Culture of Human Gastric Organoids

Using Cultrex™ UltiMatrix Basement Membrane Extract

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™ UltiMatrix RGF Basement Membrane Extract 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™.

TABLE // 01

Materials Needed for Gastric Organoid Culture

Product Name	Supplier	Catalog #
Cultrex Organoid Harvesting Solution	R&D Systems	3700-100-01
Cultrex UltiMatrix Reduced Growth Factor Basement Membrane Extract	R&D Systems	BME001-05
Advanced DMEM/F-12 Cell Culture Medium	Thermo Fisher	12634010
GlutaMax™	Thermo Fisher	35050061
HEPES	Tocris Bioscience	3173
Penicillin-Streptomycin	Various	Various
N21-MAX Supplement	R&D Systems	AR008
N-2 MAX Supplement	R&D Systems	AR009
N-Acetylcysteine	Tocris Bioscience	7874
Gastrin I (Human)	Tocris Bioscience	3006
SB 202190 (p38 MAPK inihibitor)	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
A 83-01 (ALK5 inhibitor)	Tocris Bioscience	2939
CHIR 99021 (GSK-3 inhibitor)	Tocris Bioscience	4423
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
Recombinant Human Wnt-3a	R&D Systems	5036-WN

Equipment

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

Other Required Reagents

- Distilled (DW) or deionized water (DI)
- Phosphate buffered saline (PBS)
- 1% Ammonium
- 1% BSA/PBS
- 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 // 02

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
GlutaMax™	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

Reagent Name	Solvent	Stock Solution	Preparation	Storage
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 UltiMatrix RGF Basement Membrane Extract on ice for four hours or overnight at 2 - 8 °C (on ice 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 // 03

Preparation of Gastric Organoid Culture Medium

Reagent Name	[Stock]	[Final]	Volume
Advanced DMEM/F-12 Cell culture Medium	NA	NA	46.6 mL
Recombinant Human Wnt-3a	600 µg/mL	60 ng/mL	5 µL
N21-MAX Supplement	50X	1X	1 mL
GlutaMax™	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 (ALK5 inhibitor)	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 (p38 MAPK inhibitor)	30 mM	10 µM	16.7 µL
Human Transferrin	50 mg/mL	10 µg/mL	10 µL
Gastrin I Human	100 µM	1 nM	0.5 µL
Recombinant Human EGF	500 µg/mL	50 ng/mL	5 µL
		Total	50 mL

4. Sterile filter the media.

Methods for Culturing Human Gastric Organoids

Starting Organoids from a Cryovial

a. Thaw the 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 Advanced DMEM/F12 cell culture medium. Gently pipet up and down three times using a serological pipette to resuspend the 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 the gastric organoids, and aspirate the medium.

d. Resuspend the gastric organoids in Cultrex UltiMatrix RGF Basement Membrane Extract, at 10,000 organoids per mL (500 organoids per well). Pipet up and down three times using a serological pipette to disperse the organoids in the Cultrex UltiMatrix RGF Basement Membrane Extract, and dispense 50 µL of the Cultrex UltiMatrix RGF Basement Membrane Extract/organoid mixture in the center of each well of a 24-well plate (Figure 1) or follow the guidelines in TABLE 4 to the right for other plate formats.

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

Record Keeping and Calculations for Preparing Gastric Organoid Starting/ Passaging Medium:

a. Record the total volume of Cultrex UltiMatrix RGF Basement Membrane Extract

_____

b. Record the number of wells seeded

_____

TABLE // 04

BME and Media Volumes Used in Different Multiwell Formats

Plate Format	Volume of Basement Membrane Extract/ Well	Domes/ Well	Volume of Culture Media/ Well
6-well plate	200 µL	10-15	2 mL
12-well plate	100 µL	5-7	1 mL
24-well plate	50 µL	1-3	500 µL
48-well plate	25 µL	1	250 µL
96-well plate	5 µL	1	100 µL

Figure 1. (A) Placement of Cultrex UltiMatrix RGF BME/organoid mixture in the center of a well of a 24-well plate. (B) Placement of multiple domes within a well of a 6-well plate.

c. Incubate the plate in the cell culture incubator for 25 minutes to polymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

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

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

e. Prepare Gastric Organoid Starting/Passaging Medium, as indicated in Table 5.

f. 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 UltiMatrix RGF BME/organoids to prevent their disruption.

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

TABLE // 05

Preparation of Gastric Organoid Starting/Passaging Medium

Reagent Name	[Stock]	[Final]	Calculation	Amount Added
Gastric Organoid Culture Medium	NA	NA	Total Volume	
Nicotinamide	1 M	10 µM	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	

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; See Table 6). Gastric organoids can be cultured for two weeks before passaging, depending on cell seeding density.

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

TABLE // 06

Medium Change Dates

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

Passaging or Cryobanking Organoids

a. View gastric organoids under the microscope. Each well should contain approximately 500 organoids for optimal growth. Organoid 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. Aspirate the medium without disturbing the organoids at the bottom of the wells.

c. Wash each well with 10 volumes of cold (4 °C) PBS, and aspirate without disturbing the Cultrex UltiMatrix RGF Basement Membrane Extract dome (e.g. if the well has 1x 50 µL dome, 500 µL of wash solution must be used).

d. Add 10 volumes of cold (4 °C) Cultrex Organoid Harvesting Solution to each well to depolymerize the Cultrex UltiMatrix RGF Basement Membrane Extract. In the protocol outlined here, each well contained 50 µL of Cultrex UltiMatrix RGF Basement Membrane Extract, so 500 µL of Organoid Harvesting Solution is needed per well in the plate. Domes can be scraped and gently triturated to aid the dissociation. They can also be left in the plate or transferred to a centrifuge conical tube.

e. Organoids should be incubated on ice with gentle shaking (<100 rpm) until the organoids are visually released from the matrix and the matrix is completely dissolved, anywhere from 30 minutes to 90 minutes, check every 10 minutes after the first 30 minutes. Apoptosis inhibitors like Y compound (5-10 µM final concentration for 3-4 days) may be used to avoid stress due to cold temperatures.

   Note: For this incubation, tubes or plates can be placed inside of small Styrofoam boxes or ice buckets with the lids closed on top and then placed in an orbital shaker.

f. Pipet up and down three times with a serological pipette across the well to solubilize any remaining gel.

g. After the organoids are completely released an optional step may be performed: if expansion is desired, pass the organoid solution through a 20-gauge needle into a conical tube to fragment the organoids.

h. Centrifuge the tube at 500 × g at 4 °C for 5 minutes.

i. Aspirate the supernatant but be careful not to disturb the organoid pellet.

j. Resuspend the pellet in 10 volumes of cold (4 °C) PBS or cold base media (without any growth factors, to avoid waste) as a wash.

k. Centrifuge the tube at 500 × g at 4 °C for 5 minutes.

l. Aspirate the supernatant, but be careful not to disturb the organoid pellet and then continue with the desired application (passage, fixation, etc).

For Passaging Organoids

m. Resuspend the segmented organoids in Cultrex UltiMatrix RGF Basement Membrane Extract and dispense 50 µL of the Cultrex UltiMatrix RGF Basement Membrane Extract/ organoid mixture into the center of each well of a 24-well plate to form a dome or follow the guidelines outlined in TABLE 4 for other plate formats.

   Note: The Cultrex UltiMatrix RGF Basement Membrane Extract-contained organoids should not touch the sides of the wells.

n. Incubate the plate in the cell culture incubator for 25 minutes to polymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

o. Add 500 µL of Gastric Organoid Starting/ Passaging Medium per well if using a 24-well plate or follow the guidelines outlined in TABLE 4 for other plate formats.

   Note: Medium should be gently pipetted into the corner of the well away from the Cultrex UltiMatrix RGF Basement Membrane Extract to prevent its disruption.

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

For Cryobanking Organoids

q. Passage the organoids 2-3 days before cryopreservation.

r. Resuspend the 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.

s. Place the cryovials in a freezing container, and store at < -80 °C for 24 hours.

t. Transfer the cryovials to a liquid nitrogen tank for long term storage.

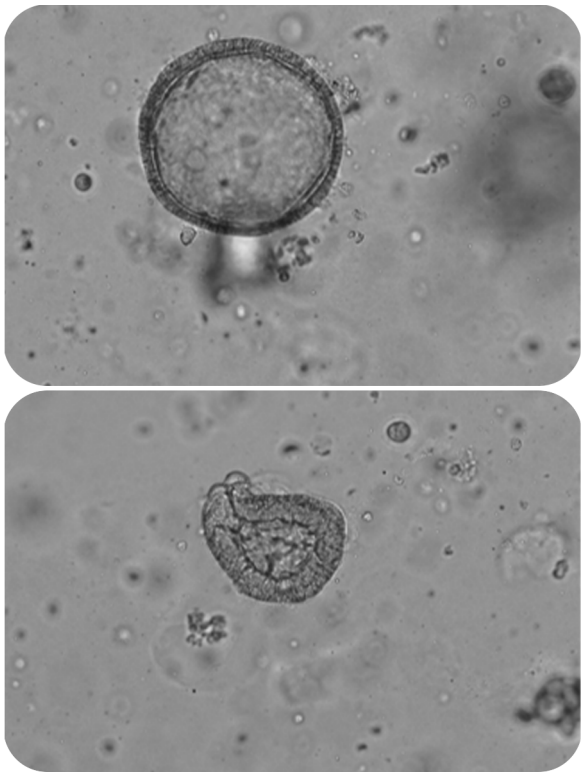

Figure 2. Undifferentiated Human Gastric Organoids. Representative brightfield images of human gastric organoids that were cultured using Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and the other reagents listed in this protocol.

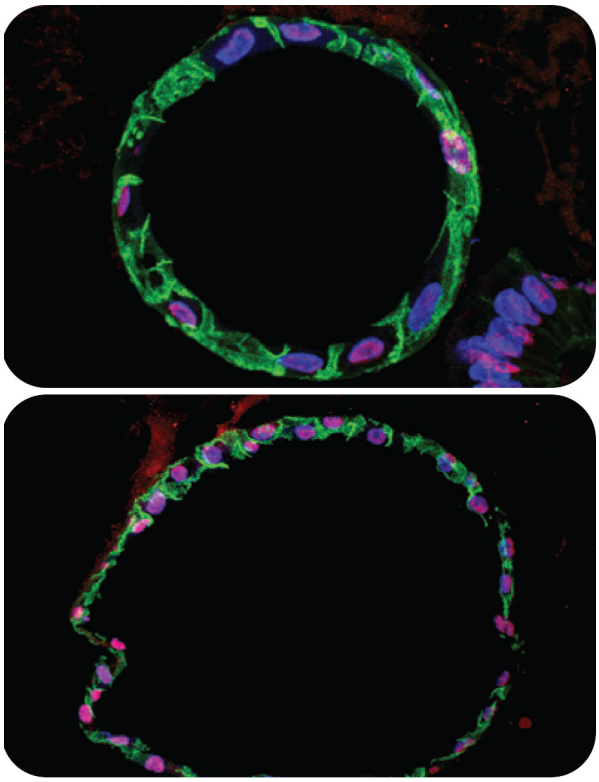

Figure 3. Immunohistochemistry of Undifferentiated Human Gastric Organoids. Human gastric organoids were cultured using Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and the other reagents listed in this protocol. Undifferentiated gastric organoids were stained using the Goat Anti-Human/Mouse E-Cadherin Antigen Affinity-purified Polyclonal Antibody (green; R&D Systems; Catalog # AF748), the Mouse Anti-Human HOXB7 Monoclonal Antibody (red; R&D Systems; Catalog # MAB8040), and counterstained with DAPI (blue; Tocris; Catalog # 5748).

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