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MD Anderson Cancer Center
Making Cancer History®

Enteroid Maintenance Protocol

Reagent

Reagent name	Supplier	Cat No.	Solvent	Stock solution	final conc
Matrigel, GFR, phenol free	Corning	356231			
GlutaMAX-I	Invitrogen	35050-061		200 mM	2 mM
Penicillin/Streptomycin	Invitrogen	15140-122		100X	1x
N2 supplement	Invitrogen	17502-048		100x	1x
B27 supplement	Invitrogen	17504-044		50x	1x
N-Acetylcysteine	Sigma-Aldrich	A9165-5G	dd H2O	500 mM	1 mM
mouse recombinant EGF	Invitrogen	PMG8043	PBS	50 microg/ml	50 ng/ml
A-83-01	Tocris	2939	DMSO	500 microM	500 nM
SB202190	Sigma-Aldrich	S7067	DMSO	10 mM	10 microM
Nicotinamide	Sigma-Aldrich	N0636	ddH2O	1M	10 mM
[Leu15]-Gastrin I	Sigma-Aldrich	G9145	PBS	10 microM	10 nM
HEPEs 1M	Invitrogen	15630-080		1M	10mM
Advanced DMEM/F12	Invitrogen	12634-028			
Recovery Cell Freezing Media	Invitrogen	12648-010			

All stock solutions and aliquot are stored in -20^0 C. Matrigel is aliquoted and stored in -20^{0}C

Equipment

	Cat No.
24 well Nunclon delta surface tissue culture dish	Thermo Scientific 142475
Refrigerated centrifuge with swing rotor	

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Sterilized filter pipette tips	
BD 1ml TB syringe	309626

Enteroid Medium

CMGF- (complete media without growth factor): Keep at 4^0C for up to 4 weeks

- 500 ml Advanced DMEM/F12 (contains albumin, and ITS)
- 5 ml Glutamax 100x
- 5 ml HEPES 1M
- 5 ml Pen/Strep

CMGF+ (complete media with growth factor): keep at 4°C for up to 2 weeks, amount listed are for 10ml

- 1.5 ml CMGF-
- 5 ml Wnt 3 conditioned media (ATCC L-Wnt3a cell line Cat #CRL-2647)
- 200 ul B27 (50x)
- 100 ul N2 (100x)
- 20 ul n-acetylcysteine (500mM)
- 2 ml Rspo-1 conditioned medium (obtained cell line from Dr. Calvin Kuo, Stanford University)
- 1 ml Noggin (obtained cell line from Dr. Muncan V. Van den Brink GR)
- 10 ul EGF (1000x final conc. 50 ng/ml)
- 10 ul Gastrin (1000x final conc. 10 nMI)
- 100 ul Nicotinamide (final conc. 10 mM)
- 10 ul A83 (TGFb type I receptor inhibitor) (1000x final conc. 500 nM)
- 10 ul SB202190 (P38 inhibitor) (1000x final conc. 10uM)

Note: For Gastroid and Ileal enteroids culture, the cells grow best using High Wnt (50% CMGF+ and 50% Wnt conditioned medium).

Differentiation media: CMGF+ without Wnt 3a, R-spondin, Nicotinamide and SB202190, reduce Noggin conditioned medium to half of the concentration.

For culturing Wnt3a, R-Spondin and Noggin secreting cell lines, please refer to separate protocol.

Procedure

Enteroids maintenance protocol v5- 11/18/16 Saved at Data (//storage/bcm-mvm-public)-Lab Estes-Protocols-Enteroids

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Revive enteroids from Liquid Nitrogen

1. Thaw matrigel aliquot overnight at 4^0C
2. Add 10ml of CMGF- into 15ml corning tube, leave tube on ice
3. Transfer frozen vial containing entroids from LiN_2 to dry ice
4. Hold vial under room temperature tap water till ice detaches from the vial wall
5. Transfer contents in the vial to 15ml tube containing 10ml cold CMGF- using 2ml pipet
6. Spin down in refrigerated swing rotor centrifuge at 80g (845rpm) at for 5 min at 4^0C
7. Take off medium and leave 15ml tube containing enteroids pellet on ice. Resuspend pellet in 120ul matrigel (enough to seed 4 wells, 30ul/well) using cold P200 pipet tips, plate enteroids as droplets in 4 wells of 24 well plate and transfer plate to 37^0C incubator. Let gel settle for 5-10minutes, add 500ul of room temperature CMGF+ to each well and culture in 37^0C incubator.
8. Refresh culture with CMGF+ every other day until it's ready to be passaged.

Passage enteroids (usually 1:3, following protocol is for one well only, repeat with other wells)

1. Usually after 6-7 days, enteroids are ready to be passaged
2. Remove medium from wells (around the solid Matrigel) with P1000 pipet
3. Add 500ul cold CMGF- to well and mechanically break up Matrigel with pipetting P1000 up and down couple of times
4. Then using 1ml syringe with needle 25Gx5/8 , up and down 1-2 times in each well, then transfer whole contents into 15ml tube (if you have more wells to passage, you can combine wells into same tube), add 2x more cold CMGF-
5. Spin down in refrigerated swing rotor centrifuge at 80g (845rpm) at for 5 min at 4^0C
6. Remove all medium. Keep tube on ice.
7. Resuspend entroids pellet in Matrigel (calculate the amount of matrigel you will need, 30ul/well) using cold P200 pipet tips.
9. Pipet 30ul/well of enteroids matrigel mixture as droplet into 24 well plate using cold P200 pipet tips. Transfer plate into 37^0C incubator. Let gel settle for 5-10minutes, add 500ul of room temperature CMGF+ to each well and culture in 37^0C incubator.
10. Refresh culture with CMGF+ every other day.

Freeze enteroids (usually after 6-7 days in culture, combine two wells of enteroids into 1 cryovial)

1. Usually after 6-7 days, enteroids are ready to be passaged.

Enteroid Maintenance Protocol

2. Remove medium from wells (around the solid Matrigel) with P1000 pipet
3. Add 500ul cold CMGF- to well and mechanically break up Matrigel with pipetting P1000 up and down couple of times
4. Spin down in refrigerated swing rotor centrifuge at 80g (845rpm) at for 5 min at 4^0C
5. Remove all medium. Keep tube on ice.
6. Resuspend enteroids into freezing medium (each cryovial using 500ul) at original ratio of 2 wells into 1 vial
7. Keep cryovial into cell freezing container and keep in -80c for at least overnight. Next day transfer vials into LiN2

Enteroids Differentiation

1. Usually after 4 days in culture, switch CMGF+ to differentiation medium for 3-5 days, refresh medium every other day.