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ADSI –CCO-SOP-1.0

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INSTRUMENTATION, REAGENTS AND MEDIA FOR COLON ORGANOID CULTIVATION

Purpose

The SOP-ADSI-1.0 was issued to describe the preparation of the solutions and medias required to generate and maintain organoids from colon cancer samples and the respective healthy tissues.

Scope

SOP 1.0 is intended to cover all resources, personnel and equipment needed to generate the medias and solutions for the generation, maintenance and cryopreservation of organoids from fresh tissue samples of colon cancer patients.

Introduction

This protocol provides a summary of all medias required to establish a functional organoid culture system.

1. Equipment

- Incubator Binder APT.line™ (150CE2)
- Laminar Air-Flow Labculture Plus ESCO, Class II BSC
- Micro Plate Reader (Mithras LB940)
- Centrifuge (4°C) Hettich Zentrifugen ROTIXA A50 RS
- Mixer at 4°C
- Thermomixer
- Brightfield microscope Motic AE31 and camera ProgRes CF cool JENOPTIKA
- Microwave
- Table centrifuge VWR Mini Star
- Analog Vortex Mixer (VWR)
- Waterbath

- 24 well tissue culture plates (Greiner bio-one #662160)
- 96-well-plate (Corning #3603)
- 10 cm petri dishes (SARSTEDT #83.3902)
- Sterile syringes, 10 ml (Braun #4606108V)
- Sterile Syringe Filters 0.45 µm PES
- pluriStrainer 100um (pluriSelect #43-50100-03)
- pluriStrainer 400um sterile (pluriSelect #43-50400-03)
- pluri-Connector Ring (pluriSelect #41-50000-03)
- cell lifter (Costar #3008)
- Neubauer - Cell Counting Chamber (A. Hartenstein ZK06)
- Sterile forceps
- Sterile scissors
- Sterile scalpel + blades
- Sterile Erlenmeyer flask (200 ml)
- Sterile 50 ml bottle
- Sterile 15 ml tubes
- Bottle Top Filter (SARSTEDT #83.1823.101)
- Sterile 50 ml tubes
- Sterile 0,5 ml, 1,5 ml, 2 ml and 5 ml tubes
- Cryovials (STAR LAB #E.3110-6122)
- Mr. Frosty freezing container (ThermoFisher Scientific #5100-0001)
- Set of pipettes 10 µl, 200 µl, 1000 µl and pipette tips
- Pipette Aid and pipettes (10 ml, 25 ml, 50 ml)
- Glass cover slips (round)
- Glass slides
- Box with crashed ice

3. Reagent

- Advanced DMEM/F-12 (Gibco-Thermo Fisher #12634028)
- Glutamax 100 x (GIBCO #35050038)
- HEPES (1 M) (GIBCO #15630080)
- Pen/Strep 100 x (GIBCO #15070063)
- DPBS, 1 x no calcium, no magnesium (Gibco #14190-094)
- Sterile water
- Freezing Medium (GIBCO-Thermo Fisher #12648010)
- 0.25 % Trypsin-EDTA (Sigma-Aldrich #T4049-100ML)
- Trypan Blue
- EDTA (Sigma Aldrich #431788-25G)
- WCM – Wnt3a conditioned media (Broutier 2016)*
- RCM – R-Spondin conditioned media (Broutier 2016)**
- NCM – Noggin conditioned media (Broutier 2016)***

*Wnt3a conditioned medium was obtained from L-Wnt3a cells. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

**R-spondin1-conditioned medium was produced from 293T-HA-Rspol-Fc producer cell line; MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

***Noggin-conditioned medium from HEK293-mNoggin-Fc cell line. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

- Matrigel (Corning #356231)
- DMSO Hybri-Max (Sigma #D2650)
- Nicotinamide (Sigma-Aldrich #N0636-100G)
- N-Acetylcyteine (Sigma-Aldrich #A9165-25G)
- B-27 (Thermo Fisher #17504044)
- A83-01 (R&D Systems Europe #2939)
- SB 202190 (Sigma Aldrich S7067-5MG)
- Primocin – 500 mg (InvivoGen Cat. Code: ant-pm-1)
- *R-Spondin (PREPROTECH #120-38) optional instead of RCM (100 ng/ml)*
- *Noggin (PREPROTECH #250-38) optional instead of NCM (1000 ng/ml)*
- *Wnt3a – if required 100 ng/ml (PEPROTECH)*
- m-EGF (PREPROTECH #315-09)
- Real Time – GloTM MT Cell Viability Assay (Promega #G9711)
- Gastrin (R&D Systems Europe #3006/1)
- Prostaglandin E2 (Sigma-Aldrich #P0409)
- STEMPRO hESC (Fisher A1000701 (zusätzl. angereichert mit FCS+Pen/Strep/L-Glu+Primocin)
- Liberase DH (Roche #5401089001)
- Fetal Bovine Serum (Biowest #S1810-500)
- Triton X-100
- BSA – Albumin Fraktion V (Roth #8076.4)

4. Reagent setup

All buffer and solutions are prepared under sterile conditions in a laminar air-flow cabinet

- **Matrigel**
  Thaw 10 ml Flask of Matrigel on ice
  Make aliquots: 10 x 1 ml in cryotubes
  Store at -20°C
- **Pen/Strep (100x)**
  (long term storage -20°C)
  Store at 4°C
- **HEPES (1 M = 100x)**
  Store at 4°C
- **Glutamax (100x)**
  (long term storage at -20°C)
  Store 4°C
- **GF- (Growth Factors – Medium = Washing Medium)**
  composition
  Advanced DMEM/F12 - 500ml
  Pen/strep (100x) - 5ml
  HEPES (1M=100x) - 5ml
  Glutamax (100x) - 5ml
- **0.25% Trypsin-EDTA**
  Thaw 100 ml and make 5 ml aliquots
  Store at -20°C
  Once thawn store at 4°C

- **Freezing medium**
  Thaw 50ml and make 5-10ml aliquots
  Store at -20°C
  Once thawn store at 4°C
- **PBS + Pens/Strep**
  10 ml PBS + 100µl Pen/Strep (100x)
  Dissolve in 10ml PBS without Calcium and Magnesium
  Filter sterilize (0.22µm)
  Make 2ml aliquots
  Store at -20°C
- **Chelation Solution**
  10ml PBS + 200µl 0.5M EDTA
- **PBS/0.1 % BSA**
  10 ml PBS + 10mg BSA
- **N-Acetyl cysteine solution**
  3ml sterile water
  245mg N-Acetyl cysteine
  Dissolve by shaking and leave at RT
  Filter sterilize 0,22µm
  Make 500 µl aliquots in Eppendorf tubes and store at -20°C
  (1250µl for 500 ml culture medium)
- **Nicotinamide (1 M = 100 x)**
  1,464 g
  Fill up to 12 ml with 1 x PBS
  Dissolve by shaking and leave at RT
  Filter sterilize 0,22 µm
  Make aliquots 500 µl and store at -20°C
  (5000 µl for 500 ml culture medium = 10 mM)
- **B27 (50 x)**
  Thaw on ice
  Prepare 2 ml aliquots
  Store at -20°C
  (1000 µl for 500 ml culture medium)
- **Primocin (500 x)**
  Store at -20°C
  (80µl for 40ml culture medium = 2x)
- **Y-27632 (10 mM = 1000 x)**
  10 mg
  Dissolve in 3122 µl sterile water
  Make 400µl aliquots (10 mM)
  (40µl in 40 ml culture medium = 10 µM)
  Store at -20°C
- **Gastrin (100 µM = 10000 x)**
  Dissolve 1mg gastrin in 4,77 ml PBS (without Calcium and Magnesium)
  Make 50 µl aliquots or smaller
  (4 µl for 40 ml culture medium = 10 n M)
  Store at -20°C
- **PGE2 (100 µM = 10000 x)**
  Dissolve 1mg PGE2 in 4,77ml PBS (without Calcium and Magnesium)
  Make 50µl aliquots or smaller
  (4µl for 40 ml culture medium = 10 nM)
  Store at -20°C
- **m-EGF (500 µg/ml = 10000 x)**
  Dissolve 500µg m-EGF in 1000 µl PBS/0.1 % BSA
  Make 50µl aliquots, store at -20°C
  (4µl in 40ml culture medium or 50µl in 500 ml culture medium = 50 ng/ml)
- **A83-01 (major stock = 50 mM and 1,5 mM = 3000 x)**
  Stock:
  Dissolve 10 mg A83-01 in 474 µl DMSO
  Make 18 µl aliquots (50 mM)
  Store at -20°C
  Pre-dilution stock:
  Quickly thaw a 18 µl major stock aliquot
  Dilute with 582 µl DMSO
  Make 13,3 µl aliquots (1,5 mM = 3000 x)
  (13,3 µl for 40ml culture medium)
  Store at -20°C
- **SB202190 (30 mM = 3000 x)**
  Dissolve 5 mg SB202190 in 503 µl DMSO
  Make 13,3 µl aliquots
  (13,3 µl for 40 ml culture medium)
  Store at -20°C
- **WCM (50%)**
  Freshly prepared (every 2 weeks)
  Store at 4°C
- **RCM**
  Store at - 20°C
  Thaw on ice
  (4 ml for the generation of 40 ml media – see media generation for details)
- **NCM**
  Store at -20°C
  Thaw on ice
  (2 ml for the generation of 40 ml media – see media generation for details)
- **PBS + Pen/Strep + Primocin:**
  500 ml DPBS
  5 ml Pen/Strep
  prepare aliquots of 50 ml (falcon tubes)
  add 100 µl of Primocin in 50ml
  Store at 4°C
- **DMEM + Pen/Strep + Primocin:**
  500 ml DMEM
  5 ml Pen/Strep
  make aliquots (50 ml tube)
  100 µl of Primocin in 50 ml
  Store at 4°C
- **DMEM + P/S + FBS:**
  500 ml DMEM

5 ml Pen/Strep
60 ml FBS
make aliquots (50 ml tube)
100 µl of Primocin in 50 ml
Store at 4°C

- **RBC Lysis Buffer 1X:**
  300 µl of RBC Lysis Buffer
  2.7 ml of sterile H2O

5. Procedure for Media preparation

5.1 Media composition for intestinal WT organoids (Colon, Rectum)

Compound	Seeding Medium (SM)	Expansion Medium (EM)	Differentiation Medium (DM)	Basal medium	Basal medium
GF- medium	x	x	x	x	
RCM	10%	10%	10%	x	
NCM	5%	5%	5%	x	
Nicotinamide	10mM	10mM		x	
N-acetyl	1,25mM	1,25mM	1,25mM	x	
B27	1x	1x	1x	x	
WCM	50%	50%			x
mEGF	50ng/ml	50ng/ml	50ng/ml		x
Y-27632 (ROCK-inh)	10µM	10µM			x
A83-01 (TGFß-inh)	500nM	500nM	500nM		x
SB202190 (P38 inh)	10µM	10µM			x
Gastrin	10nM	10nM	10nM		x
PGE2	10nM	10nM	10nM		x
Primocin	100µg/ml	100µg/ml	100µg/ml		x

Table 1: Media composition for intestinal organoids (seeding medium (SM), expansion medium (EM) and differentiation medium (DM). SM is used within the first days of organoid culture and contains apoptosis inhibitors to ensure stem cell survival. EM is used for organoid culture expansion and keeps the stemness of the cells. The DM is used to differentiate the organoids into which recapitulates the epithelial or tumor tissue of the patient.

5.2 Media generation for intestinal WT organoids

5.2.1 Seeding Medium (SM) and Expansion Medium (EM) – for intestinal WT organoids (Colon, Rectum)

Preparation of basal medium
Prepare a sterile 500ml Erlenmeyer flask. Thaw RCM, NCM, B27,

Nicotinamide and N-Acetylcysteine on ice. Add the required amounts as indicated in ***Table 2***. N-ACETYLCYSTEINE IS VERY ACIDIC – ADD AT THE END! Make 20ml aliquots in 50ml tubes. Label properly (Intestinal-SM/EM without growth factors, date, operator) and freeze at -20°C. Aliquots are stable for up to 12 months at -20°C.

Preparation of complete ready to use media

Thaw respective basal medium aliquot on ice, add growth factors (thaw at RT) as indicated in ***Table 2***. Mix thoroughly and keep at 4° C. Avoid repeated cycles of warming up. WCM must not be kept longer than 2 weeks at 4° C.

5.2.2 Differentiation Medium (DM) – for intestinal WT organoids (Colon, Rectum):

Preparation of basal medium

Prepare a sterile 1000 ml Erlenmeyer flask. Thaw RCM, NCM, B27 and N-Acetylcysteine on ice. Add the required amounts as indicated in ***Table 2***. N-ACETYLCYSTEINE IS VERY ACIDIC – ADD AT THE END! Make 20 ml aliquots in 50 ml tubes. Label properly (Intestinal DM without growth factors, date, operator) and freeze at -20°C. Aliquots are stable for up to 12 months at -20°C.

Preparation of complete ready to use media

Thaw respective basal medium aliquot on ice, add growth factors (thaw at RT) as indicated ***Table 2***. Mix thoroughly and keep at 4° C. Avoid repeated cycles of warming up. WCM must not be kept longer than 2 weeks at 4° C.

Table 2: Reagent volumes for media preparation for intestinal organoids

Compound	Seeding Medium (SM)	Expansion Medium (EM)	Differentiation Medium (DM)	Basal medium	Complete ready to use medium
GF- medium	217ml	217ml	727ml	x	
RCM	100ml	100ml	100ml	x	
NCM	50ml	50ml	50ml	x	
Nicotinamide	10ml	10ml	-	x	
N-acetyl	2,5ml	2,5ml	2,5ml	x	
B27	20ml	20ml	20ml	x	
WCM	20ml	20ml	-		x
mEGF	4µl	4µl	4µl		x
Y-27632 (ROCK-inh)	40µl	-	-		x
A83-01 (TGFß-inh)	13,3µl	13,3µl	13,3µl		x
SB202190 (P38 inh)	13,3µl	13µl	-		x
Gastrin	4µl	4µl	4µl		x
PGE2	4µl	4µl	4µl		x
Primocin	80µl	80µl	80µl		x

5.2.3 Generation of basal media and complete ready to use media for intestinal tumor organoids - Expansion Medium (M2-M6)

Preparation of basal medium

Prepare a sterile 500ml Erlenmeyer flask. Thaw RCM, NCM, B27, Nicotinamide and N-Acetylcysteine on ice. Add the required amounts as indicated in the table. N-ACETYLCYSTEINE IS VERY ACIDIC – ADD AT THE END! Make 20ml aliquots in 50ml tubes. Label properly (BM Basal Medium without growth factors, date, operator) and freeze at -20°C. Aliquots are stable for up to 12 months at -20°C.

Preparation of complete ready to use media

Thaw respective basal medium aliquot on ice, add growth factors (thaw at RT) as indicated in ***Table 3***. Mix thoroughly and keep at 4° C. Avoid repeated cycles of warming up. WCM must not be kept longer than 2 weeks at 4° C.

Compound	M2	M3	M4	M5	M6	Basal medium	Complete ready to use medium
GF- medium	217ml	217ml	217ml	217ml	727ml	x	
RCM	100ml	100ml	100ml	100ml	100ml	x	
NCM	50ml	50ml	50ml	50ml	50ml	x	
Nicotinamide	10ml	10ml	10ml	10ml	10ml	x	
N-acetyl	2,5ml	2,5ml	2,5ml	2,5ml	2,5ml	x	
B27	20ml	20ml	20ml	20ml	20ml	x	
WCM	-	-	-	-	20ml		x
mEGF		4µl		4µl	4µl		x
Y-27632	40µl	40µl	40µl	40µl	40µl		x
A83-01			13,3µl	13,3µl	13,3µl		x
SB202190		13µl	-	13µl	13µl		x
Gastrin	4µl	4µl	4µl	4µl	4µl		x
PGE2					4µl		x
Primocin	80µl	80µl	80µl	80µl	80µl		x
GF-medium	20ml	20ml	20ml	20ml	-		x

Table 3: Reagent volumes for the media preparation of tumor organoid expansion media

5.2.4 Generation of basal media and complete ready to use media for intestinal tumor organoids – Differentiation Medium (M2-M6)

Preparation of basal medium

Prepare a sterile 500ml Erlenmeyer flask. Thaw RCM, NCM, B27, and N-Acetylcysteine on ice. Add the required amounts as indicated in the table. N-ACETYLCYSTEINE IS VERY ACIDIC – ADD AT THE END! Make 40ml aliquots in 50ml tubes. Label properly

(BM-DM Basal Medium - Differentiation Medium, without growth factors, date, operator) and freeze at -20°C. Aliquots are stable for up to 12 months at -20°C.

Preparation of complete ready to use media

Thaw respective basal medium aliquot on ice, add growth factors (thaw at RT) as indicated in ***Table 4***. Mix thoroughly and keep at 4° C. Avoid repeated cycles of warming up.

Table 4: Reagent volumes for the media preparation of tumor organoid differentiation media

Compound	M2	M3	M4	M5	M6	Add to big batch	Add to aliquot
GF- medium	727ml	727ml	727ml	727ml	727ml	x	
RCM	100ml	100ml	100ml	100ml	100ml	x	
NCM	50ml	50ml	50ml	50ml	50ml	x	
N-acetyl	2,5ml	2,5ml	2,5ml	2,5ml	2,5ml	x	
B27	20ml	20ml	20ml	20ml	20ml	x	
mEGF		4µl		4µl	4µl		x
A83-01			13,3µl	13,3µl	13,3µl		x
Gastrin	4µl	4µl	4µl	4µl	4µl		x
Primocin	80µl	80µl	80µl	80µl	80µl		x

6. Applicable references

ADSI –CCO-SOP-2.0, ADSI –CCO-SOP-3.0, ADSI –CCO-SOP-4.0

7. Reference

- Fujii, M., et al. (2016). Colorectal Tumor Organoid Library Demonstrates Progressive Loss of Niche Factor Requirements during Tumorigenesis. Cell Stem Cell 18, 1-12.
- Sato, T., et al. (2011). Long-term Expansion of Epithelial Organoids from Human Colon, Adenoma, Adenocarcinoma, and Barrett's Epithelium. Gastroenterology 141, 1762-1772
- Broutier L, Andersson-Rolf A, Hindley C J, Boj S F, Clevers H, Koo B K, Huch M. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. Nat Protoc. 2016 Sep;11(9):1724-43. doi: 10.1038/nprot.2016.097. Epub 2016 Aug 25.