Grant agreement no. 668294
PHC-14-2015 'New therapies for rare diseases'

- Research and Innovation Action -

D4.4 - SOP for the cell culture methods

WP 4 – Good Manufacturing Practice (GMP) processing and scaling up

Due date of deliverable: month 60

Actual submission date: 29/04/2021

Start date of project: 01/01/2016 Duration: 72 months

Lead beneficiary for this deliverable: UCL

Last editor: Lucinda Tullie

Contributors: Isobel Massie, Laween Meran, Lucinda Tullie, Paolo De Coppi, Vivian Li

Dissemination Level		
PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

History table

Version	Date	Released by	Comments
1.0	24/5/2020	Lucinda Tullie	

Table of contents

Key word list

Human intestinal organoids
Human intestinal fibroblasts
Bioreactor

Definitions and acronyms

Acronyms	Definitions
BME	Basement membrane extract
GMP	Good manufacturing practice
SI	Small intestine
Adv DMEM	Advanced Dulbecco's Modified Eagle Medium/F12 supplemented
PBS	Phosphate buffered saline
RT	Room temperature
DMEM	Dulbecco's Modified Eagle Medium
BSA	Bovine serum albumin

1. Introduction

1.1 General context

This deliverable (4.4) refers to development of a standard operating procedure for isolating human intestinal organoids and fibroblasts in culture from tissue obtained as per WP 4.1. It also details a protocol for expansion of both organoids and fibroblasts and their subsequent seeding onto acellular scaffold for the purpose of generating intestinal mucosal grafts for either subsequent transplantation or functional testing in vitro.

INTENS aims to generate tissue engineered intestine and in order to engineer autologous intestinal grafts, robust protocols are required for isolation and expansion adult intestinal stem cells, as organoids, and stromal cells (fibroblasts) for subsequent seeding onto scaffold.

1.2 Deliverable objectives

This deliverable is relevant to the objective to standardise organoid and fibroblast isolation and culture to work towards meeting Good Manufacturing Practice (GMP) requirements. to enable clinical translation.

2. Methodological approach

Establishment of human small intestinal organoid cultures from biopsies

Materials:

Tissue biopsy/surgical tissue from paediatric small intestine
Advanced Dulbecco's Modified Eagle Medium/F12 supplemented with 10mM HEPES (Invitrogen # 15630-056), 2mM GlutaMax (Invitrogen # 35050-038), 5% penicillin-streptomycin (Invitrogen #15140-122) (AdvDMEM+++)
0.1% BSA (bovine serum albumin) in PBS
Chelation buffer 5x (see recipe below)
Chelation buffer 1x (see recipe below)
0.5M EDTA
1M DTT
Distilled water
PBS and penicillin-streptomycin 5%
PBS with Ca and Mg
BME (Cultrex)

15 and 50ml falcon tubes
10cm petri dish
Disposable scalpels
24-well cell culture plate
Y-27632 Rho kinase inhibitor (Y-27632 dihydrochloride, Tocris, cat number 1254)
Dulbecco's Modified Eagle Medium/ supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ThermoFisher), 5% penicillin-streptomycin, 1x insulin-(AdvDMEM+++)
Basal human intestinal organoid medium
Intesticult™ (Stemcell technologies)

Chelation buffer 5x:
Dissolve the following in 500ml distilled water:
Na2HPO4 at 28mM (1.97g)
KH2PO4 at 40mM (2.7g)
NaCl at 480mM (14g)
KCl at 8mM (0.38g)
Sucrose at 220mM (37.5g)
D-sorbitol at 274mM (25g)

Check pH is 7 to 7.3, pass through sterile filter and store at 4°C

Chelation buffer 1x:

Volume required (ml)	**50**	**100**	**500**
5x chelation buffer (ml) (final conc. 1x)	10	20	100
Water (ml) (final conc. 80%)	40	80	51
1M DTT (µl) (final conc. 0.5µM)	25.5	51	255
0.5M EDTA (ml) (final conc. 10µM)	1	2	10

1. Collect tissue biopsy samples in 50ml falcon with 25ml AdvDMEM+++ on ice*
   **Isolate organoids as soon as possible for maximal efficiency and at least within 24 hours*
2. Transfer tissue into petri dish and cut into 1mm^3 pieces using scalpel
3. Pre-coat 10ml pipette with 0.1% BSA and transfer tissue into 15ml falcon
4. Wash x3 with PBS by pipetting up and down* (approx. 15 times and leaving tissue to settle to bottom of falcon) until supernatant is clear
   **Take care to pre-coat each pipette with 0.1% BSA to avoid tissue pieces adhering to pipette*
5. Add 11ml of 1X chelation buffer to each 15ml falcon
6. Rotate at 15rpm at 4°C for 1 hour
7. Let tissue settle to bottom of 15ml falcon and aspirate chelation buffer
8. Add 9ml PBS and "ketchup shake' the falcon tubes until the supernatant is cloudy

9. Collect supernatant* (leaving tissue pieces at base of falcon – save these for plating for fibroblasts)
   **Option of checking for crypts by adding 10ul supernatant to a microscope slide under light microscope (10x magnification) – crypts appear as U-shaped cell clusters. If minimal crypts, to improve yield repeat steps 5-9 with residual tissue pieces in falcon.*
10. Add 4ml PBS (with Ca and Mg) to supernatant (ratio of 1 PBS:2 supernatant) to inactivate chelation buffer
11. Centrifuge at 1000rpm at 4°C for 5 mins
12. Wash pellet in AdvDMEM +++ supplemented with 5% penicillin-streptomycin
13. Centrifuge at 1000rpm at 4°C for 5 mins
14. Aspirate supernatant and resuspend pellet in undiluted BME and plate 30µl/well (3 drops) in a pre-warmed 24-well cell culture plate*
    **The volume of BME depends on the size of the pellet but from primary tissue of approximately 30mg, 300ul BME is used. Take care not to plate the crypts too densely approx. 10,000 crypts/droplet of BME. A "test" droplet of pellet suspended in BME can be plated and checked under a light microscope (x10 magnification) and, if the crypts are too dense, further BME added before further plating of BME as in step 14.*
15. Place plate upside down in 37°C 5% CO_2 incubator for 20-30mins for BME droplets to solidify
16. Add either basal human intestinal organoid culture medium or Intesticult 500µl/well supplemented with 10uM Rho Kinase inhibitor and return plate to incubator
17. Plate left over tissue pieces (from step 9) on 10cm culture plate/s
18. Leave at RT for 20 mins to ensure tissue adheres to plate
19. Add DMEM +++ and place in 37°C 5% CO_2 incubator. Fibroblasts should grow out from the tissue within 3-4 days.

Table 1: Basal human intestinal organoid culture medium:

Wnt 3a:	Conditioned medium made in-house by Stienke vd Brink from L-Wnt-3a cell line from Marc vd Wetering in DMEM 10% FBS.
Rspondin:	Conditioned medium made in-house by Stienke vd Brink from 293T-HA-Rspol-Fc cell line from Akifumi Ootani made in the Calvin Kuo in Stanford.
Noggin:	Conditioned medium made in-house
B27 supplement:	Invitrogen #175504-044
Nicotinamide:	Sigma N0636
N-Acetylcysteine:	Sigma-Aldrich A9165
TGF-ß receptor inhibitor:	Tocris #2939
p38 SB202190 inhibitor:	Sigma-Aldrich S7067
EGF:	Invitrogen Biosource PMG8043
Gastrin I:	Sigma G9145

	250µl	**1ml (µl)**	**5ml (µl)**	**10ml (µl)**	**20ml (µl)**	**50ml (µl)**
Wnt 3a conditioned media (final conc. 50%)	125	500	2.5ml	5ml	10ml	25ml
AdvDMEM +++ (final conc. 20%)	50	200	1000	2ml	4ml	10ml
Rspondin conditioned media (final conc. 20%)	50	200	1000	2ml	4ml	10ml
Noggin conditioned media (final conc. 10%)	25	100	500	1000	2ml	5ml
B27 supplement (50x) (final conc. 1x)	5	20	100	200	400	1000
Nicotinamide 1M (final conc.10mM)	2.5	10	50	100	200	500
N-Acetylcysteine 500mM (final conc.1.25mM)	0.625	2.5	12.5	25	50	125
TGF-ß receptor inhibitor (A83) 0.5mM (final conc. 500nM)	0.25	1	5	10	20	50
p38 SB202190 inhibitor 30mM (final conc. 10µM)	0.083	0.332	1.66	3.32	6.64	16.6
EGF (500ug/ml) (final conc. 50ng/ml)	0.025	0.1	0.5	1	2	5
Gastrin I 100µM (final conc.10nM)	0.025	0.1	0.5	1	2	5
Total volume (µl):	**258.508**	**1034.032**	**5170.16**	**10340.32**	**20680.64**	**51701.6**

Splitting and expansion of human intestinal organoids[1]

Materials:

AdvDMEM+++
Heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich)
TryPLE™ Express Enzyme 1x (Gibco 12604013)
BME (Cultrex)
15ml falcon tubes
24-well cell culture plate
Y-27632 Rho kinase inhibitor (Y-27632 dihydrochloride Tocris #1254)

1. Aspirate media from wells
2. Add 1ml AdvDMEM+++ to each well and disrupt the BME droplets using P1000 pipette
3. Transfer to a 15ml falcon (3 wells of 24 well plate/15ml falcon) and add AdvDMEM+++ to al total volume of 5ml
4. Centrifuge at 900rpm at 4°C for 5 mins
5. Aspirate the supernatant*
   **BME accumulates over the organoid pellet as a cloudy mesh – the key is to aspirate as much BME as possible without also removing the organoid pellet. If the BME/organoid pellet are difficult to separate then resuspend the BME/pellet in AdvDMEM+++, leave on ice for 10mins and then repeat steps 4&5*
6. Add TryPLE (1ml/15ml falcon) and resuspend the pellet
7. Place 15ml falcon in water bath at 37°C for 5-10 mins*
   **Check every 2-3mins for a "snowstorm" appearance of the TryPLE – once this appears then remove the falcon from the waterbath*
8. Cease the trypsin reaction by removing the falcon from the waterbath, placing on ice and adding 2ml AdvDMEM+++ and 10% FBS (ratio of 1:2 TryPLE: AdvDMEM+++ and FBS) Mix thoroughly with P1000
9. Centrifuge at 1000rpm at 4°C for 5 mins
10. Resuspend pellet in BME 300-400µl and plate 30µl/well (3 drops) in a pre-warmed 24-well cell culture plate *
    **Split ratio is 1:4-1:6 (of BME volume) in fully established cultures depending on confluency*
11. Place plate upside down in 37°C 5% CO_2 incubator for 20-30mins for BME droplets to solidify
12. Add organoid medium 500µl/well supplemented with 10uM Rho Kinase inhibitor – refresh the medium every 2-3 days

Table 2: BME volumes for different multi-well plates

Number of wells in plate	BME volume/well (µl)	Domes/well	Culture medium/well (ml)
6 wells	200	10-15	2
12 wells	100	5-7	1
24 wells	50	1-3	0.5
48 wells	25	1	0.25
96 wells	5-10	1	0.1

Splitting and expansion of human intestinal fibroblasts[2]

Materials:

Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% pencillin-streptomycin and 1x insulin-transferrin-selenium solution (DMEM+++)
TryPLE™ Express Enzyme 1x (Gibco 12604013)
15ml falcon tubes
T175 cell culture flasks

1. Aspirate media from flask/plate containing confluent fibroblasts
2. Add TryPLE (2ml/10cm^2 plate, 3ml/T75cm^2, 5ml/T175cm^2 flask) and place in 37°C 5% CO_2 incubator for 5-10mins*
   **During this time check under the microscope to monitor the progress of the reaction. Once fibroblasts have the appearance of round single cells under the microscope remove plate/flask from incubator and agitate/tap flasks to release fibroblasts so they are free floating*
3. To cease the trypsin reaction add DMEM+++ to the flask/plate in a 2:1 ratio with the TRyPLE (i.e. for a T175cm^2 flask add 10ml DMEM+++) – ensure they are well mixed
4. Transfer the contents of the plate/flask into a 15ml falcon and place on ice
5. Centrifuge at 1000rpm at 4°C for 5 mins
6. Aspirate the supernatant and resuspend the pellet in 3ml DMEM+++
7. Add 1ml fibroblasts suspended in DMEM+++ to new culture flask containing DMEM +++ media*
   ** Split ratio is 1:3 flasks in fully established cultures. Media volumes: 10ml for 10cm^2 plate, 15ml for T75cm^2 flask and 20ml for T175cm^2 flask*

Seeding of fibroblasts and organoids onto scaffold (decellularized native tissue)[2]

Materials

Sterilised, acellular intestinal scaffold (Generated as per WP 2.1)
Custom made mini-platforms (photo)
26 Gauge canulae (Terumo SR+DU2619PX) – remove the plastic portion of the cannula to leave the cannula needle
1ml syringe
6 well plates
Perfusion plates (Amsbio, AMS, AVP-KIT-5)
Bioreactor circuit: medium reservoir (custom made by Chem Glassware UK manufacturers); outlet tubing (Cole Parmer 224-2081); 0.22-µm air filter, peristaltic pump (Cole Parmer 224-1505), three-way stopcocks (Becton Dickinson 394601)

1. Place sterilised scaffolds (1x1cm^2) onto custom made mini-platforms (figure 1) and put in 6-well tissue culture plate/s.
2. Trypsinise SI fibroblasts (as per protocol above), resuspend in DMEM

3. Count fibroblasts: add 10µl of DMEM and fibroblasts to a hemocytometer, count the number of fibroblasts in 4x4 large squares – do this twice and take an average value. Multiply the number of fibroblasts by 10,000 to get the number of fibroblasts/ml DMEM. Aiming for 0.5 $x10^6$ fibroblasts/ml DMEM – if necessary re-centrifuge at 1000rpm at 4°C for 5 mins and resuspend pellet to achieve this
4. Aspirate 1ml DMEM (0.5 $x10^6$ fibroblasts) into 1ml syringe and attach to 26 Gauge canula needle (having already removed and disposed of the plastic cannula)
5. Seed the fibroblasts into the acellular scaffold via inserting the cannula between the mucosal and submucosal layers and injecting 250µl into lateral aspect each of the 4 sides of the scaffold. Leave the needle in situ for 10 seconds after completing fibroblast injection prior to removing it.*
   **Injection should lead to a submucosal bleb being visible – with injection of all 4 sides of the acellular scaffold these 4 blebs will coalesce (see figure 1). Expect some leakage of media on removal of the cannula needle.*
6. Add further DMEM media 10ml/well to cover seeded scaffold, place in 37°C 5% CO_2 incubator and maintain in static culture for 3 days
7. On day 3, place scaffolds (mounted on mini-platforms) into new 6 well cell culture plates
8. Trypsinise SI organoids (as per protocol above), resuspend in basal human intestinal organoid culture medium and count (as per step 3). Aiming for $1x10^6$ organoids/cm^2 of scaffold suspended in 20µl of organoid media – if 1000rpm at 4°C for 5 mins and resuspend pellet to achieve this.
9. Pipette SI organoids; $1x10^6$ organoids suspended in 20 µl media, onto the mucosal surface of the scaffold (seeding at a density of $1x10^6$ /cm^2)
10. Place in 37°C 5% CO_2 incubator for 30 minutes to facilitate organoid engraftment
11. Add 10ml basal human intestinal organoid culture medium/well to cover the scaffold and return to 37°C 5% CO_2 incubator.
12. Maintain in static culture for 4 days
13. Set up the dynamic culture circuit as per the photograph in the steile hood
14. Fill the medium reservoir with 150ml basal human intestinal organoid culture medium with penicillin-streptomycin 100U/ml
15. Transfer the scaffolds (mounted on mini-platforms) into the perfusion plate
16. Add 10ml basal human intestinal organoid culture medium to each of the wells to cover the scaffold
17. Transfer the circuits to 37°C 5% CO_2 incubator and place tubing in peristaltic pump*
    **Take care to ensure the correct orientation of the tubing to ensure that media is pumped both into and out of the perfusion plate. Labelling the tubing "IN" and "OUT" can help facilitate subsequent identification. Ensure the perfusion plates are elevated to the height of the pump to eliminate any effects of gravity upon the flow of media into and out of the perfusion plates*
18. Start peristaltic pump at 5rpm, circulating media at a rate of 3ml/min*
    **Take care to check the perfusion system daily for evidence of media levels in the perfusion plate running low/overflow. This can be adjusted by ceasing the pump action upon the OUT and IN tubes respectively to increase/decrease the media levels.*

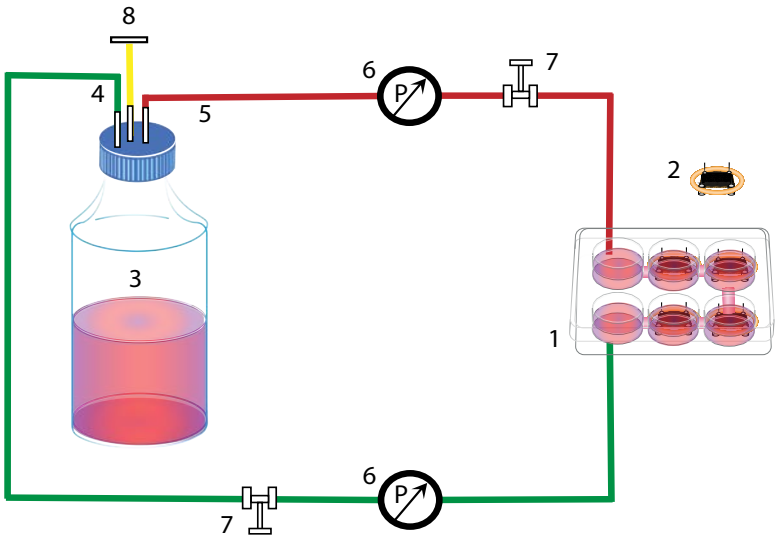
19. Leave in dynamic culture for 7 days prior to in vivo heterotopic transplantation of grafts.

Figure 1: Blank scaffolds and appearance following seeding with fibroblasts (stromal cells) and organoids[2]

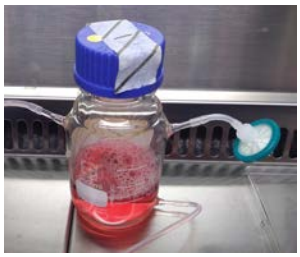
blank scaffold | stromal cells injection | organoid cell seedings

Figure 2: Set-up of bioreactor for dynamic culture[2]

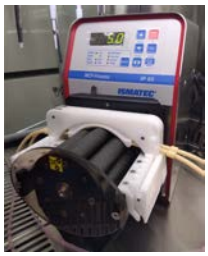
1. Perfusion plate
2. Mounted graft
3. Media Reservoir
4. Inlet tubing
5. Outlet tubing
6. Pump
7. 3-way stopcock
8. Air filter



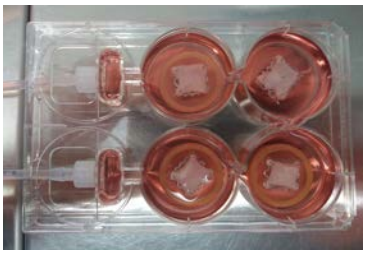
3



6



1



3. Summary of activities and research findings

Using the above protocols, we have demonstrated that paediatric intestinal organoids can be isolated from intestinal stem cells, expanded and cultured, via this method, and maintain their regional identity and differentiation potential.[2]

Furthermore, both organoids and fibroblasts can be seeded onto decellularized scaffold (WP 2.1) and cultured in both static and dynamic culture, to generate intestinal mucosal grafts. These grafts have demonstrated function in vitro (WP 3.4) and survival to 2 weeks following heterotopic transplantation in vivo.[2]

4. Conclusions and future steps

Utilising the above SOPs for organoid and fibroblast isolation, expansion and seeding we have generated successfully jejunal mucosal grafts which we have characterised and assessed function in vitro in addition to heterotopic transplantation. This is an important first step towards generating a full thickness intestinal graft.

Challenges remain in making this SOP Good Manufacturing Practice (GMP) compliant. Steps toward this include utilising extracellular matrix derived hydrogels instead of BME[3] and alternative organoid media including Intesticult Media (from StemCell Technologies) or commercially available Wnt surrogate-Fc fusion protein (U-protein express-BV N001-0.5mg)[4] instead of Wnt3a conditioned media.

5. Publications resulting from the work described (*if applicable*)

Meran, L. *et al.* Engineering transplantable jejunal mucosal grafts using patient-derived organoids from children with intestinal failure. *Nat Med* **26**, 1593-1601, doi:10.1038/s41591-020-1024-z (2020).

Giobbe, G. G. *et al.* Extracellular matrix hydrogel derived from decellularized tissues enables endodermal organoid culture. *Nat Commun* **10**, 5658, doi:10.1038/s41467-019-13605-4 (2019).

6. Bibliographical references (*if applicable*)

1 Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772, doi:10.1053/j.gastro.2011.07.050 (2011).

2 Meran, L. *et al.* Engineering transplantable jejunal mucosal grafts using patient-derived organoids from children with intestinal failure. *Nat Med* **26**, 1593-1601, doi:10.1038/s41591-020-1024-z (2020).
3 Giobbe, G. G. *et al.* Extracellular matrix hydrogel derived from decellularized tissues enables endodermal organoid culture. *Nat Commun* **10**, 5658, doi:10.1038/s41467-019-13605-4 (2019).
4 Janda, C. Y. *et al.* Surrogate Wnt agonists that phenocopy canonical Wnt and beta-catenin signalling. *Nature* **545**, 234-237, doi:10.1038/nature22306 (2017).