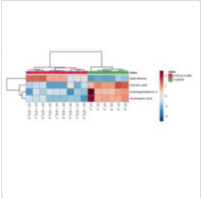

Feb 25, 2022

Protocol to secretome investigation of tumor 3D co-culture model

ANNA MARIA AP FERNANDES[1], Giulia Carli Mendes[2], Alex Rosini[3], Andrea Corazzi Pelosi[1], Leonardo Maciel[2], Luísa F Bueno [2], Lívia Maria F Silva[2], Rafael F Bredariol[2], Maycon Giovani Santana[4], Andreia de Melo Porcari[3], Denise G. Priolli[1]

[1]Postgraduate Programme Stricto Sensu in Health Science, São Francisco University, Bragança Paulista, São Paulo, Brazil;

[2]Multidisciplinary Laboratory, Medical School, Sao Francisco University, Bragança Paulista, São Paulo, Brazil.;

[3]MS4Life Laboratory of Mass Spectrometry, Health Sciences Postgraduate Program, São Francisco University, Bragança Paulista, São Paulo, Brazil;

[4]Multiprofessional Nursing Residency Program in Oncology, A.C.Camargo Cancer Center, São Paulo, Brazil.

ANNA MARIA AP FERNANDES: Giulia Carli Mendes; Alex Rosini these authors contributed equally to this work and share the first authorship;

dx.doi.org/10.17504/protocols.io.b24vqgw6

Spheroids project

ANNA MARIA FERNANDES
1 Health Sciences Postgraduate Program, São Francisco Univer...

Citation: ANNA MARIA AP FERNANDES, Giulia Carli Mendes, Alex Rosini, Andrea Corazzi Pelosi, Leonardo Maciel, LuÃÂsa F Bueno , LÃÂvia Maria F Silva, Rafael F Bredariol, Maycon Giovani Santana, Andreia de Melo Porcari, Denise G. Priolli Protocol to secretome investigation of tumor 3D co-culture model https://dx.doi.org/10.17504/protocols.io.b24vqgw6

Three-dimensional (3D) cell culture technologies, which more closely mimic the complex microenvironment of tissue, are being increasingly evaluated as a tool for the preclinical screening of clinically promising new molecules, and for the study of tissue metabolism. Studies of metabolites released into the extracellular space (secretome) allow understanding the metabolic dynamics of tissues and changes caused by therapeutic interventions. Although quite advanced in the field of proteomics, studies on the secretome of low molecular weight metabolites (< 1500 Da) are still very scarce.

We present an untargeted metabolomic protocol based on the hybrid technique of high-performance liquid- chromatography coupled with high-resolution mass spectrometry for the analysis of low-molecular-weight metabolites released into the culture medium by 3D cultures and coculture (secretoma model). For that, we analayzed HT-29 human colon carcinoma cells and 3T3-L1 preadipocytes in 3D-monoculture and 3D-coculture.

This protocol represents a possibility to list metabolites released in the extracellular environment in a comprehensive and untargeted manner, opening the way for the generation of metabolic hypotheses that will certainly contribute to the understanding of tissue metabolism, tissue-tissue interactions, and metabolic responses to the most varied interventions. Moreover, it brings potential to determine novel pathways and identify accurate biomarkers in cancer and other diseases. The metabolites indicated in our study have a close relationship with the tumor microenvironment in accordance with the literature review.

For the 3D cell culture by levitation we used Bio-Assembler™ system (Bio Science 662840, Greiner One Bio, Americana, Brazil) in the n3D Biosciences and adapted protocol published by:

- HAISLER, W. L. et al. Three-dimensional cell culturing by magnetic levitation. **Nature protocols**, v. 8, n. 10, p. 1940-1949, 2013. https://doi.org/10.1038/nprot.2013.125

DOI

dx.doi.org/10.17504/protocols.io.b24vqgw6

ANNA MARIA AP FERNANDES, Giulia Carli Mendes, Alex Rosini, Andrea Corazzi Pelosi, Leonardo Maciel, Luísa F Bueno , Lívia Maria F Silva, Rafael F Bredariol, Maycon Giovani Santana, Andreia de Melo Porcari, Denise G. Priolli 2022. Protocol to secretome investigation of tumor 3D co-culture model. **protocols.io** https://dx.doi.org/10.17504/protocols.io.b24vqgw6

Mass Spectrometry, 3D Cell Culture, Colonic Neoplasm, Biomarkers

protocol ,

Dec 20, 2021

Feb 25, 2022

56181

- HAISLER, W. L. et al. Three-dimensional cell culturing by magnetic levitation. **Nature protocols**, v. 8, n. 10, p. 1940-1949, 2013. https://doi.org/10.1038/nprot.2013.125

Trypsin EDTA **Gibco - Thermo Fischer Catalog #25-051-CI.**

Dulbecco's Modified Eagle's Medium (DMEM) **Sigma Aldrich Catalog #D5796**

Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #11360070**

Fetal Bovine Serum **Gibco - Thermo Fischer Catalog #10270106**

Gibco™ Penicillin-Streptomycin (10,000 U/mL) **Fisher Scientific Catalog #15-140-122**

Trypan Blue Solution 0.4% **Thermo Fisher Scientific Catalog #15250061**

Acetonitrile **J.T. Baker LC/MS Grade, 4 L Catalog #9829-03**

Isopropanol HPLC solvent **JT Baker Catalog #9095-02**

Water MilliQ **Contributed by users**

T25 or T75 Flask **Contributed by users**

24 Well Bio Assembler Kit **greiner bio-one Catalog #662840**

2D CELL CULTURE

1 Use human colon carcinoma (HT-29) and pre-adipocytes cells (3T3-L1) (Banco de Células do Rio de Janeiro (BCRJ; Duque de Caxias, Brazil).

2 Thaw HT-29 and 3T3-L1 cells and propagate in culture using Modified Dulbecco Eagle Medium (DMEM - Sigma D-5648, São Paulo, Brazil), supplement with [M]**100 millimolar (mM)** sodium pyruvate (Gibco -11- 360, Thermo Fisher Scientific, Waltham, Massachusetts, USA), [M]**10 % (v/v)** fetal bovine serum (Gibco 2010-09, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and [M]**1 % (v/v)** antibiotics ([M]**100 U/ml** of penicillin and [M]**10 mg/mL** of streptomycin (Gibco 15140-122, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

3 Culture cells in a humidified chamber with [M]**5 % (v/v)** CO_2 (HeraCELL 150) at **37 °C** .

4 Incubate cell cultures with **3 mL** trypsin-EDTA [M]**0.25 % (v/v)** (Gibco 25 200, Fisher Scientific, Waltham, Massachusetts, USA) at **37 °C** for three minutes to allow cell disaggregation and propagation.
Use DMEM plus 10% FBS to inactivate trypsin. Transfer the cell pellet to a new 75 cm^3 flask (T75) containing **10 mL** DMEM.

5 Change the culture medium according to the cell doubling time.

6 Determine cell viability in a Neubauer chamber using Trypan Blue (Gibco 15250061, Thermo Fisher Scientific, Waltham, Massachusetts, USA)

3D CELL CULTURE

7 Use Bio-Assembler™ system (Bio Science 662840, Greiner One Bio, Americana, Brazil) in the n3D Biosciences 24-well configuration (HAISLER et al., 2013).

Haisler WL, Timm DM, Gage JA, Tseng H, Killian TC, Souza GR (2013). Three-dimensional cell culturing by magnetic levitation.. Nature protocols.
https://doi.org/10.1038/nprot.2013.125

8 Prepare the magnetic nanoparticles by removing it from the refrigerator and thawing it at room temperature **20-25 °C** for about 15 min.

9 Culture HT- 29 cells (passage 12th) in monolayer culture at T75 flask. Determine the cell viability (>75%) in a Neubauer chamber, when the cells confluence reaches 80%-90%.

10 Add **1 µL per 10.000 cells** of magnetic nanoparticles (Nanoshuttle™- PL, Greiner) in the single cell suspension flask, homogenize gently the suspension and centrifuge **1500 rpm, 00:05:00 , 3 times** . 5m

11 Resuspend the cells and fill each well of cell-repellent 24-well plate with an amount of solution necessary to reach 7.5 x 10^3 cells, after centrifugation.

12 Complete with **250 µL** of supplemented medium Modified Dulbecco Eagle Medium (DMEM - Sigma D-5648, São Paulo, Brazil), supplement with **100 millimolar (mM)** sodium pyruvate (Gibco -11- 360, Thermo Fisher Scientific, Waltham, Massachusetts, USA), **10 % (v/v)** fetal bovine serum (Gibco 2010-09, Fisher Scientific, Waltham, Massachusetts, USA) and **1 % (v/v)** antibiotics (**100 U/ml** of penicillin and **10 mg/mL** of streptomycin) (Gibco 15140-122, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to a volume of **250 µL /well**

13 Place a magnetic coupling driver under the plate for **05:00:00** and incubate it in the humidified chamber with **5 % (v/v)** CO_2 (HeraCELL 150) at **37 °C** . 5h

14 Close the plate and place the levitation drive atop the intermediate lid to levitate the cells.

If the cells not immediately levitate gently shake the plate moving it to back and forth, until they levitate

15

Citation: ANNA MARIA AP FERNANDES, Giulia Carli Mendes, Alex Rosini, Andrea Corazzi Pelosi, Leonardo Maciel, LuÃÂsa F Bueno , LÃÂvia Maria F Silva, Rafael F Bredariol, Maycon Giovani Santana, Andreia de Melo Porcari, Denise G. Priolli Protocol to secretome investigation of tumor 3D co-culture model https://dx.doi.org/10.17504/protocols.io.b24vqgw6

Keep the magnetic coupling drive for 7 days.

16 Use the field microscopy to verify the cohesion of the structures formed.

17 Collect the culture medium whenever the exchange is necessary. To do it, use the holding drive to hold the 3D culture down while aspirating the liquid.

18 Culture 3T3-L1 cells **600 µL** in monolayer to 90% confluence and cell viability (>75%), and incubate in a supplemented medium in repellent hanging drop plate at **37 °C** / **5 % (v/v)** CO2/ **95 %** humidity and monitore until the aggregates have formed and differentiated to adipocytes.

3D CELL COCULTURE

19 Add 3T3-L1 spheroid suspension in each well of cell repellent HT-29 wells plate using a magnetic pen, 21 days after the beginning of HT-29 spheroid formation and keep it for 7 days.

EXTRACTION OF SAMPLES 10m

20 Add an aliquot of iced isopropanol **50 mg** to the culture medium **200 µL** and maintain **Overnight** at **-20 °C** , for secretome analysis.

21 Centrifuge the samples for **12500 rpm, 4°C, 00:10:00** . 10m

22 Remove a medium aliquot of **150 µL** of each sample and dry under nitrogen pressure.

23 Resuspend dry extracts in **150 µL** of an internal fluoride-phenylalanine standard solution at **200 micromolar (µM)** .

Citation: ANNA MARIA AP FERNANDES, Giulia Carli Mendes, Alex Rosini, Andrea Corazzi Pelosi, Leonardo Maciel, LuÃÂsa F Bueno , LÃÂvia Maria F Silva, Rafael F Bredariol, Maycon Giovani Santana, Andreia de Melo Porcari, Denise G. Priolli Protocol to secretome investigation of tumor 3D co-culture model https://dx.doi.org/10.17504/protocols.io.b24vqgw6

LIQUID CHROMATOGRAPHY ANALYSES

24 Perform chromatography analyses using a UPLC H-class (Waters), with an ACQUITY CSH C18 column (Waters) with dimensions 2.1 × 100 mm x 1.7 µm using a mobile phase:

Phase A) MilliQ water **Contributed by users** plus [M]**0.1 % (v/v)** Formic acid, LC-MS grade **Thermo Fisher Scientific Catalog #28905** .

Phase B) Acetonitrile **Contributed by users** .

25 Set the flow rate at 0.4 mL/min.

26 Apply the segmented gradient as follows:

A	B	C	D
Time (min)	Flow (ml/min)	A (%)	B (%)
Initial	0.400	90.0	10.0
2.00	0.400	90.0	10.0
7.00	0.400	10.0	90.0
9.00	0.400	10.0	90.0
11.00	0.400	90.0	10.0
13.00	0.400	90.0	10.0

27 Set the temperature at **20 °C** , while the injection volume is **5 µL** for positive and **2 µL** for negative modes.

MASS SPECTROMETRY ANALYSES

28 Perform the analyses using XEVO-G2XSQTOF (Waters) equipment equipped with an electrospray source.

29 Perform the analyses in the mode of positive (+) and negative (-) ionization.

Citation: ANNA MARIA AP FERNANDES, Giulia Carli Mendes, Alex Rosini, Andrea Corazzi Pelosi, Leonardo Maciel, LuÃÂsa F Bueno , LÃÂvia Maria F Silva, Rafael F Bredariol, Maycon Giovani Santana, Andreia de Melo Porcari, Denise G. Priolli Protocol to secretome investigation of tumor 3D co-culture model https://dx.doi.org/10.17504/protocols.io.b24vqgw6

30 Optimize the font parameters for better performance. Initially:

A	B	C
Parameters	Mode Positive	Mode Negative
Source temperature (°C)	140	140
Desolvation temperature (°C)	550	550
Desolvation flow (L.h-1)	900	900
Capillary (kV)	3	2.5
Sampling cone (kV)	30	40
Cone gas flow (L.h-1)	10	50

31 Acquire the spectra under the acquisition mass range of 50-1200 Da, using the MS^E approach (6 V for low energy and a 15-30 V ramp for high energy scanning).

32 Use Leucine encephalin [M] **200 Parts per Million (PPM)** in an ⊠Acetonitrile **Contributed by users** : ⊠MilliQ water **Contributed by users** 1:1 v/v) as lockmass.

33 Use a [M] **0.5 millimolar (mM)** sodium formate solution for instrument calibration.

DATA PROCESSING AND POTENTIAL IDENTIFICATION OF COMPOUNDS

34 Process the raw data with Progenesis IQ (Waters) software for peak detection, alignment, integration, deconvolution, data filtering, ion annotation, and MS^E based putative identification of compounds.

35 Use the following databases to suggest the identifications : Lipid Maps (http://www.lipidmaps.org/), LipidBlast (https://fiehnlab.ucdavis.edu/projects/LipidBlast) and Human Metabolome Database (http://www.hmdb.ca/metabolites).

36 Use the following parameters for identification : mass error of the precursor ≤ 5 ppm, mass error of the fragment ≤ 10 ppm, mass precision, and isotopic similarity.

STATISTICAL ANALYSES

37 Perform the volcano plot statistical test between all culture conditions and in the presence and absence (white samples) of the cells.

Citation: ANNA MARIA AP FERNANDES, Giulia Carli Mendes, Alex Rosini, Andrea Corazzi Pelosi, Leonardo Maciel, LuÃÂsa F Bueno , LÃÂvia Maria F Silva, Rafael F Bredariol, Maycon Giovani Santana, Andreia de Melo Porcari, Denise G. Priolli Protocol to secretome investigation of tumor 3D co-culture model https://dx.doi.org/10.17504/protocols.io.b24vqgw6

38 Rank the relevant molecular features according to the false discovered ratio (FDR) and log2 fold change (FC) values.

39 Consider only the molecular features that presented FDR value < 0.05 and log2 FC>0 when compared to the secretome of the culture medium (white), In the assignment of cell secretomes

Citation: ANNA MARIA AP FERNANDES, Giulia Carli Mendes, Alex Rosini, Andrea Corazzi Pelosi, Leonardo Maciel, LuÃÂsa F Bueno , LÃÂvia Maria F Silva, Rafael F Bredariol, Maycon Giovani Santana, Andreia de Melo Porcari, Denise G. Priolli Protocol to secretome investigation of tumor 3D co-culture model https://dx.doi.org/10.17504/protocols.io.b24vqgw6