Culture of Human Intestinal Organoids

Using Cultrex™ UltiMatrix Basement Membrane Extract

This protocol provides a procedure for subculturing normal human intestinal organoids. This protocol was modified from the submerged method described in Pleguezuelos-Manzano, C. *et al.* (2020) Curr. Protoc. Immunol. **130**: e106.

The protocol provided below is intended to culture organoids from normal human intestinal 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 Intestinal 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
Penicillin-Streptomycin	Various	Various
HEPES	Tocris Bioscience	3173
N21-MAX Supplement	R&D Systems	AR008
N-Acetylcysteine	Tocris Bioscience	7874
Nicotinamide	Tocris Bioscience	4106
Prostaglandin E2 (PGE2)	Tocris Bioscience	2296
A 83-01 (ALK5 inhibitor)	Tocris Bioscience	2939
SB 202190 (p38 MAPK inihibitor)	Tocris Bioscience	1264
Y-27632 dihydrochloride (Rho Kinase inhibitor)	Tocris Bioscience	1254
Recombinant Human Wnt-3a	R&D Systems	5036-WN
Recombinant Human R-Spondin 1	R&D Systems	4645-RS
Recombinant Human Noggin	R&D Systems	6057-NG
Recombinant Human EGF	R&D Systems	236-EG

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% BSA/PBS
- DMSO

Reagent Preparation

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

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

TABLE // 02

Preparation of Stock Solutions for Intestinal 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
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
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
Recombinant Human Wnt-3a	1% BSA/PBS	600 µg/mL	500 µg in 833 µL	–80 °C
Y-27632 dihydrochloride	DI Water	10 mM = 3.2 mg/mL	1 mg in 313 µL	4 °C
PGE2	DMSO	10mM	10 mg in 2.84 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 Intestinal Organoid Culture Medium, as indicated in Table 3.

   Note: The recipe below is for either 50 or 100 mL, but it may be scaled as desired.

TABLE // 03

Preparation of Intestinal Organoid Culture Medium

Reagent Name	[Stock]	[Final]	Volume	
Advanced DMEM/F12 Cell Culture Medium	NA	NA	46.5 mL	93 mL
GlutaMAX™	100X	1X	500 µL	1 mL
Penicillin-Streptomycin	100X	1X	500 µL	1 mL
HEPES 1M	100X	1X	500 µL	1 mL
N21-MAX Supplement	50X	1X	1 mL	2 mL
Nicotinamide	1M	10 mM	500 µL	1 mL
N-Acetylcysteine	500 mM	1.25 mM	125 µL	250 µL
Recombinant Human Wnt-3a	100 µg/mL	100 ng/mL	50 µL	100 µL
Recombinant Human R-Spondin 1	1 mg/mL	1 µg/mL	50 µL	100 µL
Recombinant Human Noggin	200 µg/mL	100 ng/mL	25 µL	50 µL
Recombinant Human EGF	500 µg/mL	50 ng/mL	5 µL	10 µL
Prostaglandin 2 (PGE2)	10 mM	1 µM	5 µL	10 µL
A 83-01 (ALK5 inhibitor)	20 mM	500 nM	1.25 µL	2.5 µL
SB 202190 (p38 MAPK inhibitor)	100 mM	10 µM	5 µL	10 µL
		Total	50mL	100mL

4. Sterile filter the media.

Methods for Culturing Human Intestinal 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 intestinal organoids, and aspirate the medium.

d. Resuspend intestinal 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 below for other plate formats.

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

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

Record Keeping and Calculations for Preparing Organoid Starting/Growth Medium

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

_____

b. Record the number of wells seeded

_____

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 Intestinal Organoid Starting/Passaging Medium needed

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

e. Prepare Intestinal Organoid Starting/Growth Medium, as indicated in Table 5.

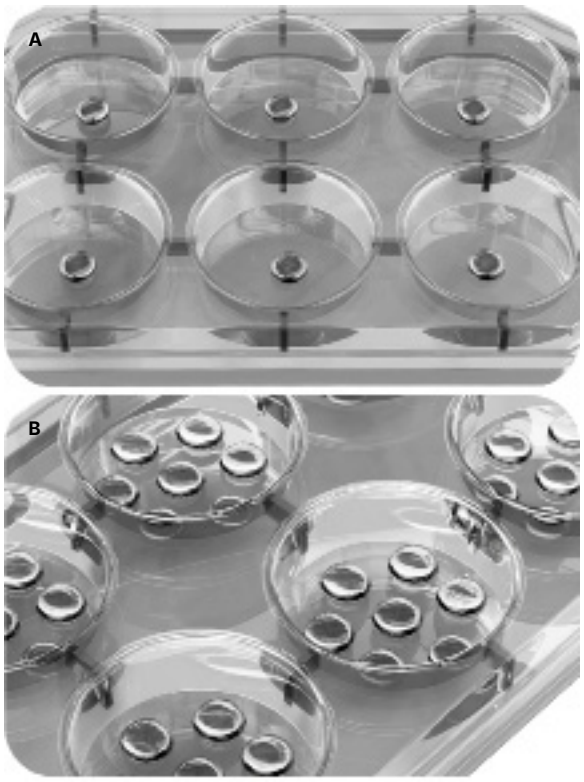

Figure 1. (A) Placement of Cultrex UltiMatrix RGF BME/organoid mixture in the center of a well of a 24-well plate. (B) Placement of multiple domes within a well of a 6-well plate.

f. Add 500 µL of Intestinal Organoid Starting/ Growth 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 Intestinal Organoid Starting/Growth Medium

Reagent	[Stock]	[Final]	Calculation	Amount Added
Intestinal Organoid Culture Medium	NA	NA	Total Volume	
Y-27632 dihydrochloride	10 mM	10 µM	Total Volume / 1,000	

Intestinal Organoid Culture Maintenance

The culture medium should be aspirated from each well and replaced with fresh Intestinal Organoid Culture Medium every other day (i.e. Monday, Wednesday, and Friday; See Table 6). Intestinal organoids can be cultured for one to 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

e. View the intestinal 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.

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

g. 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).

h. 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.

i. 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.

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

k. 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.

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

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

n. 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.

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

p. 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

q. 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.

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

s. Add 500 µL of Intestinal Organoid Starting/ Growth 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.

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

For Cryobanking Organoids

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

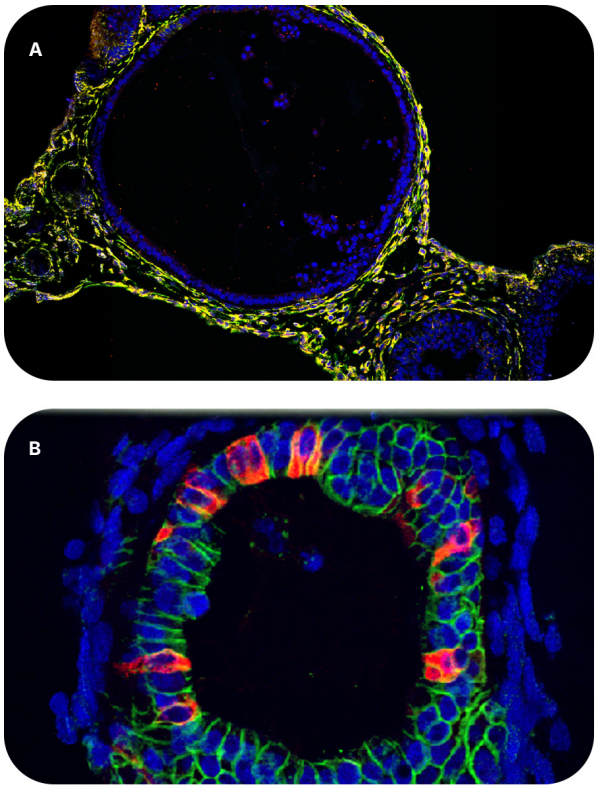

Figure 2. Characterization of iPSC-derived Human Intestinal Organoids. iPSC-derived human intestinal organoids were cultured using Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and the other reagents listed in the intestinal organoid culture recipe shown. (A) Human intestinal organoids were stained using a Rat Anti-Human/Mouse/Rat Vimentin Monoclonal Antibody (green; R&D Systems, Catalog # MAB2105) and a Goat Anti-Human/Mouse Desmin Antigen Affinity-purified Polyclonal Antibody (red; R&D Systems, Catalog # AF3844) to visualize myofibroblast cells and counterstained with DAPI (blue; Tocris Bioscience, Catalog # 5748). (B) Human intestinal organoids were stained using a Goat Anti-Human/Mouse E-Cadherin Antigen Affinity-purified Polyclonal Antibody (green; R&D Systems, Catalog # AF748), a Mouse Anti-Human MUC2 Monoclonal Antibody (red, Novus Biologicals, Catalog # NBP2-44431) and counterstained with DAPI (blue; Tocris Bioscience, Catalog # 5748).

v. Resuspend the segmented organoids in 90% FBS, 5% DMSO, and 10 µM Y-27632 dihydrochloride, and dispense 500 µL of the organoid mixture into each labeled cryovial.

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

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

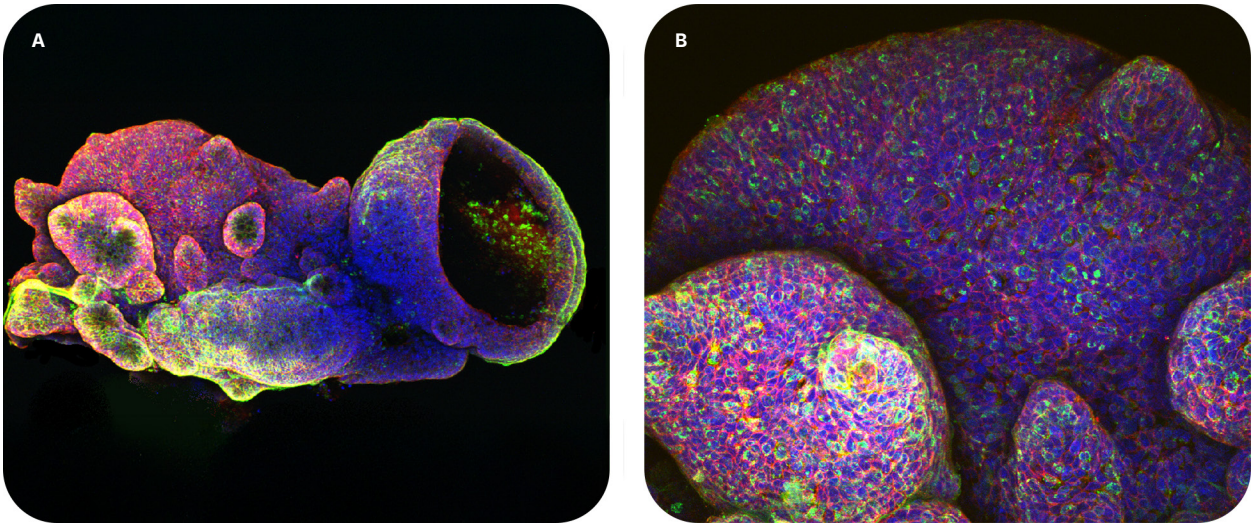

Figure 3. Characterization of Adult Stem Cell-derived Human Descending Colon Organoids. Adult stem cells isolated from human descending colon were embedded in Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and cultured in growth medium for 30 days. (A) Organoids were fixed and stained with a Mouse Anti-Human MUC2 Monoclonal Antibody (green; Novus Biologicals, Catalog # NBP2-44431), to visualize intestinal goblet cells, and counterstained with a Goat Anti-Human/Mouse E-Cadherin Antigen Affinity-purified Polyclonal Antibody (red; R&D Systems, Catalog # AF748) and DAPI (blue; Tocris Bioscience, Catalog # 5748). The image shown was taken at 10x magnification. (B) Organoids were fixed and stained with a Mouse Anti-Human Chromogranin A Monoclonal Antibody (green; R&D Systems, Catalog # MAB90981) to visualize enteroendocrine cells, and counterstained with a Goat Anti-Human/Mouse E-Cadherin Antigen Affinity-purified Polyclonal Antibody (red; R&D Systems, Catalog # AF748) and DAPI (blue; Tocris Bioscience, Catalog # 5748). The image shown was taken at 20x magnification.

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