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Duke - Isolation, Culture, and Maintenance of Patient-Derived Tumor Biopsy V.2

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

NCI PDMC consortium

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ABSTRACT

This protocol is split into 3 sections: collecting tumor cells, passaging, and cryopreserving organoids.

Phase 1:
Aim: Collect viable cells from biopsy samples
Tumor organoid: Colorectal Cancer
Source: Human primary tissue

Phase 2:
Aim: Passage and expand organoid samples

Phase 3:
Aim: Organoid cryopreservation
Tumor organoid: Colorectal Cancer
Source: Human primary tissue

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MATERIALS

NAME	CATALOG #	VENDOR
PBS		
HEPES	BP310-500	Fisher Scientific

NAME	CATALOG #	VENDOR
FBS		Invitrogen - Thermo Fisher
MACS 15 mL Tube Rack	130-091-052	Miltenyi Biotec
Collagenase	C5138	Sigma
Cell strainer 70um filter	352350	Falcon
HBSS	14060040	Gibco - Thermo Fischer
B-27™ Supplement (50X), minus antioxidants	10889038	Thermo Fisher
Externally and Internally Threaded Cryogenic Storage Vials	12567501	Thermo Fisher
Advanced DMEM/F-12	12634010	Thermo Fisher
Recovery™ Cell Culture Freezing Medium	12648010	Thermo Fisher
Antibiotic-Antimycotic (100X)	15240062	Thermo Fisher
L-Glutamine	21051040	Thermo Fisher
Cryogenic Box Divider, vertical, 2mL with 100-cells	4000014	Thermo Fisher
N-Acetyl-L-cysteine	A9165	Sigma Aldrich
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix	356231	Corning
Rock Inhibitor Y-27632 Dihydrochloride	1254	Tocris
N2 supplement (100x supplement)	17502048	Gibco, ThermoFisher

MATERIALS TEXT

Razor blades (brand new)
Ice
Petri Dish (100X15 mm)
15 ml tube
Ethanol 70%
Tweezers
Ice
Ethanol 70%
Biosafety cabinet
Basal media (L-glutamine, HEPES, B27, N2, AA, NACE)
Basal media (L-glutamine, HEPES, B27, N2, AA, NACE) human (1mM), A-83-01 (500 uM), Rock inhibitor Y-27631 (10 mM), SB 202190 (30 mM))

Basal media

Supplements	Vendor	Catalog #	Solvent	Stock Solution	Working concentration	Store
Advantage DMEM/F-12	ThermoFisher	12634-010	NA	NA	NA	4 °C. Protect from light
L-Glutamine 200 mM (100X)	ThermoFisher	25030-081	NA	200mM	2 mM	(-)20°C. Protect from light
HEPES (1M)	ThermoFisher	15630080	NA	1 M	10 mM	4 °C
B-27™ Supplement (50X), minus vitamin A	ThermoFisher	12587010	NA	50X	1X	(-)20°C. Protect from light
N-2 Supplement (100X)	ThermoFisher	17502048	NA	100X	1X	(-)20°C. Protect from light
Antibiotic-Antimycotic or Anti-Anti (100X)	ThermoFisher	15240-062	NA	10000/10000/25 U/ml	100 U/ml	(-)20°C
NACE (N-Acetyl-L-cysteine)	Sigma-Aldrich	A9165-5G	Sterile Water	500mM	1mM	4 °C

* Basal media must store at 4°C afterward all supplements are added

Basal Media Formulation

Basal media + small molecules

Small molecules	Vendor	Catalog #	Solvent	Stock Solution	Working concentration	Store
Basal media (500ml)			NA	NA	NA	4 °C
[Leu15]-Gastrin I human	Sigma-Aldrich	G9145- 1MG	Sterile Water	100 uM	10 nM	(-)20°C
A-83-01	Sigma-Aldrich	SML0788-5MG	DMSO	500 uM	500 nM	(-)20°C
SB202190	Sigma-Aldrich	S7067-5MG	DMSO	30 mM	10 uM	4 °C
Y-27632	STEMCELL TECHNOLOGIES	72304	PBS	10 mM	10 uM	(-)20°C

* Basal media + small molecules can be frozen afterward all growth factors are added

Formulation of Basal Media + Small Molecules

Small molecules-Preparation

Growth Factor	Vendor	Final Concentration in Media	Stock Solution	Solvent	Amount of solvent	Store
[Leu15]-Gastrin I human	Sigma-Aldrich	10 nM	1 mM	Sterile Water	48.07 uL	(-)20°C
A-83-01	Sigma-Aldrich	500 nM	500 uM	DMSO	474.47 uL	(-)20°C
SB202190	Sigma-Aldrich	10 uM	30 mM	DMSO	503 uL	(-)20°C
Y-27632	STEMCELL TECHNOLOGIES	10 uM	10 mM	PBS	1500 uL	(-)20°C

Small Molecule Preparation

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Transport media

Materials	Vendor	Catalog #	Solvent	Stock Solution	Working concentration	Store
Advantage DMEMF-12	Thermo Fisher	12634-010	NA	NA	NA	4 °C. Protect from light
Penicillin Streptomycin	Thermo Fisher	15140-122	NA	10000/10000 U/ml	100 U/ml	(-)20°C
Rock inhibitor Y-27632	Stemcell Technologies	72304	PBS	10 mM	10 uM	(-)20°C

* Transport media must keep on ice or 4°C afterward all materials are added

Transport Media Formulation

Cell digestion solution

Materials	Vendor	Catalog #	Solvent	Stock Solution	Working concentration	Store
Liberase™ TH Research Grade	Sigma-Aldrich	5401135001	HBSS buffer	2.5 mg/ml	50 ug/ml	(-)20°C
Hank's Balanced Salt Solution	ThermoFisher	24020117	NA	NA	NA	Room temperature

Cell Digestion Solution

EQUIPMENT

NAME	CATALOG #	VENDOR
MACSmix™ Tube Rotator	130-090-753	

Disassociation of Tumor Cells

1 Store tissue samples in cold transport media (**10 mL**). **Keep samples on ice at all time and process within** **00:45:00**

2 Transfer the tumor biopsy sample (1-2 cm^3) and transport media into a petri dish and remove remnant non-tumor tissue with sterile tweezers.

The size of the tissue sample will affect the cells yield and the end of the protocol. Make sure to get enough tissue sample (~ 1-2cm^3)

3 Transfer the tumor tissue to a new petri dish and cut the sample into small pieces with a sterile razor blade (<2 mm^2).

4 Add **5 mL** of cold sterile PBS to the dish and transfer the tumor fragments and PBS to a 15 ml centrifuge tube, pipetting up and down for three times.

5 Allow the tissue fragments to settle by gravity for **00:01:00** **On ice** , remove and discard the supernatant

6 Resuspend the tissue fragments in **5 mL** HBSS buffer plus Collagenase and transfer to a fresh 15 ml tube.

7 Place the tube in

MACSmix™ Tube Rotator

Miltenyi Biotec 130-090-753

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and incubate the enzymatic digestion at **37 °C** for **01:30:00** .

The time of cell digestion will affect the cells yield and the end of the protocol. Make sure to incubate for enough time

8 Quench the enzymatic digestion by adding **1 mL** of cold, sterile FBS to the reaction and proceed immediately to plating for organoid culture.

Plating Cancer Cells for 3D Organoid Culture

9 Warm up a 24-well flat bottom plate in the incubator and cool the 10% FBS solution on ice.

10 Strain the quenched enzyme solution through a 70 um cell strainer into a 15 ml tube using a reducing adaptors assembly. Use one cell strainer, reducing adaptor and tube per sample.

11 Wash the cells twice in **5 mL** of 10% FBS by pipet up and down at least 10 times then centrifuging the sample for 3 minutes at 2000g to pellet the cells.

12 Resuspend the cell pellet in 1 ml basal media and determine the cellular yield using a hemocytometer.

For instructions on using hemocytometer:
Hemocytometer Protocol.docx

13 Transfer the desired cell sample to a fresh tube and **2000 x g, Room temperature 00:07:00** to pellet the cells. Discard the supernatant. Do not remove the pellet.

The following steps are for plating 4 x **50 µl** culture domes. If fewer or additional culture domes are required based on the counts in step 5, adjust the volume of Matrigel and PBS.

14 Thoroughly resuspend the cells in **40 µl** of cold PBS by pipetting up and down 10 times. Avoid introducing bubbles.

15 Add **160 µl** of Matrigel to the cell solution and mix by pipetting up and down 10 times. Avoid introducing bubbles.

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Keep the Matrigel **On ice** all the time, at room temperature will start to polymerization

16 Gently plate **50 µl** domes of the Matrigel-cell suspension in the center of the 4 central wells in the pre-warm 24 well flat bottom plate.

17 Carefully transfer the plate to **37 °C** incubator and incubate for at least **00:30:00** to allow domes to solidify (polymerize).

18 Gently add **1 mL** of basal media + small molecules to each well by pipetting the media gently down the wall of the well.

19

Optional: Add sterile PBS to the unused wells of the 24 well plate to limit evaporation.

20 Place the lid on the culture plate and return it to the tissue culture incubator.

21 Perform a full media change every **48:00:00** to expand organoids. Observe cancer organoids daily and replace the media every 2 days after cancer cell isolation. Proceed to the next phases (drug screening, passage organoids and cryopreservation) after 4 or 5 days.

Passaging Cancer Organoids

22

Passage must be performed between 7 or 10 days depending on organoid size and density, passaging helps to avoid the organoid overgrowth and keeps the culture healthy and expanding.

Carefully remove media from each well.

23 Gently add **1 mL** cold PBS to each well by pipetting the PBS gently down the wall of the well.

24 Carefully remove and discard PBS from each well.

25 Add **1 mL** of accumax on the top of the dome in each well and perform mechanical detach from the bottom (gently scrape).

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26 Carefully transfer the plate to 37°C incubator. Incubate at **37 °C** for **00:10:00**

27 Collect and transfer the accumax-cell suspension to a 15 ml tube.

28 Add **2000 µl** of FBS to get 10% FBS and pipette up and down the accumax-cell suspension at least 10 times.

29 Centrifuge the sample for **200 x g, Room temperature 00:05:00** to pellet the cells. Do not remove the pellet.

30 Remove the 24 well flat bottom plate from the **37 °C** incubator.

31 Add **40 µl** of PBS to the sample tube. Pipette up and down 10 times to thoroughly resuspend the pellet. Avoid introducing bubbles.

32 Add **160 µl** of Matrigel to the sample tube. Pipette up and down 10 times to thoroughly resuspend the pellet. Avoid introducing bubbles.

33 Gently add **50 µl** of Matrigel-cell suspension in 4 central wells of a pre-warm 24 well plate. This protocol uses a 1:2 split ratio on passage organoid between 7 to 10 days after plating, or when the density reaches 150 organoids per well.

34 Carefully transfer the plate to 37°C incubator. Incubate at **37 °C** for at least **00:30:00** to allow domes to solidify (polymerization).

35 Gently add **1 mL** of conditioned media to each well by pipetting the media gently down the wall of the well.

36 Place the lid on the culture plate and incubate at **37 °C** and 5% CO_2.

37 Every 2 days perform a full media change.

Cryopreserving Cancer Organoids

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38 Carefully remove media from each well and add 1 ml of recovery cell culture freezing medium per well.

39 Scrape the Matrigel off the bottom of the wells with a 1,000 ul pipette and transfer the cancer organoids into a one cryovial. Label with date and tissue source or any other specifications.

40 Place each tube in a freezing container and incubate the tubes at **-80 °C** for at least 1 day.

41 Transfer the frozen cryovials to a liquid nitrogen storage tank. Cancer organoids can be kept in liquid nitrogen storage for at least 3 years.

Recovery of Frozen Organoids

42 Remove the cryovials from storage and thaw them quickly in a **37 °C** water bath.

43 Collect the organoids with a 1,000 µl pipette into a 15-ml centrifuge tube.

44 Add **10 mL** of basal media and spin the organoids down at **200 x g, 4°C 00:05:00**

45 Remove and discard the supernatant and suspend the organoids with Matrigel.

46 The appropriate volume of Matrigel depends on the number of the cancer organoids. In most cases splitting one vial (1000 µl) of preserved organoids to 4-6 wells of a 24 well flat bottom plate is enough.

47 Gently add **50 µl** of Matrigel-cell suspension in 4 central wells of a pre-warm 24 well plate.

48 Carefully transfer the plate to 37°C incubator. Incubate at **37 °C** for at least **00:30:00** to allow domes to solidify (polymerization).

49 Gently add **1 mL** of conditioned media to each well by pipetting the media gently down the wall of the well.

50 Place the lid on the culture plate and incubate at 37°C and 5% CO_2.

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