Human Gastric Organoid Culture Protocol

This protocol provides a procedure for subculturing normal human gastric organoids. This protocol was modified from the submerged method described in Bartfeld, S. *et al.* (2015) Gastroenterology **148**:126.

The protocol provided below is intended to culture organoids from normal human gastric tissues using Cultrex® Organoid Qualified Basement Membrane Extracts as a scaffold. The majority of reagents used in this protocol were sourced from the Bio-Techne brands of R&D Systems and Tocris Bioscience.

MATERIALS

Table 1. Materials needed for gastric organoid culture.

Reagent name	Supplier	Catalog #
Cultrex Organoid Harvesting Solution	R&D Systems	3700-100-01
Cultrex Reduced Growth Factor Basement Membrane Extract (RGF BME), Type 2	R&D Systems	3533-005-02*
Advanced DMEM/F-12 Cell Culture Medium	Thermo Fisher	12634-010
Glutamine	Tocris Bioscience	5823
HEPES	Tocris Bioscience	3173
N21-MAX Supplement	R&D Systems	AR008
N-2 MAX Supplement	R&D Systems	AR009
N-Acetylcysteine	Tocris Bioscience	5619
TrypLE™ Express	Thermo Fisher	12604-021
Fetal Bovine Serum (FBS)	Thermo Fisher	26140-079
Gastrin I (Human)	Tocris Bioscience	3006
SB 202190 (p38 MAPK Inhibitor)	Tocris Bioscience	1264
Nicotinamide	Tocris Bioscience	4106
Human Insulin, Solution	Sigma-Aldrich	I9278
Human Transferrin	Sigma-Aldrich	T8158
Y-27632 dihydrochloride (Rho Kinase Inhibitor)	Tocris Bioscience	1254
Recombinant Human EGF	R&D Systems	236-EG
Recombinant Human R-Spondin 1	R&D Systems	4645-RS
Recombinant Human Noggin	R&D Systems	6057-NG
Recombinant Human FGF-10	R&D Systems	345-FG
A 83-01 (ALK5 inhibitor)	Tocris Bioscience	2939
CHIR 99021, (GSK-3 Inhibitor)	Tocris Bioscience	4423
Recombinant Human Wnt-3a	R&D Systems	5036-WN

*Cultrex RGF BME, Type 2 (Catalog # 3533-005-02) is recommended for robust organoid cultures and Cultrex RGF BME, Type R1 (Catalog # 3433-005-R1) is recommended for difficult to grow organoid cultures.

EQUIPMENT

1. Cell culture incubator (37 °C, 5% CO_2)
2. Cell culture hood with laminar flow
3. Centrifuge with refrigeration and swinging bucket rotor
4. 37 °C water bath
5. Ice bucket
6. Laboratory refrigerator
7. Mini cell scraper, sterile
8. Pipet aid and serological pipettes (5 mL)
9. Micropipettes and tips (2–200 µL)
10. Conical tubes, 10 mL and 50 mL, sterile
11. Cell strainer, 100 µm, sterile
12. 24-well plate, tissue-culture treated, sterile
13. Vacuum pump
14. Medium filtration unit, 0.1 µm, 500 mL, sterile
15. Syringe, 50 mL, sterile
16. Syringe filter, 0.2 µm, sterile
17. Cell culture waste container

OTHER REQUIRED REAGENTS

1. Distilled (DW) or deionized water (DI)
2. Phosphate buffered saline (PBS)
3. 1% Ammonium
4. 1% BSA/PBS
5. DMSO

REAGENT PREPARATION

Use aseptic technique at all times during this protocol. This protocol is optimized for human gastric organoids; organoids from other tissues may have different culture requirements.

1. Prepare stock solutions for gastric organoid culture, as indicated in Table 2:

Table 2. Preparation of stock solutions for gastric organoid culture medium.

Reagent name	Solvent	Stock solution	Preparation	Storage
N-Acetylcysteine	DI water	500 mM = 81.6 mg/mL	200 mg in 2.4 mL	4 °C
Glutamine	PBS	200 mM = 29.2 mg/mL	1.46 g in 50 mL	4 °C
Gastrin I (Human)	1% Ammonia with sonication	100 µM = 210 µg/mL	500 µg in 2.38 mL	–20 °C
Recombinant Human EGF	1% BSA/PBS	500 µg/mL	200 µg in 400 µL	–80 °C
Recombinant Human R-Spondin 1	1% BSA/PBS	1 mg/mL	1 mg in 1 mL	–80 °C
Recombinant Human Noggin	1% BSA/PBS	100 µg/mL	100 µg in 1 mL	–80 °C
Recombinant Human FGF-10	1% BSA/PBS	100 µg/mL	100 µg in 1 mL	–80 °C
A 83-01	DMSO	25 mM = 10.54 mg/mL	10 mg in 949 µL	–20 °C
SB 202190	DMSO	30 mM = 9.9 mg/mL	5 mg in 505 µL	4 °C
Nicotinamide	DW	1 M = 122.12 mg/mL	6.1 g in 50 mL	4 °C
Human Transferrin	DW	50 mg/mL	100 mg in 2 mL	–20 °C
Recombinant Human Wnt-3a	1% BSA/PBS	600 µg/mL	500 µg in 833 µL	–80 °C
Y-27632 dihydrochloride	PBS	10 mM = 3.2 mg/mL	1 mg in 313 µL	4 °C
CHIR 99021	DMSO	20 mM = 9.3 mg/mL	10 mg in 1.08 mL	–20 °C

2. Thaw Cultrex RGF BME, Type 2 on ice for four hours or overnight in the refrigerator.
3. Prepare Gastric Organoid Culture Medium, as indicated in Table 3:

   Note: *The recipe below is for 50 mL, but it may be scaled as desired.*

Table 3. Preparation of Gastric Organoid Culture Medium.

Reagent	[Stock]	[Final]	Volume
Advanced DMEM/F-12 Cell culture Medium	NA	NA	21.6 mL
Recombinant Human Wnt-3a	600 µg/mL	60 ng/mL	5 µL
N21-MAX Supplement	50X	1X	1 mL
Glutamine	200 mM	2 mM	500 µL
HEPES	1 M	10 mM	500 µL
Penicillin/Streptomycin	100X	1X	500 µL
N-2 MAX Supplement	100X	1X	500 µL
A 83-01	25 mM	2 µM	4 µL
N-Acetylcysteine	500 mM	1 mM	100 µL
Recombinant Human FGF-10	100 µg/mL	200 ng/mL	100 µL
Recombinant Human R-Spondin 1	1 mg/mL	1 µg/mL	50 µL
Recombinant Human Noggin	100 µg/mL	100 ng/mL	50 µL
Human Insulin	10 mg/mL	7.5 µg/mL	37.5 µL
SB 202190	30 mM	10 µM	16.7 µL
Human Transferrin	50 mg/mL	10 µg/mL	10 µL
Gastrin I Human	100 µM	1 nM	5 µL
Recombinant Human EGF	500 µg/mL	50 ng/mL	5 µL
		Total	50 mL

4. Sterile filter the media.

PROTOCOLS

1. Starting Organoids from a Cryovial

   a. Thaw cryovial containing organoids in a 37 °C water bath.

   Note: *The contents should thaw in 2–3 minutes; do not allow the cryovial to remain at 37 °C any longer than is necessary.*

   b. Transfer the contents of the cryovial to a 15 mL conical tube and add 9 mL of Gastric Organoid Culture Medium. Gently pipet up and down three times using a serological pipette to resuspend organoids.

   Note: *Organoids may be counted at this time if needed to determine seeding volumes.*

   c. Centrifuge the vial at 500 × g for 3 minutes to pellet gastric organoids, and aspirate medium.

   d. Resuspend gastric organoids in Cultrex RGF BME, Type 2 at 10,000 organoids per mL (500 organoids per well). Pipet up and down three times using a serological pipette to disperse organoids in the RGF BME, and dispense 50 µL of the Cultrex RGF BME, Type 2/organoid mixture in the center of each well of a 24-well plate (Figure 1).

   Note: *The Cultrex BME-contained organoids should not touch the sides of the well.*

   Record total volume of Cultrex RGF BME, Type 2 _____

   Record the number of wells seeded _____

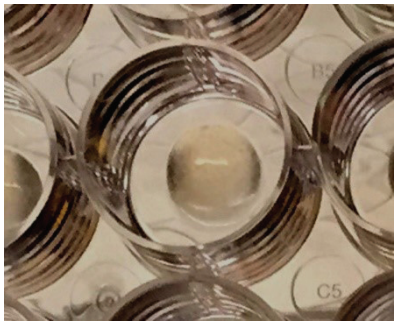

Figure 1. Placement of Cultrex RGF BME, Type 2/ organoid mixture in the center of the well of a 24-well plate.

   e. Incubate the plate in the Cell culture incubator for 25 minutes to polymerize the Cultrex RGF BME, Type 2.

   f. Calculate the volume of Gastric Organoid Starting/Passaging Medium needed

$$\frac{\text{\_\_\_\_\_}}{\text{Number of well}} \times \frac{0.5\ \text{mL}}{\text{Number of well}} = \frac{\text{\_\_\_\_\_}}{\text{Total Volume (mL)}}$$

   g. Prepare Gastric Organoid Starting/Passaging Medium, as indicated in Table 4:

Table 4. Preparation of Gastric Organoid Starting/Passaging Medium.

Reagent	[Stock]	[Final]	Calculation	Amount Added
Gastric Organoid Culture Medium	NA	NA	Total Volume	
Nicotinamide	1 M	10 mM	Total Volume / 100	
Y-27632 dihydrochloride	10 mM	10 µM	Total Volume / 1,000	
CHIR 99021	20 mM	2.5 µM	Total Volume / 8,000	

   h. Add 500 µL of Gastric Organoid Starting/Passaging Medium per well.

   Note: *Medium should be gently pipetted into the corner of the well away from the Cultrex RGF BME/organoids to prevent their disruption.*

   i. Return plate containing organoid cultures to the cell culture incubator to promote organoid growth.

2. Gastric Organoid Culture Maintenance

   The culture medium should be aspirated from each well and replaced with fresh Gastric Organoid Culture Medium every other day i.e., Monday, Wednesday, and Friday). Gastric organoids can be cultured for two weeks before passaging (see Table 5).

   Note: *Medium should be gently aspirated from and pipetted into the corner of the well away from the Cultrex RGF BME/organoids to prevent their disruption.*

Table 5. Medium Change Dates

	Change 1	Change 2	Change 3	Change 4	Change 5
Record Date					

3. Passaging Organoids

   a. View gastric organoids under the microscope. Each well should contain approximately 500 organoids for optimal growth. Organoids cultures exhibiting rapid growth may be split 1:4 during passaging, while slow growing cultures may benefit from a 1:1 split. Make this determination prior to harvesting to estimate reagent needs prior to starting.

      Note: *Organoid density is important for optimal growth; too many organoids will strain culture resources, while too few organoids lack paracrine signaling necessary to sustain growth.*

   b. Transfer the 24-well plate containing gastric organoids from the cell culture incubator to the cell culture hood.

   c. Scrape the entire surface of each well using the cell scraper to dislodge the Cultrex RGF BME, Type 2-contained organoids located in the center of each well.

   d. Transfer organoids to 15 mL conical tube(s); cultures of identical tissue and treatment may be combined, if desired.

   e. Centrifuge the tube at 500 × g for 3 minutes at room temperature.

   f. Aspirate the medium without disturbing the Cultrex RGF BME, Type 2-contained organoids at the bottom of the tube.

   g. Add 10–20 volumes of TrypLE Express to each well to digest the Cultrex RGF BME, Type 2. Each well contained 50 µL of RGF BME-2, so 500 µL of TrypLE Express will be needed per well in the conical tube. For example, one full plate uses 24 wells × 500 µL = 12 mL.

   h. Pipet the TrypLE/Cultrex RGF BME, Type 2/organoids up and down five times with a serological pipette to mix.

   i. Place the conical tube(s) in a 37 °C water bath for 12 minutes to digest the Cultrex RGF BME, Type 2.

      Note: *Most of the Cultrex RGF BME, Type 2 should be visibly digested during this incubation; however, some small amount may remain. The presence of small amount of gel (<100 µL) is acceptable.*

   j. Add 1/10 volume of FBS to each conical tube to quench the TrypLE Express reaction.

   k. Pipet up and down three times with a serological pipette to mix.

   l. Pipet the organoid mixture through a cell strainer at 100 µm to segment organoids.

   m. Centrifuge the tube at 500 × g at room temperature for 3 minutes.

   n. Aspirate medium, but be careful not to disturb the organoid pellet.

   o. Resuspend segmented organoids in Cultrex RGF BME, Type 2, and dispense 50 µL of the mixture in the center of each well of a 24-well plate.

      Note: *The Cultrex RGF BME-contained organoids should not touch the sides of the well.*

   p. Incubate the plate in the cell culture incubator for 25 minutes to polymerize Cultrex RGF BME, Type 2.

   q. Add 500 µL of Gastric Organoid Starting/Passaging Medium per well.

      Note: *Medium should be gently pipetted into the corner of the well away from the Cultrex RGF BME, Type 2/organoids to prevent their disruption.*

   r. Return plate containing organoid cultures to the cell culture incubator to promote organoid growth.

4. Cryobanking Organoids

   a. View gastric organoids under the microscope. Each well should contain approximately 500 organoids for optimal growth. Organoids cultures exhibiting rapid growth may be split 1:4 during passaging, while slow growing cultures may benefit from a 1:1 split. Make this determination prior to harvesting to estimate reagent needs prior to starting.

      Note: *Organoid density is important for optimal growth; too many organoids will strain culture resources, while too few organoids lack paracrine signaling necessary to sustain growth.*

   b. Transfer the 24-well plate containing gastric organoids from the cell culture incubator to the cell culture hood.

   c. Scrape the entire surface of each well using the cell scraper to dislodge the Cultrex RGF BME, Type 2-contained organoids located in the center of each well.

   d. Transfer organoids to conical tube(s); cultures of identical tissue and treatment may be combined, if desired.

   e. Centrifuge the tube at 500 × g for 3 minutes at room temperature.

   f. Aspirate the medium without disturbing the organoids at the bottom of the tube.

   g. Add 10–20 volumes of TrypLE Express to each well to digest the Cultrex RGF BME, Type 2. Each well contained 50 µL of Cultrex RGF BME, Type 2, so 500 µL of TrypLE Express will be needed per well in the conical tube. For example, one full plate uses 24 wells × 500 µL = 12 mL.

   h. Pipet the TrypLE/Cultrex RGF BME, Type 2/organoids up and down five times with a serological pipet to mix.

   i. Place the conical tube(s) in a 37 °C water bath for 12 minutes to digest the Cultrex RGF BME, Type 2.

      Note: *Most of the Cultrex RGF BME, Type 2 should be visibly digested during this incubation; however, some small amount may remain. The presence of small amount of gel (<100 µL) is acceptable.*

   j. Add 1/10 volume of FBS to each conical tube to quench the TrypLE Express reaction.

   k. Pipet up and down three times with a serological pipet to mix.

   l. Centrifuge the tube at 500 × g at room temperature for 3 minutes.

   m. Aspirate medium, but be careful not to disturb the organoid pellet.

   n. Resuspend segmented organoids in 90% FBS, 10% DMSO, and 10 µM Y-27632 dihydrochloride, and dispense 500 µL of the organoid mixture into each labeled cryovial.

   o. Place cryovials in a freezing container, and store at ≤–70 °C for 24 hours.

   p. Transfer the cryovials to a liquid nitrogen dewar for long term storage.

DATA EXAMPLES

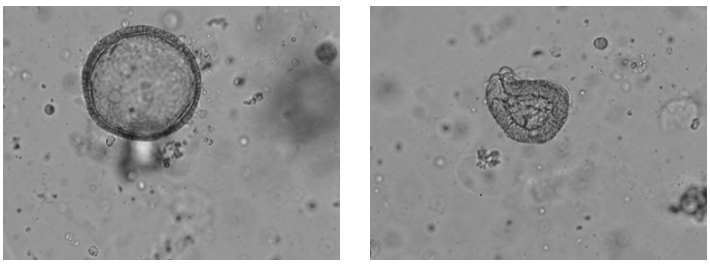

Figure 1. Undifferentiated Human Gastric Organoids. Representative brightfield images of human gastric organoids that were cultured using Cultrex RGF BME, Type 2 and the Bio-Techne reagents listed in this protocol.

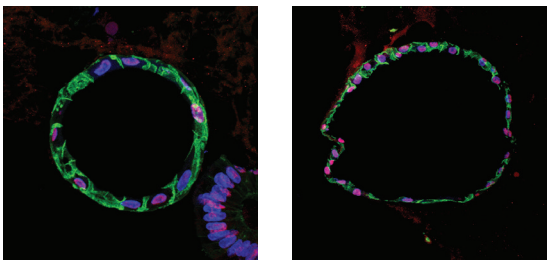

Figure 2. Immunohistochemistry of Undifferentiated Human Gastric Organoids. Human gastric organoids were cultured using Cultrex RGF BME, Type 2 and Bio-Techne reagents listed in this protocol. Undifferentiated colon organoids were stained using the Human/Mouse E-Cadherin Antibody (green; R&D Systems; Catalog # AF748), the Human HOXB7 Antibody (red; R&D Systems; Catalog # MAB8040), and counterstained with DAPI (blue; Tocris; Catalog # 5748).

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