumps\ in\ 10\ \mu L}{10\ \mu L}$$
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. Add 0.25 mL mTeSR™1 or mTeSR™ Plus per well.

13. Gently shake the tube to ensure cell aggregates are evenly distributed. Add the appropriate volume for 4000 clumps (calculated in step 11) to the 24-well plate prepared in step 12. If necessary, top up the well to 0.5 mL with medium. 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 plate is in the incubator. Do not disturb the plate for 24 hours.

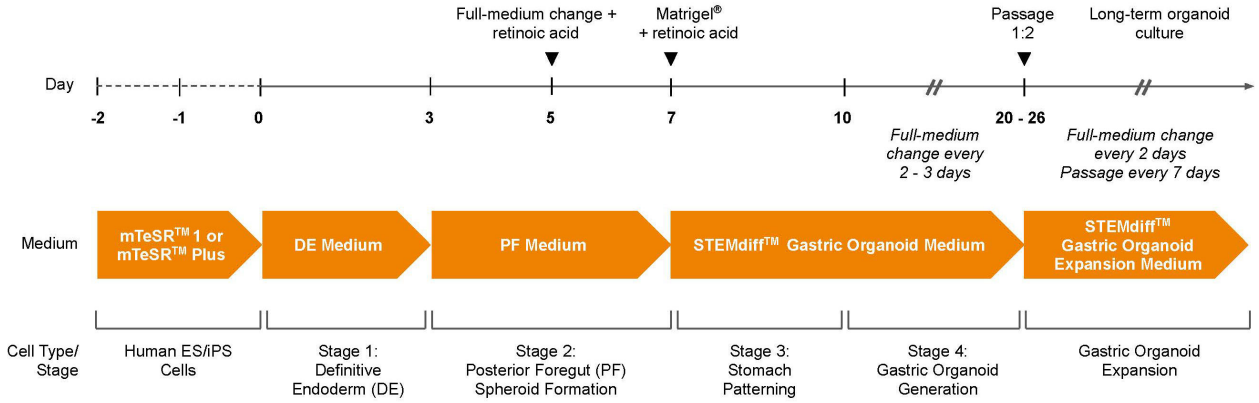
    Note: A starting seeding density of 4000 clumps has demonstrated reproducible results; however, an initial experiment is recommended to determine the optimal clump seeding density for the cell line being used. Seed a range of clump densities (e.g. 4000, 5000, and 6000 clumps per well), and initiate differentiation of each density on the same day.

14. Proceed to section 5.0 for differentiation.

5.0 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 4.0). 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 mL of fresh mTeSR™1 or mTeSR™ Plus (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.

5.1 Protocol Diagram



STEMdiff™ gastric organoid workflow in which hPSC cultures progress through a four-stage differentiation process using stage-specific, specialized media to generate and fully differentiate human gastric organoids that can be expanded long term and cryopreserved.

Endoderm differentiation is initiated on Day 0. By Day 3 of the protocol, cultures display characteristics typical of definitive endoderm (DE), and posterior foregut (PF) differentiation is initiated. During PF differentiation, cells form spheroids that are released from the monolayer into the culture medium (Day 7). These spheroids are collected, embedded in Corning® Matrigel®, and cultured in STEMdiff™ Gastric Organoid Medium to pattern and mature into gastric organoids. Organoids can be fully differentiated if reseeded in STEMdiff™ Gastric Organoid Medium, or repeatedly passaged to maintain cultures and then cryopreserved if cultured in STEMdiff™ Gastric Organoid Expansion Medium.

5.2 Preparation of Media

Use sterile technique to prepare media for differentiation. There are two medium formulations required; prepare DE Medium on **Day 0**, and PF Medium on **Day 3**, as indicated in the protocols. Refer to Table 2 for medium components, volumes, and preparation & storage. Volumes indicated are for one well of a 24-well plate, for supporting 3 days of differentiation for Stage 1 and 4 days of differentiation for Stage 2; if preparing other volumes, adjust accordingly.

Table 2. Preparation of Media for Differentiation

MEDIUM	COMPONENT	VOLUME	PREPARATION & STORAGE
Day 0			
DE Medium (1.5 mL)	STEMdiff™ Endoderm Basal Medium	1.5 mL	Mix thoroughly. If not used immediately, store at 2 - 8°C for up to 1 week.
	STEMdiff™ Definitive Endoderm Supplement CJ	15 µL	
Day 3			
PF Medium (2 mL)	STEMdiff™ Endoderm Basal Medium	2 mL	Mix thoroughly. Store at 2 - 8°C.
	STEMdiff™ Gastrointestinal Supplement PK	20 µL	
	STEMdiff™ Posterior Foregut Supplement	20 µL	

5.3 Stage 1: Differentiation to Definitive Endoderm (DE)

1. **Day 0**: Prepare the volume of DE Medium required for Day 0, Day 1, and Day 2 (1.5 mL/well), as follows:
   a. 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.
   b. Thaw the 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.
   c. Combine components as indicated in Table 2.
2. Aliquot the volume of DE Medium required for Day 0 use (0.5 mL/well) and warm to room temperature. Store remaining DE Medium at 2 - 8°C.
3. Aspirate medium from hPSCs (prepared in section 4.0). Add 0.5 mL DE Medium per well dropwise down the side of the well of a tilted plate.
4. Incubate at 37°C with 5% CO_2 and 95% humidity for a maximum of 24 hours.
5. **Day 1**: Aliquot the volume of DE Medium required for Day 1 use (0.5 mL/well) and warm to room temperature. Store remaining DE medium at 2 - 8°C.
6. Aspirate medium from cells. Add 0.5 mL DE Medium per well dropwise down the side of the well of a tilted plate.
7. Incubate at 37°C with 5% CO_2 and 95% humidity for 24 hours.
8. **Day 2**: Warm the remaining DE Medium to room temperature.
9. Aspirate medium from the cells. Add 0.5 mL DE Medium per well dropwise down the side of the well of a tilted plate.

10. Incubate at 37°C with 5% CO_2 and 95% humidity for 24 hours.
11. **Day 3**: Cells have formed a confluent monolayer and are ready to be assayed for the formation of definitive endoderm (section 10.1). Replicate wells can be carried forward for differentiation into posterior foregut (section 5.4).

    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. A high number of cells in the wells at this stage induces quick consumption of medium nutrients. To ensure optimal differentiation yield, perform medium changes within or close to 24 hours. For optimal performance, do not exceed this time. After 72 hours of incubation, confluent monolayers of tightly packed endodermal cells will form.

5.4 Stage 2: Differentiation to Posterior Foregut (PF)

1. **Day 3**: Prepare the volume of PF Medium required for Day 3 - Day 7 (0.5 mL/well), as follows:
   a. Thaw Supplement PK and Posterior Foregut Supplement on ice. 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.

   b. Combine components as indicated in Table 2.

      Note: On Day 5, 2 µM retinoic acid must be added to the remaining PF Medium (step 5c).
2. **Day 3 - 4**: Aliquot a sufficient volume of PF Medium (0.5 mL/well) and warm to room temperature (15 - 25°C). Store remaining PF Medium at 2 - 8°C.
3. Aspirate medium from cells (prepared in section 5.3) and replace with 0.5 mL of PF Medium.
4. Incubate at 37°C with 5% CO_2 and 95% humidity for 24 hours.
5. **Day 5**: Perform a full-medium change as follows:

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

   a. Observe monolayer under a microscope. Three-dimensional (3D) structures may be visible as early as Day 4, but will be even more visible by Day 5 of differentiation. Free-floating PF spheroids will appear on Day 5 - 7 of differentiation.

      Note: If spheroids are formed between Day 4 and 5, they will be floating on top of the monolayer. Since they are not correctly patterned as PF spheroids yet, they can be discarded by performing a medium change.

   b. Aliquot a sufficient volume of PF Medium (1 mL/well) and warm to room temperature. Store remaining PF Medium at 2 - 8°C.
   c. Add 2 µM retinoic acid (RA) to PF Medium. Mix thoroughly.

      Note: RA is light sensitive; to minimize light exposure, work in a biosafety cabinet with the light off when adding RA to the medium and adding the medium to the culture plate.

   d. Aspirate medium from cells. Add 1 mL of PF Medium + RA.
   e. Incubate at 37°C with 5% CO_2 and 95% humidity for 48 hours. Observe monolayer under a microscope daily. Free-floating PF spheroids will appear on Day 6 - 7.
6. Place a 24-well tissue culture-treated plate in the 37°C incubator. This will be required on Day 7 (section 6.1.3).
7. **Day 7**: Proceed to section 6.0 to collect and assess spheroids.

   Note: If there are no free-floating spheroids, but the monolayer shows clear formation of ridges, perform a full-medium change as described in steps 5b - e, then incubate at 37°C for an additional 1 - 2 days. If there are no spheroids or ridges visible, terminate the experiment.

6.0 Generation of Human Gastric Organoids

The following protocols are for collecting spheroids (generated in section 5.4), embedding spheroids in Matrigel® domes (**Day 7**), and maturation of spheroids to gastric organoids (**Day 10** to **Day 20 - 26**).

6.1 Stage 3: Patterning and Generation of Human Gastric Organoid Cultures

6.1.1 Setup

Perform the following tasks at least one hour before embedding spheroids in Matrigel® domes:

- Place a sufficient volume of Matrigel® (Corning Catalog #356231) on ice (50 µL/dome)
- Place a box of 200 µL pipette tips at -20°C

6.1.2 Spheroid Collection and Counting

1. On **Day 7**, coat a sterile 24-well clear flat-bottom plate (not pre-warmed) with 0.25 mL/well Anti-Adherence Rinsing Solution. Rinse each well with sterile 0.5 mL DMEM/F-12 with 15 mM HEPES.
2. Using a 1 mL pipettor, transfer the free-floating spheroids (generated between Days 6 - 7 in section 5.4) and the medium from each well of the culture plate into individual wells of the 24-well plate prepared in step 1. Do not aspirate the monolayer. Discard the plate containing the monolayer.

   Note: Some spheroids might still be attached to the monolayer. For optimal collection from the culture plate, slowly pipette the medium on the wall of the culture well a few times to completely detach the spheroids from the monolayer. Try not to aspirate or disturb the monolayer.

   Note: If a number of spheroids are still attached to the monolayer, differentiation to PF (Stage 2) can be extended by performing a full-medium change as described in section 5.4 steps 5b - e, then incubate at 37°C for an additional 1 - 2 days.

   a. Count the number of lifted spheroids per well by inspecting the plate under the microscope.
   b. Coat sterile 15 mL conical tubes with 2 mL Anti-Adherence Rinsing Solution per tube. Rinse each tube with 2 mL of sterile DMEM/F-12 with 15 mM HEPES.
   c. After counting, collect spheroids from the counting plate and transfer to a tube prepared in step b. If necessary, pool wells to obtain > 50 spheroids per tube.

Note: The number of spheroids may range from dozens to several hundred per well. It is recommended to pool the wells if the number of spheroids per well is < 50. From this step onward, there is sufficient medium included in the differentiation kit to support full differentiation (Day 34) in up to 14 wells, or for organoid maturation (Day 26) and establishment of organoid culture in Expansion Medium (P1) in up to 16 wells.

Note: A PF spheroid is a cell aggregate with the potential to give rise to one human gastric organoid. Multiple fused spheroids should be counted as one unit, which will generate one human gastric organoid. Spheroids will typically have the shape of a sphere. In most of the spheroids, an epithelium surrounding a central cell mass can be recognized (refer to Figure 1).

Note: Spheroids can be cryopreserved as organoid progenitors in CryoStor® CS10; refer to section 9.0.

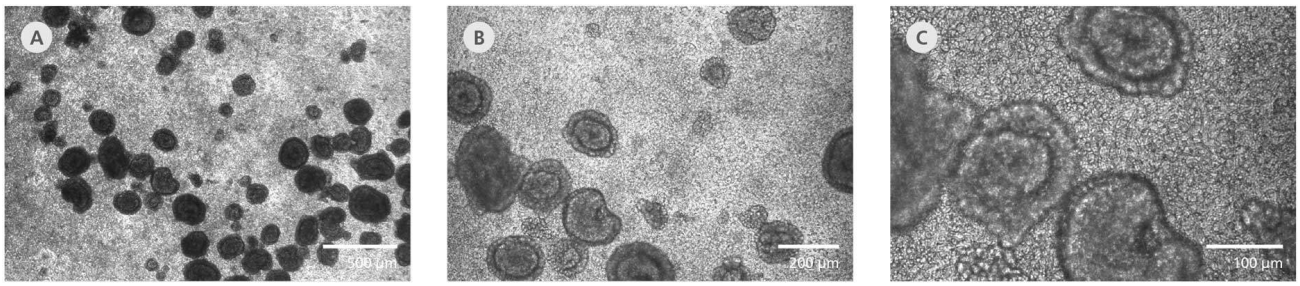


Figure 1. Posterior Foregut (PF) Cultures with Successful Spheroid Formation and Release

(A-B) Representative images of Day 7 differentiation cultures demonstrate robust formation of PF spheroids, which bud off the cell monolayer into the culture medium. **(C)** Typical spheroid morphologies of an outer polarized epithelium surrounding an inner cell mass. Magnifications: 5X, 10X, and 20X (left to right).

3. Thaw the entire bottle of STEMdiff™ Gastric Organoid Medium at room temperature (15 - 25°C). Alternatively, place at 2 - 8°C overnight on Day 6. Mix thoroughly. Warm to room temperature before use.

   Note: If not used immediately, store the volume required for up to five medium changes (1.5 mL/well) at 2 - 8°C for up to 2 weeks. Aliquot and store the remaining medium 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 1 week. Do not re-freeze.

6.1.3 Embedding Spheroids in Matrigel® Domes

1. Once all spheroids (collected in section 6.1.2) have settled to the bottom of the conical tube by gravity, carefully aspirate and discard supernatant.

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

2. Remove the 200 µL pipette tips from the freezer and place in the biosafety cabinet. Remove the 24-well plate from the incubator (from section 5.4 step 6).

3. Using a pipettor with a cold 200 µL pipette tip, add cold Matrigel® to the tube as indicated in Table 3. 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.

   Table 3. Volume of Matrigel® for Embedding Spheroids

NUMBER OF SPHEROIDS	VOLUME OF MATRIGEL® (µL)	NUMBER OF DOMES
50 - 100	50	1
101 - 150	100	2
151 - 200	150	3
201 - 250	200	4
251 - 300	250	5

4. Using the same pipette tip, gently transfer 50 µL of embedded spheroids into the center of one well of the warm 24-well plate, as described below.

   Note: Work quickly to prevent gelling of Matrigel®.

   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 away from the well.

   Note: Dispensing Matrigel® too quickly will flatten the dome.

e. Repeat step 4 for remaining wells.

5. Place the lid on the dish. Incubate at 37°C with 5% CO_2 and 95% humidity for 15 - 25 minutes to allow Matrigel® to solidify.

   Note: Expect a lot of mesenchyme to grow at the base of the Matrigel® dome and attach to the plate. To minimize this, and to obtain cultures more enriched with epithelial organoid structures, invert the plate after 2 minutes.

6. Aliquot a sufficient volume of STEMdiff™ Gastric Organoid Medium (0.5 mL/well) and warm to room temperature. Add 2 µM retinoic acid (RA). Mix thoroughly.

   Note: RA is only required at the embedding stage, and not for subsequent medium changes with STEMdiff™ Gastric Organoid Medium.

   Note: RA is light sensitive; to minimize light exposure, work in a biosafety cabinet with the light off when adding RA to the medium and adding the medium to the culture plate.

7. Add 0.5 mL of STEMdiff™ Gastric Organoid Medium + RA carefully to the side of the well (avoid disturbing the dome; remember to work in the dark). Place the lid on the dish. Incubate at 37°C with 5% CO_2 and 95% humidity for 3 days.
8. On **Day 10**, perform a full-medium change as described in section 6.2.

6.2 Stage 4: Maturation of Spheroids to Gastric Organoids

On Day 10, and every 2 - 3 days until Day 20 - 26, perform a full-medium change as described below. Refer to Figure 2 for representative images of gastric organoids at Days 10, 20, and 26.

1. Aliquot a sufficient volume of STEMdiff™ Gastric Organoid Medium (without RA) (0.5 mL/well) and warm to room temperature (15 - 25°C).

   Note: If using a frozen aliquot of medium (frozen on Day 7), thaw at room temperature. Alternatively, on the day before the medium change, thaw overnight at 2 - 8°C. After thawing aliquots, use immediately or store at 2 - 8°C for up to 1 week. Do not re-freeze.

2. Remove medium from the well. Add 0.5 mL STEMdiff™ Gastric Organoid Medium carefully to the side of the well. Incubate at 37°C with 5% CO_2 and 95% humidity.
3. On **Day 20 - 26**, proceed to section 7.0 for gastric organoid differentiation or section 8.0 for expansion of gastric organoid cultures.

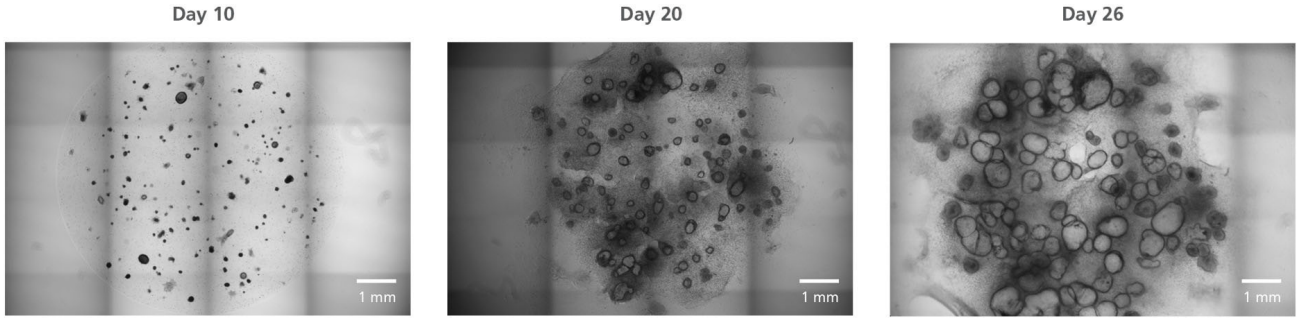


Figure 2. Generation of Human Gastric Organoids Using STEMdiff™ Gastric Organoid Differentiation Kit

Embedded PF spheroids cultured in STEMdiff™ Gastric Organoid Medium mature into gastric organoids surrounded by mesenchyme. Stage and day of the protocol (parentheses) are indicated below each image. Magnification: 5X.

7.0 Gastric Organoid Differentiation

Perform the following protocol once the majority of gastric organoids (generated in section 6.2) show a cystic morphology and a clear lumen formation (Figure 2c) - no earlier than **Day 20** and no later than **Day 26**.

Note: This protocol is designed to reduce the density of the gastric organoids in order for them to grow larger and continue to develop and differentiate. It is recommended to reduce the number of organoids to ~5 - 10 per well and to reduce the amount of mesenchyme in the cultures to allow for enrichment of the epithelial tissue.

7.1 Setup

Perform the following tasks at least one hour before passaging:

- Place a vial of Matrigel® (Corning Catalog #356231) on ice (50 µL/dome)
- Place a box of 200 µL pipette tips at -20°C
- Place a bottle of DMEM/F-12 with 15 mM HEPES on ice
- Place a 24-well tissue culture-treated plate in the 37°C incubator (overnight or longer incubation is optimal)

7.2 Passaging Gastric Organoids for Differentiation

1. **Day 20 - 26**: Aspirate medium from the dome cultures.
2. Using a 1 mL pipettor, add 1 mL of cold DMEM/F-12 with 15 mM HEPES to the dome containing organoids. Detach the Matrigel® dome from the bottom of the well by pushing it with the pipette tip.
3. Using a cut 200 µL pipette tip, vigorously pipette up and down in order to break up the dome and separate the organoids, but do not break the organoids into fragments. Using a 1 mL pipettor, transfer the suspension to one well of a sterile 6-well plate.

   Note: If mesenchyme is tightly attached to the organoids, try to remove it by microdissection. Try not to remove it entirely, as mesenchyme is still required to support full differentiation of the organoids (Figure 3).
4. Add an additional 2 mL DMEM/F-12 with 15 mM HEPES to the well, then gently swirl the plate for ~2 minutes. This should encourage the desired organoids to move toward the centre of the well, and the less-desired mesenchyme to move toward the edges.
5. Using a cut 200 µL pipette tip, select 5 - 10 organoids and place them into a sterile 1.7 mL micro-centrifuge tube. Repeat for as many wells required.
6. Using a 200 µL pipettor, carefully remove and discard the supernatant.
7. Remove the 200 µL pipette tips from the freezer and place in the biosafety cabinet.
8. Once all organoids have settled to the bottom of the tube by gravity, carefully aspirate and discard supernatant.

   Note: It is critical to remove as much supernatant as possible.
9. Remove the 24-well plate from the incubator.

Perform steps 10 - 11 quickly to prevent gelling of Matrigel®:

10. Using a pipettor with a cold and cut 200 µL pipette tip, add 50 µL of cold Matrigel® to the organoid pellet. Gently distribute organoids into the Matrigel® by pipetting up and down several times.

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

11. Using the same pipette tip, gently transfer embedded organoids into the center of one well of the warm 24-well plate 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 away from the well.

       Note: Dispensing Matrigel® too quickly will flatten the dome.
    e. Repeat steps 10 - 11 for the remaining wells.
12. Place the lid on the dish. Incubate cultures at room temperature (15 - 25°C) for 15 - 25 minutes to allow Matrigel® to solidify.
13. Aliquot a sufficient volume of STEMdiff™ Gastric Organoid Medium (0.5 mL/well) and warm to room temperature.
14. Add 0.5 mL of STEMdiff™ Gastric Organoid Medium 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.
15. Perform a full-medium change every 2 days by removing medium then adding fresh STEMdiff™ Gastric Organoid Medium as described in steps 10 - 11. Incubate at 37°C with 5% CO_2 and 95% humidity.
16. On **Day 34**, organoids are ready for downstream assays and analysis.

    Note: On Day 34, gastric organoids can be passaged into a new Matrigel® dome as single organoids (as described in section 7.0) to continue differentiation and volume expansion (refer to Figure 3c & 3d). Use cut 1 mL pipette tips only.

    Note: Gastric organoids can also be passaged in STEMdiff™ Gastric Organoid Expansion Medium by mechanical dissociation, as described in section 8.0.

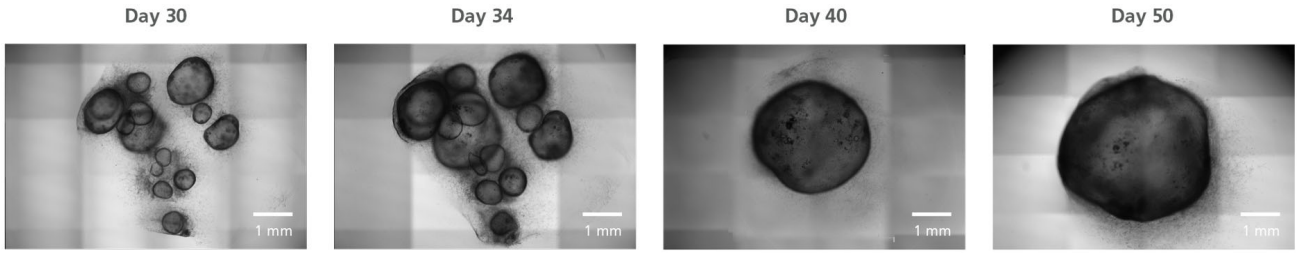


Figure 3. Differentiation of Human Gastric Organoids Using STEMdiff™ Gastric Organoid Differentiation Kit

Gastric organoids fully differentiate in STEMdiff™ Gastric Organoid Medium. Differentiated organoids will show a characteristic cystic morphology surrounded by mesenchymal cells. On Day 34, it will also be possible to observe formation of buds in the epithelium facing the lumen of the organoid. Stage and days of the protocol (parentheses) are indicated below each image. Magnification: 5X.

8.0 Gastric Organoid Expansion

Gastric organoids (generated in section 6.2) should be passaged for expansion once the majority of organoids show a cystic morphology and a clear lumen formation (Figure 2c), and no earlier than **Day 20**.

Note: Optimal performance of organoid expansion has been obtained by passaging organoids no later than Day 26. However, organoids retain the potential to give rise to expansion cultures after Day 30 of differentiation.

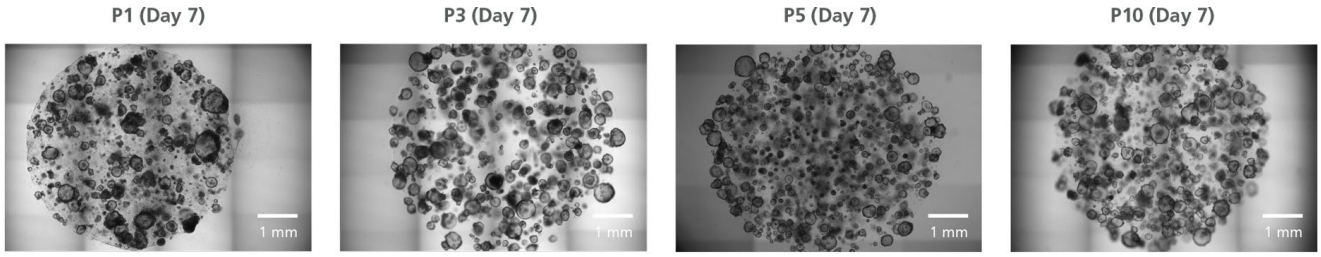


Figure 4. Generation of Human Gastric Organoids Using STEMdiff™ Gastric Organoid Differentiation Kit

Embedded posterior foregut spheroids cultured in STEMdiff™ Gastric Organoid Medium mature into gastric organoids. On Day 20 - 26 of maturation, organoids can be repeatedly passaged every 7 days into STEMdiff™ Gastric Organoid Expansion Medium. At the end of each passage, the Matrigel® dome should contain multiple organoids that enrich in epithelial components with passaging progression, while losing the mesenchyme characteristic of early passages. Passage number, as well as days post-embedding in the passage (parentheses), are indicated below each image. Magnification: 5X.

8.1 Preparation of Media

1. On the day of passaging, when organoids have matured (refer to Figure 4), use sterile technique to prepare STEMdiff™ Gastric Organoid Expansion Medium, as follows:
   a. Thaw STEMdiff™ Gastric Organoid Expansion Supplement 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.
   b. Thaw an aliquot of STEMdiff™ Gastric Organoid Medium (previously frozen on Day 7) at room temperature (15 - 25°C). Alternatively, thaw the entire bottle of STEMdiff™ Gastric Organoid Medium (if starting with a new STEMdiff™ Gastric Organoid Expansion Kit) at room temperature or overnight at 2 - 8°C. Mix thoroughly.

      Note: If the aliquot is not used immediately, store at 2 - 8°C for up to 2 weeks. If a new bottle of STEMdiff™ Gastric Organoid Medium is opened, 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 1 week. Do not re-freeze.
   c. Combine components as indicated in Table 4. Volumes indicated are sufficient for three medium changes for one well; if preparing other volumes, adjust accordingly.

Table 4. Preparation of STEMdiff™ Gastric Organoid Expansion Medium

MEDIUM	COMPONENT	VOLUME	PREPARATION & STORAGE
STEMdiff™ Gastric Organoid Expansion Medium (1.5 mL)	STEMdiff™ Gastric Organoid Medium	1.5 mL	Mix thoroughly. If not used immediately, store at 2 - 8°C for up to 1 week.
	STEMdiff™ Gastric Organoid Expansion Supplement	15 µL	

8.2 Setup

Perform the following tasks at least one hour before passaging:

- Place a vial of Matrigel® (Corning Catalog #356231) on ice (50 µL/dome)
- Place a box of 200 µL pipette tips at -20°C
- Place a bottle of DMEM/F-12 with 15 mM HEPES on ice
- Place a 24-well tissue culture-treated plate in the 37°C incubator (overnight or longer incubation is optimal)

8.3 Passaging Gastric Organoids for Expansion

Note: It is critical to pre-wet any pipette tip or tube with Anti-Adherence Rinsing Solution and then wash with D-PBS (Without Ca++ and Mg++) or DMEM/F-12 with 15 mM HEPES to minimize loss of organoid fragments due to adherence to plasticware. Up to seven 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. Pre-wet 15 mL conical tubes (one 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++) or DMEM/F-12 with 15 mM HEPES 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.
2. Aspirate medium from the dome cultures.
3. Using a 1 mL pipettor, add 1 mL of cold DMEM/F-12 with 15 mM HEPES to the dome containing organoids. Break up the dome by pipetting directly onto the dome a few times until the dome breaks and detaches.
4. Using a 1 mL pipettor, transfer the suspension to a pre-wetted 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 steps 3 - 4. Mesenchymal cells might obstruct the pipette tip and impede transfer. If this happens, pipette the medium up and down in the well to start fragmentation of the organoids and allow complete transfer to the 15 mL conical tube.
5. Add 1 mL of cold DMEM/F-12 with 15 mM HEPES to the organoids.
6. Incubate the tube on ice for at least 5 minutes per single well harvested in a 15 mL tube.

   Note: Incubation on ice is needed to dissolve Matrigel®. Wells of organoids can be pooled in the same tube for passaging; a longer incubation time will be required with a greater number of pooled wells. Evaluate Matrigel® dissolution by visual inspection of the tube.
7. Centrifuge at 300 x *g* for 5 minutes at room temperature. Using a 1 mL pipettor, carefully remove and discard the supernatant.
8. Add 1 mL of DMEM/F-12 with 15 mM HEPES. Break up organoids by pipetting up and down 15 times to obtain a homogeneous organoid fragment suspension (fragment size 100 - 500 µm).

   Note: Pipetting up and down 15 times typically generates the desired organoid fragment size. However, depending on the mesenchymal content of the cultures, full fragmentation of this fraction might be difficult to obtain. If this happens, fragments of mesenchyme can still be included in the same Matrigel® dome for the first passage. Mesenchymal content will be reduced in subsequent passages.
9. Add 1 mL of DMEM/F-12 with 15 mM HEPES.

10. Centrifuge at 300 x *g* for 5 minutes at room temperature. Using a 1 mL pipettor, carefully remove and discard supernatant.

    Note: An additional washing step may be performed if necessary, by repeating steps 9 - 10. It is critical to remove as much supernatant as possible at the end of this washing step. If multiple wells have been pooled together in the same tube, a cloudy phase may be visible (containing Matrigel® and single cells) above the organoid fragment pellet. Use a 200 µL pipettor to carefully remove this cloudy phase without removing any organoid fragments.

11. Remove the 200 µL pipette tips from the freezer and place in the biosafety cabinet. Remove the 24-well plate from the incubator.
12. Determine desired organoid density based on split ratio.

    Note: Each organoid fragment can give rise to a new gastric organoid. Keep a conservative strategy in determining split ratio, avoiding splitting higher than 1:2 at early passages (P1 and P2) and 1:4 at later passages.

Perform steps 13 - 14 quickly to prevent gelling of Matrigel®:

13. Using a pipettor with a cold 200 µL pipette tip, add 50 µL of cold (2 - 8°C) Matrigel® per seeded dome/well 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.

14. Using the same pipette tip, draw up the 50 µL suspension, then gently transfer into the center of one well of the warm 24-well plate 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 away from the well.

       Note: Dispensing Matrigel® too quickly into the culture dish will flatten the dome.

    e. Repeat steps 13 - 14 for remaining pellets.
15. Place the lid on the dish. Incubate at 37°C with 5% CO_2 and 95% humidity for 15 - 25 minutes to allow Matrigel® to solidify.

    Note: Expect organoids fragments to migrate at the base of the Matrigel® dome and attach to the plate. To minimize this and obtain higher-quality organoid cultures, invert the plate after 2 minutes.

16. Warm a sufficient volume of STEMdiff™ Gastric Organoid Expansion Medium (0.5 mL/well) to room temperature. Store the remaining medium at 2 - 8°C.
17. Add 0.5 mL of STEMdiff™ Gastric Organoid Expansion Medium 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.
18. Perform a full-medium change every 2 days by removing medium, then following steps 16 - 17.
19. Passage organoid cultures every 7 days, depending on organoid density, size, and morphology (refer to Figure 5).

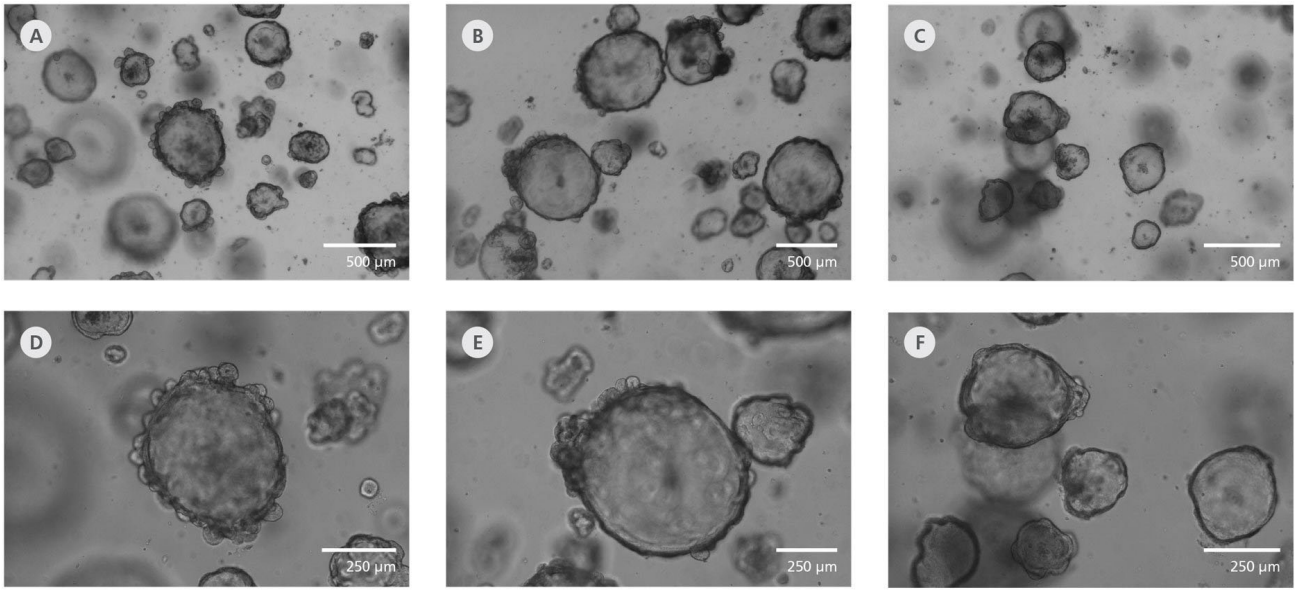


Figure 5. Organoid Morphology in STEMdiff™ Gastric Organoid Expansion Medium

Representative images of organoids cultured in STEMdiff™ Gastric Organoid Expansion Medium, magnifications 5X (**A-C**) and 10X (**D-F**). Organoids can have different morphologies: **(A,D)** Cystic body core completely surrounded by small buds on the basolateral side, **(B,E)** Cystic body core partially surrounded by small buds on the basolateral side, and **(C,F)** Cystic body core with one or no buds on the basolateral side. On Day 7 of passage, healthy organoids usually measure ~500 µm in diameter and show this spectrum of phenotypes, indicating the optimal timepoint for downstream analysis or passaging. Avoid extending cultures further than this maturation point, as organoids will start to accumulate dead cells in the lumen that will affect their morphology and regeneration capacity after passaging. Organoid phenotype may vary between hPSC lines. Examples of optimal culture density and growth are shown in Figure 4. Adjust passaging timing according to the culture density and organoid maturation stage. If at Day 7 cultures are not confluent, but organoids show phenotypes and size described here, they can be passaged by applying a low split ratio (1:1 or 1:2) to facilitate enrichment of organoids during the next passage. If on Day 7 cultures are confluent and organoids are small and dark, even if not showing a mature morphology they can be passaged by applying a high split ratio (1:3 or 1:4); this will allow organoids more space to mature and will improve morphology during the next passage.

9.0 Cryopreserving PSC-Derived Human Gastric Organoids and Spheroids

9.1 Cryopreserving Gastric Organoids

Note: It is critical to pre-wet any pipette tip or tube with Anti-Adherence Rinsing Solution and then wash with D-PBS (Without Ca++ and Mg++) or DMEM/F-12 with 15 mM HEPES to minimize loss of organoid fragments due to adherence to plasticware. Up to seven 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.
2. Using a 1 mL pipettor, add 1 mL of cold DMEM/F-12 with 15 mM HEPES to the dome containing organoids. Break up the dome by pipetting directly onto the dome a few times until the dome breaks and detaches. Transfer the suspension to a pre-wetted 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 2.
3. Add 1 mL of cold DMEM/F-12 with 15 mM HEPES to the organoid suspension.
4. Incubate the tube on ice for at least 5 minutes per well harvested in a 15 mL tube.

   Note: Incubation on ice is needed to dissolve Matrigel®. Wells of organoids can be pooled in the same tube for cryopreservation; a longer incubation time will be required with a greater number of pooled wells. Evaluate Matrigel® dissolution by visual inspection of the tube.
5. Using a 1 mL pipettor, pipette the suspension up and down ~5 - 10 times to break up organoids until a homogeneous fragment suspension has been generated.

   Note: Avoid breaking up fragments into single cells with extended pipetting.
6. Centrifuge at 300 x *g* for 5 minutes at room temperature (15 - 25°C). Place the tube on ice for 5 minutes.
7. 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. If multiple wells have been pooled together in the same tube, 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.
8. 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.
9. 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.
10. 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:

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

11. 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 13).

12. Centrifuge at 200 x *g* for 5 minutes at room temperature. Carefully remove and discard the 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 12.

13. 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.
14. 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.

15. 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).

9.2 Thawing Gastric Organoids

Note: It is critical to pre-wet any pipette tip or tube with Anti-Adherence Rinsing Solution and then wash with D-PBS (Without Ca++ and Mg++) or DMEM/F-12 with 15 mM HEPES to minimize loss of organoid fragments due to adherence to plasticware. Remove the vial containing organoid fragments from the liquid nitrogen and place the vial immediately into a 37°C water bath.

1. 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.

2. Using a 2 mL serological pipette, carefully wash the fragments with 2 mL of room temperature (15 - 25°C) DMEM/F-12 to completely thaw the organoid fragment suspension.
3. Transfer the washed fragments to a 15 mL conical tube.
4. Centrifuge at 200 x *g* for 5 minutes at room temperature. Carefully remove and discard the supernatant.

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

   Note: We recommend embedding 250 - 1000 fragments. If needed, resuspend the fragments in 2 mL of DMEM/F-12, then aliquot the fragment suspension into a new 15 mL conical tube so that each tube contains the desired number of organoid fragments for embedding; repeat step 4.

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

5. Refer to section 8.3 steps 13 - 19 for embedding organoid fragments in Matrigel® domes and organoid maintenance.

   Note: For the first 2 days of culture, add 10 µM Y-27632 to STEMdiff™ Gastric Organoid Expansion Medium.

During the thawed passage, organoids 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. After passaging the previously cryopreserved organoids, the passaging schedule will return to every 7 - 10 days, depending on organoid density, size, and morphology.

9.3 Cryopreserving Gastric Spheroids

Note: It is critical to pre-wet any pipette tip or tube with Anti-Adherence Rinsing Solution and then wash with D-PBS (Without Ca++ and Mg++) or DMEM/F-12 with 15 mM HEPES to minimize loss of spheroids due to adherence to plasticware. Volumes indicated are for harvesting organoids from one well of a 24-well plate. If pooling multiple wells, adjust volumes accordingly.

1. Refer to section 6.1.2 for spheroid collection and counting on **Day 7** of the protocol.
2. After counting, collect spheroids from the counting plate and transfer to a 15 mL tube. If necessary, pool wells to obtain 500 spheroids per tube.

   Note: Spheroid survival after thawing may be variable between different hPSC lines. Optimize freezing conditions by pooling different numbers of spheroids per vial to determine which freezing density results in optimal recovery.
3. Incubate the tube on ice for at least 5 - 10 minutes. Let spheroids sink by gravity.
4. Using a pipettor, carefully remove as much supernatant as possible.

   Note: It is critical to remove as much supernatant as possible, without removing any spheroids.
5. Add cold CryoStor® CS10 to the pellet; use 1 mL for each cryovial to be prepared. Gently distribute spheroids by pipetting up and down several times.
6. Using a 1 mL pipettor, transfer 1 mL of fragment suspension into a labeled cryovial. Place the cryovial into a freezing container.
7. 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).

9.4 Thawing Gastric Spheroids

Note: It is critical to pre-wet any pipette tip or tube with Anti-Adherence Rinsing Solution and then wash with D-PBS (Without Ca++ and Mg++) or DMEM/F-12 with 15 mM HEPES to minimize loss of organoid fragments due to adherence to plasticware. Remove the vial containing spheroids from the liquid nitrogen and place the vial immediately into a 37°C water bath.

1. Observe thawing process closely and retrieve cryovial from the water bath before spheroids are completely thawed.

   Note: The thawing process should take no longer than 1 minute.
2. Using a 2 mL serological pipette, carefully wash the fragments with 2 mL of room temperature (15 - 25°C) DMEM/F-12 to completely thaw the organoid fragment suspension.
3. Transfer the washed fragments to a 15 mL conical tube.
4. Centrifuge at 200 x *g* for 5 minutes at room temperature. Carefully remove and discard the supernatant.

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

   Note: We recommend embedding 500 spheroids per well.
5. Refer to section 6.1.3 for embedding spheroids in Matrigel® domes.

   Note: For the first 3 days of culture, add 10 µM Y-27632 to STEMdiff™ Gastric Organoid Medium + RA.
6. For maturation of spheroids to organoids, refer to section 6.2.

   Note: Organoid growth may become visible after 5 - 7 days of culture.

10.0 Characterization of Differentiation Cultures

10.1 Characterization of DE Cells

Purity of definitive endoderm (DE) 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.

10.2 Characterization of PF Cells

Purity of posterior foregut (PF) 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-SOX2 antibody

  e.g. Rabbit SOX2 antibody, clone EPR3131 (abcam Catalog #ab92494)
- 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.

10.3 Characterization of Human Gastric Organoids

Purity of human gastric organoid cultures can be assessed by immunocytochemistry after labeling with the following antibodies:

- Anti-SOX2 antibody

  e.g. Rabbit SOX2 antibody, clone EPR3131 (abcam Catalog #ab92494)
- 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-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-SOX9 antibody

  e.g. Anti-Sox9 Antibody (Millipore Sigma Catalog #AB5535)
- Anti-Claudin18 antibody

  e.g. Anti-CLDN18 Antibody (Sigma Catalog #HPA018446)
- Anti-PDX1 antibody

  e.g. Anti-PDX1 Antibody, clone EPR22002 (abcam Catalog #ab219207)
- Anti-MUC6

  e.g. Anti-Gastric Mucin/MUC6 (abcam Catalog #ab212648)
- Anti-PGA antibody

  e.g. Anti-PGA5, clone 4G9 (antibodies-online Catalog #ABIN518824)
- Anti-MUC5AC

  e.g. Anti-MUC5AC, clone 45M1 (Thermo Fisher Scientific Catalog #MA5-12178)

Results may vary depending on cell line used.

11.0 Troubleshooting

PROBLEM	POSSIBLE CAUSE	SOLUTION
Low efficiency of definitive endoderm induction	Low-quality hPSC starting culture	Ensure high quality of undifferentiated hPSC starting culture by removing all hPSC colonies that have spontaneously differentiated from maintenance cultures (section 4.0).
Cells lift off during definitive endoderm induction	• Cell density on Day 0 too high (> 90%) • 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 (or less) for the first 3 days of definitive endoderm induction
No spheroid formation during posterior foregut differentiation	Low cell density on Day 3	Seed 3 different cell clump densities (4000, 5000, and 6000 clumps per well) and start definitive endoderm induction of all three seeding densities on the same day, once one density has reached 85 - 90% confluence. Continue with posterior foregut induction of endoderm monolayers (Day 3), which show the most compact and overcrowded cell densities. Discontinue 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 posterior foregut 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 4.0).
Matrigel® dome collapses after plating onto culture plate	• Non-suitable cell culture plasticware for Matrigel® dome cultures • Low protein content of Matrigel® lot • Diluted Matrigel® due to residual medium	• A pre-warmed 24-well tissue culture-treated plate is recommended for organoid dome cultures • Use a Matrigel® lot (Corning Catalog #356231) with a minimum protein content of 8 mg/mL • Remove as much residual medium as possible from pelleted spheroids (section 6.1.3) • Don't use Matrigel® aliquots that have undergone multiple freeze/thaw cycles
Spheroids sink to the bottom of the plate and attach while embedding in Matrigel®	Diluted Matrigel® due to residual medium	Remove as much residual medium as possible from pelleted spheroids; after embedding, invert plate after 2 minutes in the incubator (section 6.1.3).
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 6.1.3).
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 8.3) • Seed a greater number of fragments
Low quantities of differentiated gastric cell types	Organoids were harvested too early	Keep organoids in culture for 10 - 12 days without passaging. Perform a full-medium change every 3 - 4 days using 0.5 mL of fresh STEMdiff™ Gastric Organoid Expansion Medium. Extended organoid culture will enrich for differentiated gastric cell lineages.

12.0 References

1. McCracken K et al. (2014) Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. Nature 516 (7531): 400–4.
2. Bartfeld S et al. (2015) In vitro expansion of human gastric epithelial stem cells and their responses to bacterial infection. Gastroenterology 148(1): 126–36.

TECHNICAL MANUAL

Generation of Human Gastric Organoids Using STEMdiff™ Gastric Organoid Differentiation Kit

STEMCELL™ TECHNOLOGIES

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