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We use this protocol and it's working

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Derivation of organoids from frozen tumour material V.1

In 1 collection

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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.

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, oesophagus and pancreatic tumour tissue. Success rates were similar to derivation from fresh tissue for colon and pancreas. However, success rates were very low for oesophagus. We therefore do not routinely freeze oesophagus tumour tissue.
- You can make up your own freezing media (10% DMSO in media). Or you can use pre-prepared freezing media. We use RecoveryTM 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 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

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°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

MATERIALS

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

Equipment

- 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

Note

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 START INSTRUCTIONS

- 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

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.

Safety information

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

Note

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).

Note

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). Aspirate 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.

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.

Note

If there is a lot 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.

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 cryovial(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 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.

Note

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 remaining 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.

Note

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).

Note

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.

Note

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).

- 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 μ L primocin
- 10 μ L penicillin-streptomycin
- 1 μ L 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.