TREVIGEN®

GASTRIC ORGANOID CULTURE PROTOCOL

THIS PROTOCOL PROVIDES THE PROCEDURE FOR SUBCULTURING NORMAL HUMAN GASTRIC ORGANOIDS WHICH WAS DERIVED FROM THE SUBMERGED METHOD AS DESCRIBED IN BARKER N, ET AL. LGR5+VE STEM CELLS DRIVE SELF-RENEWAL IN THE STOMACH AND BUILD LONG-LIVED GASTRIC UNITS IN VITRO. CELL STEM CELL. 2010 2015/04/28;6(1):25-36.

Materials

Table 1. Materials needed for gastric organoid culture.

Reagent name	Supplier	Cat No.
Cultrex® Reduced Growth Factor Basement Membrane Extract, Type 2 (RGF BME-2)	Trevigen	3533-005-02
Advanced DMEM/F-12 Cell Culture Medium	Invitrogen	12634-010
GlutaMAX-I	Invitrogen	35050-079
HEPES, 1M solution	Invitrogen	15630-080
Penicillin/Streptomycin	Invitrogen	15140-122
B27 supplement	Invitrogen	17504-044
N2 supplement	Invitrogen	17502-048
TrypLE	Invitrogen	12604-021
Fetal Bovine Serum (FBS)	Invitrogen	26140-079
N-Acetylcysteine	Sigma-Aldrich	A9165
[Leu15]-Gastrin I Human	Sigma-Aldrich	G9145
SB 202190 (p38 MAPK inhibitor)	Sigma-Aldrich	S7067
Nicotinamide	Sigma-Aldrich	N0636
Human Insulin, Solution	Sigma-Aldrich	I9278
Human Transferrin	Sigma-Aldrich	T8158
Y-27632 dihydrochloride (Rho Kinase Inhibitor)	Sigma-Aldrich	Y0503
Recombinant Human EGF	Peprotech	AF-100-15
Recombinant Human R-spondin 1	Peprotech	120-38
Recombinant Mouse Noggin	Peprotech	250-38
Recombinant Human FGF10	Peprotech	100-26
A83-01 (ALK5 inhibitor)	R&D Systems	2939
Chir 99021, (GSK-3 inhibitor)	R&D Systems	4423
Wnt3A-Conditioned Medium	see L Wnt-3A (ATCC CRL-2647)	

Equipment

1. Cell Culture Incubator (37 °C, 5% CO2)
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. Cell Scraper, Sterile
8. Pipet Aid and Serological Pipets (5 ml)
9. Micropipets 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. Cell Culture Waste Container

Method

Note: Use aseptic technique at all times during this protocol. Reagents and cultures should only be opened within the Cell Culture Incubator to prevent contamination. This protocol is optimized for gastric organoids; organoids from other tissues may have different culture requirements.

1. Prepare stock solutions for gastric organoid culture:

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

Reagent name	Solvent	Stock solution	Preparation
N-Acetylcysteine	DI water	500 mM = 81.6 mg/ml	816 mg in 10 ml
[Leu15]-Gastrin I Human	1% BSA/PBS	100 µM = 210 µg/ml	500 µg in 2.38 ml
Recombinant Human EGF	1% BSA/PBS	500 µg/ml	100 µg in 200 µl
Recombinant Human R-spondin 1	1% BSA/PBS	1 mg/ml	500 µg in 500 µl
Recombinant Mouse Noggin	1% BSA/PBS	100 µg/ml	100 µg in 1 ml
Recombinant Human FGF10	1% BSA/PBS	100 µg/ml	100 µg in 1 ml
A83-01	DMSO	25 mM = 10.54 mg/ml	10 mg in 949 µl
SB 202190	DMSO	30 mM = 9.9 mg/ml	5 mg in 505 µl
Nicotinamide	DW	1 M = 122.12 mg/ml	6.1 g in 50 ml
Human Transferrin	DW	50 mg/ml	100 mg in 2 ml
Wnt3A-Conditioned Medium	Advanced DMEM/F12, 10% FBS, PSQ	2X	NA
Y-27632 dihydrochloride (Rho Kinase Inhibitor)	PBS	10 mM = 3.2 mg/ml	1 mg in 313 µl
Chir 99021 (GSK-3 inhibitor)	DMSO	20 mM = 9.3 mg/ml	10 mg in 1.08 ml

2. Thaw RGF BME-2 on ice for four hours or overnight in the refrigerator.

3. Prepare Gastric Organoid Culture Medium:
   Note: The recipe below is for 500 ml, but it may be scaled as desired. Medium is stable for three months at 4 °C.

Table 3. Preparation of Gastric Organoid Culture Medium.

Reagent	[Stock]	[Final]	Volume
Advanced DMEM/F-12 Cell Culture Medium	NA	NA	211 ml
Wnt3A-Conditioned Medium	2X	1X	250 ml
B27 supplement	50X	1X	10 ml
GlutaMAX-I	200 mM	2 mM	5 ml
HEPES	1 M	10 mM	5 ml
Penicillin/Streptomycin	100X	1X	5 ml
N2 supplement	100X	1X	5 ml
Nicotinamide	1 M	10 mM	5 ml
N-Acetylcysteine	500 mM	1 mM	1 ml
[Leu15]-Gastrin I Human	100 µM	10 nM	500 µl
Recombinant Human R-spondin 1	1 mg/ml	1 µg/ml	500 µl
Recombinant Mouse Noggin	100 µg/ml	100 ng/ml	500 µl
Recombinant Human FGF10	100 µg/ml	100 ng/ml	500 µl
A83-01	500 µM	500 nM	500 µl
Human Insulin	10 mg/ml	7.5 µg/ml	375 µl
SB 202190	30 mM	10 µM	167 µl
Human Transferrin	50 mg/ml	10 µg/ml	100 µl
Recombinant Human EGF	500 µg/ml	50 ng/ml	50 µl
		Total	**500 ml**

4. Filter medium using the Medium Filtration Unit connected to the Vacuum Pump.
5. Prepare Gastric Organoid Starting/Passaging Medium:
   Note: The recipe below is for 50 ml, but it may be scaled as desired. Medium is stable for three months at 4 °C.

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

Reagent	[Stock]	[Final]	Volume
Gastric Organoid Culture Medium	NA	NA	49.9 ml
Y-27632 dihydrochloride (Rho Kinase Inhibitor)	10 mM	10 µM	50 µl
Chir 99021 (GSK-3 inhibitor)	20 mM	2.5 µM	6.25 µl
		Total	**50 ml**

6. Filter medium using the Medium Filtration Unit connected to the Vacuum Pump.

Starting Organoids from a Cryovial

7. 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.**
8. 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 pipet to resuspend organoids. **Note: Organoids may be counted at this time if needed to determine seeding volumes.**
9. Centrifuge the vial at 500 x g for 3 minutes to pellet gastric organoids, and aspirate medium.
10. Resuspend gastric organoids in RGF BME-2 at 10,000 organoids per ml (500 organoids per well). Pipet up and down three times using a serological pipet to disperse organoids in the RGF BME-2, and dispense 50 µl of the RGF BME-2/organoid mixture in the center of each well of a 24 well plate. **Note: The hydrogel containing organoids should not touch the sides of the well.**
11. Incubate the plate in the Cell Culture Incubator for 25 minutes to polymerize the RGF BME-2 hydrogel.
12. 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 hydrogel to prevent disruption of the hydrogel.**
13. Return plate containing organoid cultures to the Cell Culture Incubator to promote organoid growth.

Gastric Organoid Culture Maintenance

14. The culture medium should be aspirated from each well and replaced with fresh Gastric Organoid Culture Medium every Monday, Wednesday, and Friday, unless passaging (see below). **Note: Medium should be gently aspirated from and pipetted into the corner of the well away from the hydrogel to prevent disruption of the hydrogel.**

Passaging Organoids

15. 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.**
16. Transfer the 24 well plate containing gastric organoids from the Cell Culture Incubator to the Cell Culture Hood.
17. Scrape the entire surface of each well using the Cell Scraper to dislodge the RGF BME- 2 hydrogel containing organoids located in the center of each well.
18. Transfer organoids to 15 ml conical tube(s); cultures of identical tissue and treatment may be combined, if desired.
19. Centrifuge the tube at 500 *x g* at room temperature for 3 minutes.
20. Aspirate the medium without disturbing the RGF BME- 2 hydrogel containing organoids at the bottom of the tube.
21. Add 10-20 volumes of TrpLE Express to each well to digest the RGF BME-2 hydrogel. Each well contained 50 µl of RGF BME-2, so 500 µl of TrpLE Express will be needed per well in the conical tube. For example, one full plate uses 24 wells x 500 µl = 12 ml.
22. Pipet the TrpLE/RGF-BME-2/organoids up and down five times with a serological pipet to mix.
23. Place the conical tube(s) in a 37 °C water bath for 12 minutes to digest the RGF BME-2 hydrogel. **Note: Most of the RGF BME-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.**
24. Add 1/10 volume of FBS to each conical tube to quench the TrpLE Express reaction.
25. Pipet up and down three times with a serological pipet to mix.

26. Pipet the organoid mixture through a Cell Strainer at 100 µm to segment organoids.
27. Centrifuge the tube at 500 *x g* at room temperature for 3 minutes.
28. Aspirate medium, but be careful not to disturb the organoid pellet.
29. Resuspend segmented organoids in RGF BME-2, and dispense 50 µl of the RGF BME-2/organoid mixture in the center of each well of a 24 well plate. **Note: The hydrogel containing organoids should not touch the sides of the well.**
30. Incubate the plate in the Cell Culture Incubator for 25 minutes to polymerize RGF BME-2.
31. 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 hydrogel to prevent disruption of the hydrogel.**
32. Return plate containing organoid cultures to the Cell Culture Incubator to promote organoid growth.