Interreg Italia-Österreich
European Regional Development Fund
EUROPEAN UNION
p-care

SOP –UNITS-5.0

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Issued:
18/04/2022
Revised:

GENERATION OF 3D CULTURES FROM PLEURAL EFFUSION OR ASCITIC FLUIDS INCLUDING FACS ANALYSIS

Purpose

The SOP-UNITS was issued to describe how to generate and characterize 3D cultures from cancer-derived ascites and pleural effusions. In addition, this SOP contains the instructions for the isolation of tumour cells from fluids trough FACS sorting.

Scope

The SOP is intended to cover all resources, personnel and equipment needed to generate and characterize 3D cultures from cancer-derived ascites and pleural effusions.

Introduction

Cancer patients bearing for example breast, ovarian, lung cancer, or peritoneal metastases are likely to develop ascites and pleural effusions, that provide liquid biopsies offering information about cancer prognosis (Sorolla et al., Cancers 2021). After being utilized for diagnostic purposes, excess material from these liquid biopsies is often discarded, despite they could provide a readily-available, cost-effective source of tumor cells for generating cancer models to support precision medicine applications in the future.

Based on prior experience (Campaner et al., Cancers 2021; Hoffmann et al. The EMBO Journal 2020; Mazzochi et al. ACS Biomater. Sci. Eng. 2019), a procedure has been developed to obtain both 3D cultures of viable cancer cells from cancer-derived pleural effusion and ascitic fluid and a molecular characterization of cell type identity, since the starting material typically contains many different cell types. Here the procedures to obtain such cultures and their molecular characterization is described in detail.

1. Cell culture media, reagents and solutions

- Penicillin-Streptomycin Solution 100X (Euroclone, cat. no. ECB3001D)
- Primocin (Invivogen, cat.no. Ant-pm-1)
- FGF7 (Peprotech, cat. no. 100-19)
- FGF10 (Peprotech, cat.,no 100-26)
- Noggin (Peprotech, cat. no. 120-10C)
- Heregulin β-1 (Peprotech, cat. no. 100-03)
- R-spondin 3 (R&D system, cat. no. 3500-RS-025/CF)
- EGF (Cell Guidance Systems, cat. no. GFH26)
- A83-01 (Tocris, cat. no. 2939)
- Y-27632 (Selleckchem, cat. no. S1049)
- SB202190 (Sigma-Aldrich, cat. no.S7067)
- B27 supplement (Gibco, cat. no. 17504-44)
- N-2 supplement (Gibco REF 17502-048)
- N-Acetylcysteine (Sigma-Aldrich, cat. no. A9165)
- Nicotinamide (Sigma-Aldrich, cat. no. N0636)
- GlutaMax100X (Invitrogen, cat. no. 12634-034)
- Hepes (Invitrogen, cat. no. 15630-056)
- PBS-1× (Life Technologies, cat. no. 14190-094)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- FBS (Euroclone, cat. no. ECS0180L)
- Advanced DMEM/F-12 (Ad-DF, Gibco cat. no. 12634-010)
- Red Blood Cell Lysis Solution (10×), Miltenyi #130-094-183
- BSA (Sigma-Aldrich, Albumin Fraction V, cat. no. 3117332001
- EpCAM-FITC (BD, FITC-mouse anti-human EpCAM, clone EBA-1, cat: 347197)
- CD31-APC/Cy7 (Biolegend, APC/Cy7 anti-human CD31, clone WM59, cat. 303119)
- CD45-APC/Cy7 (BD, APC/Cy7, mouse anti-human CD45, clone 2D1, cat: 557833)

2. Equipment

- Pipette aid, serological pipettes (Euroclone cat no. EPS05N; EPS10N)
- Pipettes p20, p200, p1000 (Pipetman, Gilson)
- Multichannel pipettes (Pipetman, Gilson)
- Pipette tips 1000 microL (Sarstedt, Ref. 70.3050.205)
- Pipette tips 200 microL (Sarstedt, Ref. 70.3030.200)
- Blood collection tubes VACUETTE® 4ml K3EDTA, PREMIUM screw cap tube, pink cap (Greiner Bio-One, Ref. 454099)
- Conical centrifuge tubes (Euroclone, cat. no. ET5015B; ET5050B)
- Microcentrifuge tubes (Euroclone, cat. no. ET3415)
- 100 mm Petri dish (Greiner Bio-one, cat. no. 633181)
- 0.22 micron pore size sterile filter units (Millex®-GP Ref. SLGP033RS)
- 40 micron sterile Cell Strainer (FALCON, Ref. 352340)
- Ultra-low attachment U-shaped 96-Well plates (Costar Ref. 7007)
- Ice
- Refrigerated Centrifuge (SIGMA 3-16K)
- High-speed centrifuge (Eppendorf centrifuge 5810R)
- Cell culture incubator with 5% CO_2, 37°C (Thermo, Model 3111)
- CKX31 Inverted Microscope (Olympus Life Sciences)
- Cell Sorter BD FACS ARIA™ II

3. Reagent setup

- Ad-DF basal medium Ad-DF supplemented with 1X Glutamax, 50 µg/mL Primocin, 1X Penicillin/Streptomycin, 10mM Hepes.
- Growth medium composition changes depending on cancer origin. Here below are two examples.

  Growth medium for cells isolated from breast cancer-derived fluids is Ad-DF basal medium supplemented with:

Medium component	Final concentration
B27 supplement	1X
Nicotinamide	5 mM
N-Acetylcystein	1.25 mM
R-spondin 3	250 ng/mL

Heregulin β-1	5 nM
Noggin	100 ng/mL
FGF-7	5 ng/mL
FGF-10	20 ng/mL
EGF	5 ng/mL
A83-01	500 nM
SB202190	500 nM
Y-27632	5 µM

Growth medium for cells isolated from ovarian cancer-derived fluids is Ad-DF basal medium supplemented with:

Medium component	**Final concentration**
B27 supplement	1X
N2 supplement	1X
Nicotinamide	5 mM
R-spondin 3	250 ng/mL
FGF-10	100 ng/mL
EGF	10 ng/mL
SB202190	500 nM
Y-27632	5 µM

- FACS buffer PBS supplemented with 1% BSA

4. Procedure

4.1 Collection and processing of Pleural Effusion (PE) and ascitic fluid

- Collect the pleural effusion/ascitic fluid in anticoagulant-treated tubes (typically EDTA) and store them immediately on ice. 30-50 mL is the minimal volume to be collected. "Pleural fluid samples for white blood cell (WBC) and differential cell counts were sent in an anticoagulant-treated tube (ethylenediaminetetraacetic acid [EDTA] or citrate) and analyzed up to 2 hours after thoracocentesis." (See *Figure 1*).

- In the lab, transfer the freshly acquired fluid to a 50 ml conical tube on ice. For large volume samples, use sterile 500 ml Nalgene centrifuge bottles.
- Centrifuge the fluid for 5 min at 530 x g at 4°C and collect the pellet. Keep the supernatant as additive for organoid growth after processing, as described below. (See *Figure 1*)
- If the pellet contains red blood cells (observed in the top layer of the cell pellet), resuspend the pellet in 5 – 10 ml of Miltenyi 's Red Blood cell lysis solution according to manufactures instructions. Block reaction by adding basal medium. Centrifuge at 530 X g for 5 min at room temperature. (See *Figure 1*)
- Repeat this step until the blood cells are no longer visible.
- Resuspend the pellet in 5 – 10 ml of 1X PBS.
- Centrifuge at 530 X g for 5 min at room temperature.
- Resuspend the pellet in 5 – 10 ml PBS and assess its content by plating 25 µl into a dish and examine the cellular organization under a light microscope (aggregates and single cells). The organization of tumor cells in pleural effusions/ascites varies significantly, ranging from organoid-like aggregates to fully dispersed single cells. In addition, the number of tumor and immune cells is highly variable. Both the cellular organization (based on microscopy) and cell content (based on Lin- (CD31 neg; CD45 neg); EpCam+ markers analysis by FACS) should be recorded for each effusion, as described here below.

4.2 Processing of supernatant

According to the volume, the supernatant of the pleural effusion or the ascites obtained following the first centrifugation for the derivation of primary cells is transferred in 50 ml polypropylene tubes and centrifuged at (1900 x g) 3000 rpm for 30 minutes at RT in order to remove all residual cells.The supernatant is collected and transferred into new 50 ml polypropylene tubes, and further filtered using sterile filters (0.22 micron pore size) while the residual pellet is discarded. The filtrate is directly used for experiments, or collected as working aliquots in 15 ml tubes and stored at -80°C.

4.3 3D culture of cancer cells from PE/AF

- At this stage, cells are ready to be plated in ultra-low attachment plates (U-bottom) to generate 3D cultures as spheroids. If required, FACS isolation of cancer cells (using the same markers used for characterization) can be performed before plating (See *Figure 2*).
- Count cells (if aggregates are visible, count them as a cell) to estimate the total number of isolated cells. Save 50.000 cells for FACS analysis.
- The remaining cells are resuspended in growth media (which differs depending on cancer origin) at a concentration of 0.25*106 cells/mL. Add patient-specific supernatant (processed as described above) to the media at 10% final concentration.
- Using the multichannel, plate 200 µL of cell suspension each well, which results in 50.000/well cells plating.

4.4 FACS mediated cells analysis and sorting

Typically, these samples contain high levels (>50%) of lineage positive cells, however these cells are not likely to form spheroids in 3D cultures. In our experience, FACS sorting is not improving the efficiency of spheroid generation (See *Figure 4*).

- Centrifuge at 530 x g at room temperature for 5 min and eliminate supernatant
- Proceed to FACS to analyze cell composition/isolate Lin- (CD31neg; CD45 neg); EpCam+ cells. Cancer cells are isolated based on the expression of the epithelial marker EpCam.
- Prepare an antibody mix as follows:
  - 12,5 µL EpCAM-FITC
  - 5µL CD31-APC/Cy7
  - 5µL CD45-APC/Cy7
- Resuspend pellet in 300 µL of FACS buffer
- Add antibody mix and mix well
- Incubate in the dark 30 min on ice.
- Wash in 5 mL PBS
- Centrifuge at 530 x g at room temperature for 5 min.
- Discard supernatant

- Resuspend pellet in 1 ml of PBS and proceed to FACS analysis. If sorting is needed, resuspend pellet in 1 mL or 2 mL of sorting solution (1xPBS, Ca2+/Mg2+-free, 1mM EDTA, 25mM HEPES pH 7, 1% BSA) filter through a 40 micron Cell Strainer and proceed to FACS.

5. Figures

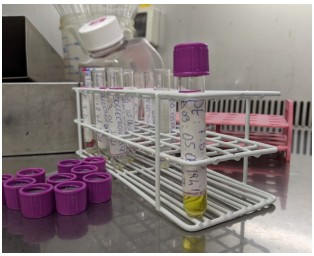
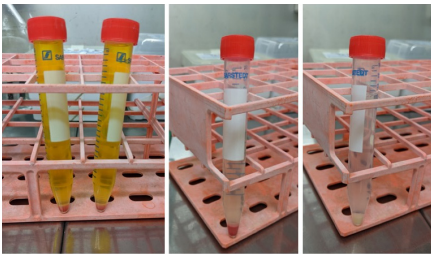


Figure 1. Representative images of fluid collected in EDTA tubes (top), and, from left to right, cell pellet after centrifugation, washing with PBS, and red-blood cells removal (bottom).



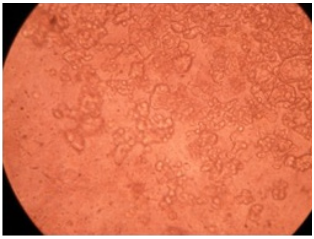
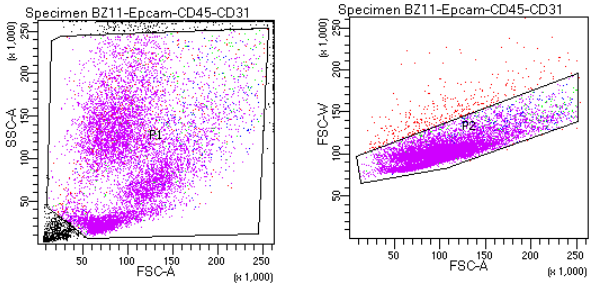
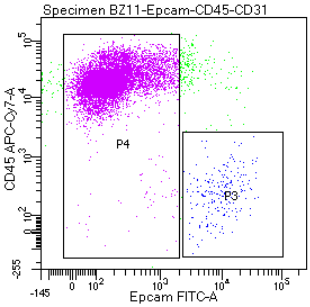


Figure 2. Representative microscope image of 3D spheroid cultures from a high grade serous carcinoma-derived ascitic fluid.





Tube: Epcam-CD45-CD31

Population	#Events	%Parent	%Total
All Events	12,271	####	100.0
P1	10,000	81.5	81.5
P2	9,629	96.3	78.5
P3	217	2.3	1.8
P4	9,166	95.2	74.7

Figure 3. Representative plots and population percentage analysis of FACS sorted Lin- EpCAM + cells isolated from a high-grade serous carcinoma-derived ascitic fluid. P3 is the EpCAM+ cancer population while P4 is the Lin+ population.

6. References

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- Hoffmann et al. Stable expansion of high-grade serous ovarian cancer organoids requires a low-Wnt environment. The EMBO Journal 2020
- Mazzochi et al. Pleural Effusion Aspirate for Use in 3D Lung Cancer Modeling and Chemotherapy Screening. ACS Biomater. Sci. Eng. 2019
- Sorolla et al. Diving into the Pleural Fluid: Liquid Biopsy for Metastatic Malignant Pleural Effusions. Cancers 2021