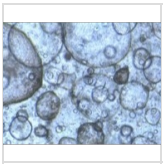

Version 1

Aug 03, 2020

Derivation of organoids from frozen tumour material V.1

Hazel Rogers[1], Laura Letchford[1], Sara Vieira[1], Maria Garcia-Casado[1], Mya Fekry-Troll[1], Charlotte Beaver[1], Rachel Nelson[1], Hayley Francies[1], Mathew Garnett[1]

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1 Works for me dx.doi.org/10.17504/protocols.io.biiakcae

Cellular Generation and Phenotyping

Hazel Rogers

ABSTRACT

This protocol describes the cryopreservation and subsequent derivation of organoid models from tumour tissue. It has been developed by the organoid derivation team within the Cellular Generation and Phenotyping Group at the Wellcome Sanger Institute. We have used the process to derive organoids from colon and pancreas tumours. In our experience success rates were very low for derivation from frozen oesophageal tumour tissue. The team has extensive experience in organoid derivation and have successfully banked over 100 models.

DOI

dx.doi.org/10.17504/protocols.io.biiakcae

PROTOCOL CITATION

Hazel Rogers, Laura Letchford, Sara Vieira, Maria Garcia-Casado, Mya Fekry-Troll, Charlotte Beaver, Rachel Nelson, Hayley Francies, Mathew Garnett 2020. Derivation of organoids from frozen tumour material. **protocols.io** dx.doi.org/10.17504/protocols.io.biiakcae

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GUIDELINES

General Information and tips

- We use 5 ml Eppendorf tubes to help with sterility. However, if you do not have access to these tubes any alternative sterile tubes of appropriate volume can be used.
- We have experience of deriving organoid models from frozen colon, oesphagus and pancreatic tumour tissue. Success rates were similar to derivation from fresh tissue for colon and pancreas. However, succes rates were very low for oesophagus. We therefore do not routinely freeze oesphagus tumour tissue.
- You can make up your own feezing media (10% DMSO in media). Or you can use pre-prepared feezing media. We use Recovery™ Cell Culture Freezing medium (Gibco). This should be aliquoted to avoid multiple freeze/thaws.
- We recommend using glass rather than plastic petri dishes for tissue dissection as tissue can get stuck in

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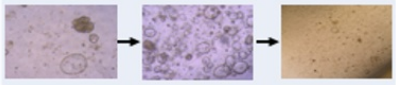
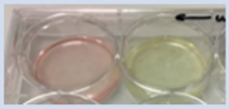
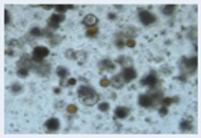
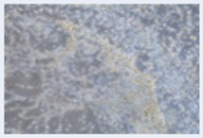
grooves cut into the plastic dish.

- Plate digested cells as close together as possible.
- Be very cautious at initial passages after derivation. Organoids can grow well for a few passages and then significantly drop off. We generally keep organoids in the same number of wells or reduce the area plated in if growth is slow or some cellular material has died.
- Not all derivations will be successful. Listed below are some common reasons we see for failure.

Reason	How do you know?	What does it look like?
Lack of cells	Insufficient starting material. No/minimal organoid formation seen.	
No organoid formation	Viable cells present but do not form organoids.	
Unable to propagate	Organoids form but unable to expand.	
Growth has dropped off	Organoids were present and expanding but have since stopped reforming.	
Bacterial contamination	Media turns very yellow and probably also cloudy. Small bacteria can be seen under high magnification.	
Fungal contamination	May manifest as very round, bright yellow dots within BME2 droplets or white colonies on top. Media may turn yellow but can remain clear.	
Mycoplasma contamination	Cannot be detected visually although organoid growth may be affected. Repeatedly test positive for mycoplasma.	-
Fibroblast contamination	Excessive fibroblast outgrowth takes over the culture.	

Trouble Shooting

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Problem	Possible Solution
BME2 is setting too quickly whilst plating.	Try keeping your solution in a cooling rack whilst plating.
Cells plated too densely resulting in BME breaking up.	Harvest cells, BME2 and media in a tube. Pipette to break up BME2. Spin, then aspirate supernatant. If a lot of BME2 is left (grey haze above pellet) re-suspend in ice cold PBS, then repeat spin. If this does not work, re-suspend in TrypLE and incubate at 37^{o}C for a few minutes before spinning. Re-suspend cell pellet in appropriate amount of BME2 and re-plate.
One well is contaminated but rest of plate looks normal.	Aspirate media from contaminated well. Add 2 ml chlorohexidine gluconate and leave for 30 min. Aspirate entire contents of well and wash out with PBS. Keep an eye on remaining wells for the next few days.
Culture is taken over by fibroblasts.	Use a P1000 pipette to harvest organoids and media in a tube and wash wells with PBS. Fibroblasts tend to attach to the surface of the culture plate so should be left behind when the organoids are harvested. Spin then aspirate supernatant. Re-suspend in TrypLE and continue with passaging protocol.

MATERIALS

NAME	CATALOG #	VENDOR
Falcon 15 mL Polystyrene Conical Tube	352095	Fisher Scientific
DPBS (no Ca, no Mg)	14190144	Thermofisher
Penicillin Streptomycin	15140 122	Invitrogen - Thermo Fisher
Recovery™ Cell Culture Freezing Medium	12648010	Thermo Fisher
Collagenase, Type II, powder	17101015	Thermo Fisher
ART™ Barrier Specialty Pipette Tips, 1000, wide bore	2079GPK	Thermo Fisher
Nunc™ Biobanking and Cell Culture Cryogenic Tubes, 1.8mL, 48mm, external thread, printed	375418	Thermo Fisher
Cultrex® Reduced Growth Factor Basement Membrane Matrix Type 2 (BME 2)	3533-010-02	Trevigen
Falcon 50mL Conical Centrifuge Tubes	14-432-22	Fisher Scientific
Costar 6-well Clear TC-treated Multiple Well Plates Bulk Packed Sterile	3506	Corning
Eppendorf Tubes 5.0 ml	0030122321	Eppendorf
Anumbra Glass Petri Dish 100x15mm	PET1008	Scientific Laboratory Supplies Ltd
Surgical Scalpel Blade No. 21	0507	Swann Morton
Cell Strainers 100 µm pore size	732-2759	VWR international Ltd
Pestle for Cell Strainer	Z742105	Sigma – Aldrich
Primocin	ant-pm-1	InvivoGen
Y-27632 dihydrochloride	Y0503	Sigma – Aldrich

MATERIALS TEXT

Equipment

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- Sterile cell culture hood
- Centrifuge
- 1000 µl and 200 µl pipettes and tips
- Pipetteboy
- Stripettes
- **37 °C** waterbath
- **37 °C** humidified incubator (5% CO2)
- Light microscope
- Tube rotator
- Cell freezing container

SAFETY WARNINGS

For full safety information refer to individual COSHH and MSDS forms

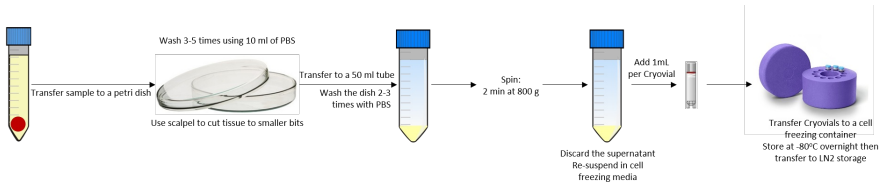
- **Primocin** can cause possible respiratory and skin sensitisation.
- **Penicillin Streptomycin** can cause possible respiratory and skin sensitisation. May also damage fertility or the unborn child.
- **Rock inhibitor (Y-27632)** is harmful if swallowed, inhaled or splashed on skin.
- Organoids derived from **primary samples** may contain uncharacterised adventitious agents, including blood-borne viruses.

BEFORE STARTING

- Prepare **100 mg/ml** collagenase stock. Re-suspend **1 g** collagenase II in **10 mL** PBS. Aliquots can be stored at **-20 °C** for up to one year.
- Place a cell freezing container (such as CoolCell™ or My Frosty™) in the fridge so it is chilled to **4 °C** before commencing work.
- Thaw appropriate amount of freezing media (**1 mL** per cryovial) and keep at **4 °C** until use.

Process Diagram

1



Cryopreservation

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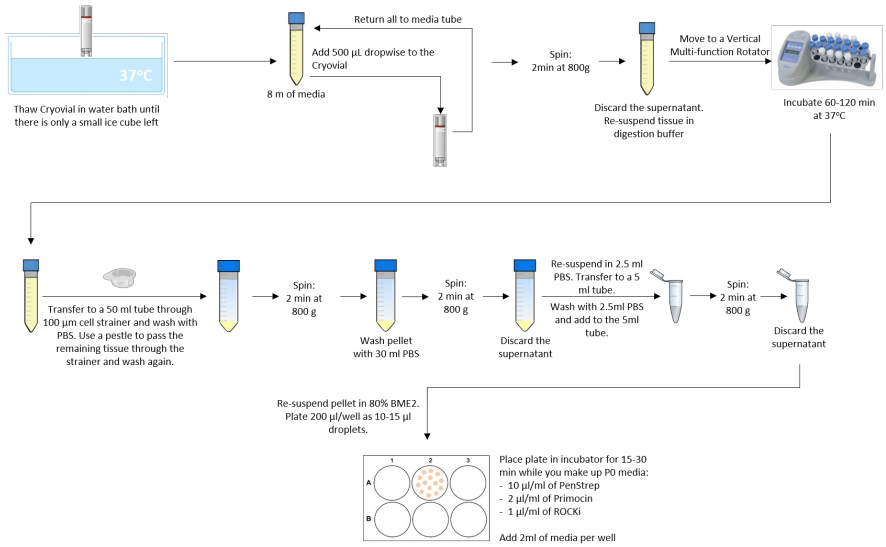

Derivation from cryopreserved tissue

Cryopreservation of primary tissue

2 Pour or pipette tissue, and media sample has been transported in, into a glass petri dish.

If tissue has unknown infection status, only open the container the sample has been transported in within a microbiological safety cabinet.

We recommend using glass rather than plastic petri dishes as tissue can get stuck in grooves cut into the plastic dish whilst cutting up the sample.

3 Aspirate as much media as possible. Add **10 mL** PBS to wash the tissue sample. Aspirate PBS and repeat wash at least two more times (we perform 3 washes for pancreas and oesophagus and 5 washes for colon).

If tissue is breaking up, making aspiration difficult without losing the sample, transfer tissue and media back to the 15 mL tube and centrifuge (800 x g 2 min). Asiprate supernatant and re-suspend in PBS to wash. Repeat these steps for appropriate number of washes.

4 Add **10 mL** of fresh PBS. Cut the sample up into as small pieces as possible (1-2 mm) using a scalpel.

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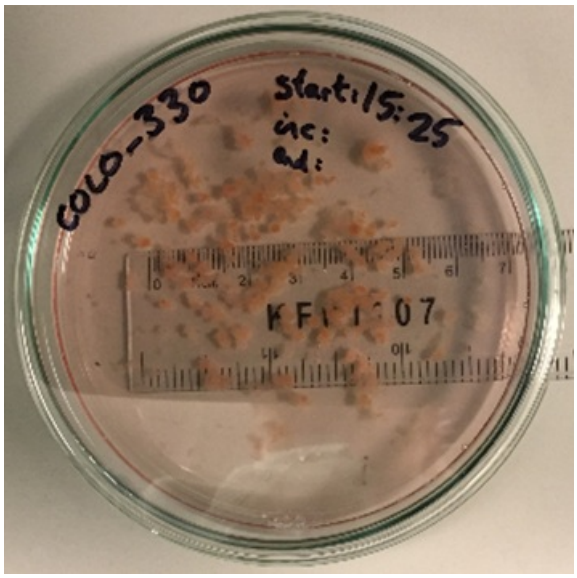
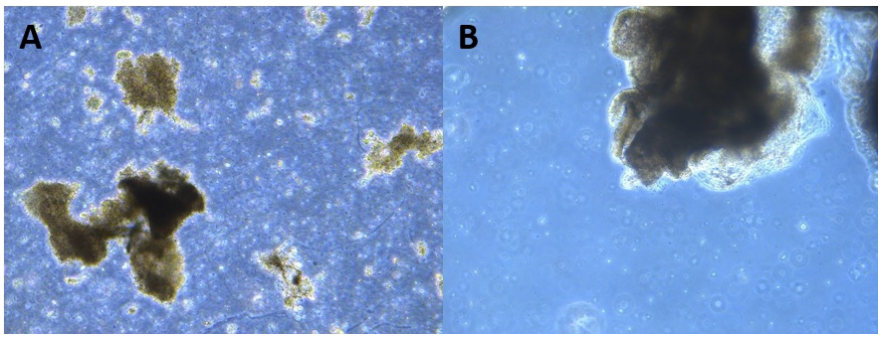


Tissue cut into small pieces



Examples of tissue samples with high (A) and low (B) cellularity (post cutting with a scalpel)

5 Transfer tissue and PBS to a **15 mL** tube. Centrifuge **800 x g** for 2 minutes.

6 Aspirate supernatant and re-suspend pellet in **1 mL** freezing medium (chilled to **4 °C**) and transfer to a cryovial.

If there is alot of tissue, split across more than one cryovial. This is to ensure there is sufficient volume of freezing media to protect all tissue pieces during freezing.

7 Place cryovial(s) in a cell freezing container (chilled to **4 °C**) and transfer immediately to a **-80 °C** freezer.

8 After 24-72 hours transfer cryovials to liquid nitrogen storage.

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Derivation from cryopreserved tissue

9 Before starting:

- Thaw BME2 aliquot overnight at **4 °C** and dilute 4:1 with appropriate organoid media (tissue specific) to make an 80% stock
- Ensure cell culture plates have been stored overnight in **37 °C** incubator
- Pre-warm organoid culture media to **Room temperature**
- Prepare digestion buffer:

Reagent	Stock Concentration	Volume
Organoid Media	-	9.5 ml
Collagenase	100 mg/ml	0.5 ml
Primocin	50 mg/ml	0.02 ml
Penicillin Streptomycin	100X	0.1 ml
Rock inhibitor (Y-27632) (10 mM)	10 mM	0.01 ml

10 Aliquot **8 mL** appropriate culture media into a **15 mL** tube.

11 Thaw cyrovial(s) of frozen tissue at **37 °C** until only a small ice crystal remains.

12 Add **500 µl** organoid media dropwise to the cryovial(s). Then transfer everything to the **15 mL** tube containing the remainder of the prepared media.

13 Centrifuge at **800 x g** for 2 minutes.

14 Aspirate the supernatant. Re-suspend the pellet in **10 mL** freshly prepared digestion buffer.

15 Place sample in a tube rotator and incubate at **37 °C** for 60-120 minutes.

16 Following incubation, assess tissue fragments under a microscope to confirm sufficient digestion. The sample should look cloudy to the eye and appear as single cells or small clumps under a microscope.

17 Transfer digested sample to a **50 mL** tube through a **100 µm** cell strainer. Use a pestle to pass any remaining

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tissue through the strainer. Wash the **15 mL** tube with **10 mL** PBS and add to the **50 mL** tube through the cell strainer. Repeat the wash step.

18 Centrifuge at **800 x g** for 2 minutes.

19 Aspirate supernatant and re-suspend pellet in **30 mL** PBS. Repeat spin at **800 x g** for 2 minutes.

When aspirating, you do not need to worry about getting too close to the pellet at this stage. The purpose of these washes is to dilute out any remainging Collagenase from the digestion buffer.

20 Aspirate supernatant and re-suspend pellet in **2.5 mL** PBS. Transfer to a **5 mL** tube (or **15 mL** tube). Wash **50 mL** tube with another **2.5 mL** PBS and transfer to **5 mL** tube. Repeat spin at **800 x g** for 2 minutes.

Transferring to a smaller volume tube helps with re-suspension in a small volume of BME2 in the next step.

21 Aspirate as much supernatant as possible. Re-suspend cell pellet in appropriate amount 80% BME2 (**200 µl** per well of a 6 well plate).

BME2 must be dispensed as quickly as possible as it will begin to set at room temperature. A cool block could be used to help keep the temperature down while plating.

Volume of BME2 to re-suspend in must be determined from size of cell pellet. Aim to plate cells as close together as possible. If unsure re-suspend in a small volume. Pipette one or two 15 µl - 20 µl droplets and check under the microscope. If too dense increase BME2 volume.

22 Using a P200 pipette, dispense organoid/BME2 suspension as small **15 µl** - **20 µl** droplets into a 6 well plate (seed **200 µl** per well).

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23 Place in a **37 °C** incubator (5% CO2) for 15-30 minutes to allow BME2 to set.

24 Prepare media containing antibiotics and Y-27632 (rock inhibitor). Add volumes below per ml of appropriate culture media:

- **2 µl** primocin
- **10 µl** penicillin-streptomycin
- **1 µl** Y-27632 (10mM stock)

25 Add **2 mL** of appropriate prepared media per well of a 6 well plate.

26 Return to incubator. Media change twice a week until ready to passage. Keep in media containing antibiotics and Y-27632 until first passage.

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