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PROTOCOLS FOR HARVESTING HUMAN BREAST MATERIAL TO OBTAIN VIABLE CELLS FOR DOWNSTREAM APPLICATIONS

This document describes the steps for processing of patient-derived material to obtain viable breast cancer cells with the purpose of preparing them for downstream applications.

In Part 1, we provide two different protocols for harvesting viable breast cancer cells derived from patient material, depending on the source material originating from ascites/pleural effusion or tissue.

In Part 2, we provide three different protocols for how to process the harvested breast (cancer) cells for downstream applications: Viably freezing cells and establish Patient-derived organotypic cultures (PDOs) or xenografts (PDXs).

In Part 3, we provide a protocol to maintain PDO cultures and an overview of culture media and required equipment, materials and reagents.

We provide a quick manual for each procedure as well as a more detailed protocol with tips and considerations.

PART 1: Harvest Viable Breast Cancer Cells From Patient Ascites And Pleural Effusions Or From Patient Tissue

- Protocol 1: Harvest viable breast (cancer) cells from ascites or pleural effusions
- Protocol 2: Harvest viable breast (cancer) cells from tissue (surgical breast resection/biopsy)

PART 2: Process Harvested Viable Breast Cancer Cells For Downstream Applications: Cryo-Protective Freezing Or Establish Patient-Derived Organotypic Cultures/Xenografts

- Protocol 3: Establish Patient-derived organoids (PDOs) from breast (cancer) cells
- Protocol 4: Establish Patient-derived xenografts (PDXs) from breast (cancer) cells by Intraductal injections in recipient mice
- Protocol 5: Viably freezing breast cells derived from ascites/pleural effusions, tissue (PDX) or organotypic cultures (PDO)

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PART 3: Additional Information

- Protocol 6: Culturing Of Patient-derived Organotypic Cultures (PDOs)
- Info 1: Overview Of Cell Culture Media
- Info 2: Overview Of Required Equipment, Materials And Reagents

PROTOCOL 1: Harvest Viable Breast (Cancer) Cells From Ascites Or Pleural Effusions

1) Sterilize all surfaces according cell culture guidelines
2) Depending on the viscosity of the sample, dilute the cell sample with the appropriate culture medium or PBS + 2% FCS
   a) Effusion fluids can usually be used directly
   b) Bone marrow or whole blood are typically diluted 1:1

For large volumes of ascites or pleural effusion fluids
First spin down the cells for 5 minutes @ 450 x g, aspirate supernatant and resuspend cell pellet in appropriate culture medium or PBS + 2% FCS

3) Transfer the liquid containing the tumor cells in a conical tube
   a) For very small volumes (< 4 mL), top up the sample to 4 mL

Harvest the mononuclear cells using Ficoll-Paque plus:

4) Gently put a pipet containing the Ficoll-Paque in the tube containing the tumor cells and **SLOWLY (!!)** pipet the Ficoll-Paque underneath the cell suspension (ratio Ficoll:cell suspension is max 2:3).
   Take care **NOT TO MIX THE TWO LAYERS**
5) Centrifuge for 20 - 30 minutes at 800 x g with the **BRAKE OFF (!!!)**
6) **CAREFULLY** remove the tubes from the centrifuge and harvest the cancer cells by inserting the pipette directly through the upper phase to the mononuclear cells at the interface
   Take care **NOT TO MIX THE LAYERS**
7) Transfer the interphase containing the mononuclear cells to a clean tube
   Take care **NOT TO TRANFER ANY OF THE OTHER LAYER(S)**
8) Wash the harvested cells twice with serum-containing culture medium (max. volume per tube)

Optional: Remove fibroblasts from cell suspension (see detailed protocol)

9) Centrifuge at 450 x g for 5 minutes
10) Process the cell pellet according the guidelines for the desired downstream application described in Part 2: Protocols 3-5

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PROTOCOL 2: Harvest Viable Breast (Cancer) Cells from Tissue (Surgical Breast Resection/Biopsy)

1) Sterilize all surfaces according cell culture guidelines
2) Collect harvested tissue in appropriate culture medium without serum, with P/S (@20°C) in 15 mL conical tube
3) Place the tumor in a 6-10 cm culture dish and add some drops of medium. **KEEP THE TUMOR WET**
4) Remove necrotic tissue using sterile scalpel blades
5) Depending on the size of the tumor and number of downstream applications, divide the tumor into big pieces; 1 piece for each downstream application
   Make sure the tumor pieces **DO NOT DRY OUT**
6) Place tumor piece in a drop of culture medium without serum, with P/S in a 6-10 cm culture dish
7) Mince the tumor piece into small pieces (1-2 mm) using sterile scalpel blades
8) Process the cell pellet according to the guidelines for the desired downstream application described in Part 2: Protocols 3-5

PROTOCOL 3: Establish Patient-Derived Organoids (PDOs) From Breast (Cancer) Cells

1) Sterilize all surfaces according cell culture guidelines

For source materials ascites/pleural effusion (protocol 1)

2) After centrifugation of the cell suspension at 450 x g for 5 minutes
3) Proceed directly to Step 13

For source material tissue pieces (protocol 2)

4) After mincing the tumor piece into small pieces (1-2 mm) using sterile scalpel blades
5) Collect all tumor pieces in 15 mL conical tube with 10-15 mL cold appropriate culture medium without serum, with P/S
6) Centrifuge for 5 minutes @ 300 x g
7) **CAREFULLY** aspirate medium
8) Incubate tumor pieces in 3-5 mL digestion mix for 1 hr @ 37°C on orbital shaker
   Check regularly; exact incubation time may depend on the tissue

Optional: DNase treatment (see detailed protocol)

9) Shear tumor suspension **(VIGOROUSLY!)**, first by 5 mL pipet, then P1000
   Make sure you cut off the tip of a P1000 tip to prevent clogging
10) Collect the suspension in a 15-50 mL conical tube with 70-100 µm strainer
    a) Recollect tumor pieces that did not pass through the strainer in a new tube

b) Shear again using a P1000 and collect the suspension in the tube with strainer from step 8
c) Repeat the shearing if necessary

11) Add FCS (2-5% final) to the suspension and centrifuge for 5 minutes @ 400 x g

12) Aspirate medium

Optional: Red blood cell lysis (see detailed protocol)

Optional: Remove fibroblasts from cell suspension (see detailed protocol)

13) Depending on the size of the cell pellet, resuspend in appropriate volume of cold culture medium
Typically, 50-200 µL suffices (see Protocol 6 for more details)
14) Add 3-4 parts of Matrigel/BME to 1 part cell suspension (e.g. 150-200 µL to 50 µL)
Make sure to **KEEP EVERYTHING ICE-COLD**
15) Plate 3 drops (10-15 µL) of the cell-Matrigel/BME-mix per well of a pre-warmed 24-well plate
16) Flip the plate upside-down and let the gels polymerize for 30-45 minutes @ 37°C in a CO_2-incubator
17) Add 600 µL of appropriate growth medium per well
18) See Protocol 6 for maintaining the PDO culture

PROTOCOL 4: Establish Patient-Derived Xenografts (PDXs) From Breast (Cancer) Cells By Intraductal Injections In Recipient Mice

1) Record the body weight of the mouse
2) Anesthetize the mouse using isoflurane (4-5% w/v) by inhalation and apply eye lubricant
During the procedure, the mouse will continue to be anesthetized using inhaled isoflurane via a nose cone. Carefully monitor the mouse for changes in respiratory and heart rate and adjust the level of isoflurane accordingly
3) Inject an analgesic
4) Secure the mouse on the 37°C warming pad and place under a binocular. Due to the anesthesia, the mouse might lose body warmth, therefore the mouse should be kept on a warming pad during the procedure
5) Use fine micro-dissection tweezers dipped into sterile oil so that the hairs of the mouse can be pushed down, making the nipple visible
6) With the same fine micro-dissection tweezers remove any dead skin that covers the nipple opening
7) After removing the dead skin, try to find the nipple opening with the fine micro-dissection tweezers
8) Load an amount of cell suspension into a 50 µl syringe with a 33G blunt needle affixed. Depending on the nipple of interest, the amount might differ

9) Hold the nipple with the fine tweezer and lift it slightly into position for the injection.
   Inject the cell suspension slowly to minimize potential damage caused by rapidly moving fluid within the ductal lumens
   Maintain the injection rate approximately 40 µl/min
10) Observe the injection site. There should be no signs of trauma to the nipple region or surrounding tissue. Swelling in the area surrounding the nipple likely indicates a mammary fat pad injection rather than a successful intraductal injection
11) Remove the mouse from the nose cone and let it recover

PROTOCOL 5: Viably Freezing Breast Cells Derived From Ascites/Pleural Effusions, Tissue (PDX) Or Organotypic Cultures (PDO)

1) Sterilize all surfaces according cell culture guidelines

For source materials ascites/pleural effusion (protocol 1) or PDO (protocol 3)

2) After centrifugation of the cell suspension at 450 x g for 5 minutes
3) Carefully aspirate supernatant
4) For home-made cryo-protective freezing solution:
   a) Resuspend cell pellet in appropriate volume of cold cell culture medium
      Aim for freezing 1-2 x 10^6 cell per mL
   b) Put on ice for at least 15 minutes
   c) Add cold freezing solution **DROP-WISE** (ratio 1:1) while swirling the cell suspension.
      Take care to **KEEP EVERYTHING ON ICE** while mixing

   For commercial cryo-protective freezing solution:
   a) Add appropriate volume of cold commercial freezing solution **DROP-WISE** to cell pellet, while swirling the cell suspension
      Aim for freezing 1-2 x 10^6 cell per mL
      Take care to **KEEP EVERYTHING ON ICE** while mixing
      If cell pellet does not dissolve, resuspend mildly using a pipette or consider resuspending the cell pellet in a small volume of culture medium prior to adding commercial freezing solution
6) Transfer your cells in freezing solution to pre-labeled, cold and sterile cryogenic vials
7) Transfer your cryogenic vials to a freezing container and place in a -80°C freezer immediately
8) After 24 hrs. transfer the vials to the liquid N_2 tank for long-term storage

For source material tissue pieces (protocol 2)

9) After mincing the tumor piece into small pieces (1-2 mm) using sterile scalpel blades
10) Add 0.5 mL of complete freezing medium (home-made or commercial) to pre-labeled, cold and sterile cryogenic vials
11) Transfer 6-10 tumor pieces to individual cryogenic vials

12) Transfer your vials to a freezing container and place in a -80°C freezer immediately
13) After 24 hrs. transfer the vials to the liquid N_2 tank for long-term storage

PROTOCOL 6: Culturing Of Patient-derived Organotypic Breast Cultures (PDO)

This is a quick default manual for culturing of Patient-derived Organotypic breast cultures. When processing new cultures, the exact details for several of the steps depend on the nature and sensitivity of the cells. These details should be determined empirically and by trial-and-error. Please follow the detailed protocol for tips and consideration for many of these steps.

1) Sterilize all surfaces and tools according cell culture guidelines
2) Aspirate cell culture medium from the multi-well plate
3) Resuspend the Matrigel/BME gels with PDOs in 5-10 mL cold AdvDF+++ in a 15 mL conical tube
4) Collect material from 1-3 wells of a 24-well plate per 15 mL conical tube
5) Centrifuge for 5 minutes @ 450 x g and aspirate medium
6) Resuspend PDOs in 150-250 µL TrypLE per conical tube
   Mix well
7) Place at 37°C for 1-4 minutes, mix cells in between by pipetting
   Confirm successful trypsinization by viewing under a microscope
   Aim for small fragments (See the detailed protocol for more details)
8) Dilute the TrypLE by adding 3-10 mL of cold AdvDF+++ to the conical tube
9) Centrifuge for 5 minutes @ 450 x g @ 4°C
10) Check the cell pellet and BME/Matrigel plug under the microscope
    If needed, resuspend and centrifuge again to further dilute out the BME remnants (770 x g = max.)
11) Aspirate most supernatant and BME remnants.
12) Determine how much of the cell suspension you require for splitting
13) Depending on the size of the cell pellet, resuspend in appropriate volume of cold culture medium
    Typically, 50-200 µL suffices (see detailed protocol for more info)
14) Add 3-4 parts of Matrigel/BME to 1 part cell suspension (e.g. 150-200 µL to 50 µL)
    Make sure to **KEEP EVERYTHING ICE-COLD**
15) Plate 3 drops (10-15 µL) of the cell-Matrigel/BME-mix per well of a pre-warmed 24-well plate
16) Flip the plate upside-down and let the gels polymerize for 30-45 minutes @ 37°C in a CO_2-incubator
17) Add 600 µL of appropriate growth medium per well
18) Refresh medium every 2-3 days
19) Re-plate organoids when the culture demands it or after max. 14 days (whichever comes first)

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INFO 1: Overview Of Cell Culture Media

AdvDF +++

	[Stock]	50 mL	500 mL	[Final]
Adv. DMEM/F12	1x	48.5 mL	500 mL	
P/S	100x	500 µL	5 mL	1x
Ultra-Glutamine	100x	500 µL	5 mL	1x
HEPES	1 M	500 µL	5 mL	10 mM

PDO-TM-HUB: Patient-derived Organoid Tumor Mamma HUB

	[Stock]	50 mL	[Final]
AdvDF+++	1x	43.5 mL	
NAC	500 mM	125 µL	1.25 mM
B27	50x	1 mL	1x
Primocin	50 mg/mL	50 µL	50 µg/mL
Noggin-CM	1 µg/mL	5 mL	100 ng/mL
R-spondin-3*	625 µg/mL	20 µL	250 ng/mL
Nicotinamide	1 M	500 µL	10 mM
FGF-7	10 µg/mL	25 µL	5 ng/mL
FGF-10	100 µg/mL	10 µL	20 ng/mL
A83-01	5 mM	5 µL	500 nM
Neuregulin	75 µg/mL (10 µM)	25 µL	37.5 ng/mL (5 nM)
Y-27632 (ROCKi)	10 mM	25 µL	5 µM
SB202190	5 mM	5 µL	500 nM
hEGF**	100 µg/mL (16.13 µM)	2,5 µL	5 ng/mL (0.8 nM)

PDO-TM-Ocello: Patient-derived Organoid Tumor Mamma-Ocello

	[Stock]	500 mL	40 mL	[Final]
Adv. DMEM/F12	1x	500 mL		
Ultra-Glutamine	100x	5 mL		1x
20x Ocello PDX	100x	25 mL		1x
Neuregulin**	75 µg/mL (10 µM)	-	5.3 µL	10 ng/mL (1.3
hEGF**	100 µg/mL (16.13	-	4 µL	10 ng/mL (1.6

*First make 500 mL Ocello Base medium w/o growth factors and store these as 40 mL aliquots @-20°C to prolong shelf life. For working stocks of Ocello complete medium add Neuregulin and hEGF.
** These are reference concentrations that may differ between PDOs, change if necessary.

INFO 2: Overview Of Required Equipment, Materials And Reagents

Equipment:

- Biosafety/Laminar flow Cabinet
- CO_2-incubator
- Pipettors, P1000, P200, P20, P10
- Centrifuge
- Aspiration needle
- Microscope and binocular
- Warming pad
- Isofluorane:Oxygen respirator device
- Scale

Materials:

- 70% ethanol solution
- 15 mL and 50 mL conical tubes
- Cryo-protective freezing container (e.g. CoolCell or Mr. Frosty)
- Micro-dissection tweezers
- Scalpels
- Sterile pipette (filter) tips
- Tissue culture plate (60 mm or 100 mm)
- Tissue culture multi-well plate (24-wells or 12-wells)

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Reagents:

- Advanced DMEM:F12 (Gibco 12-634-028)
- A83-01 (Sigma SML0788-5MG)
- B-27 supplement (50x) (Gibco 17504001)
- Basement Membrane Extract (BME) (Cultrex 3533-005-02)
- Dimethyl sulfoxide (DMSO) (Sigma Aldrich D2650)
- EGF Human Recombinant (PeproTech AF-100-15)
- Fetal Bovine Serum
- FGF-7 Human Recombinant (PeproTech 100-19)
- FGF-10 Human Recombinant (PeproTech 100-26)
- Ficoll-Paque plus (VWS 17-1440-02P)
- HEPES (Lonza BE17-737E)
- Matrigel Growth Factor reduced Basement Membrane Matrix (Corning 356231)
- N-Acetyl-L-Cysteine (Sigma-Aldrich A9165-5G)
- Neuregulin/Heregulin-Beta1 Human Recombinant (PeproTech 100-03)
- Nicotinamide (Sigma N0636-100G)
- Noggin-CM (home-made)
- Penicillin-Streptomycin (100x) (ThermoFisherScientfic 15140122)
- Primocin (Invivogen ant-pm1)
- RBC Lysis buffer (1x) (Invitrogen 00-4333)
- Recovery Cell Culture Freezing Medium (Gibco 11560446)
- R-spondin-3 Human Recombinant (PeproTech 120-44)
- SB202190 (Sigma-Aldrich S7067-5MG)
- TrypLE Express (1x) with Phenol Red (Gibco 11558856)
- Ultra-Glutamine (Lonza BE17-605E/U1)
- Y-27632 (ApexBio A3008)