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Version 2

# Protocol to secretome investigation of tumor 3D co-culture model V.2

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**We use this protocol and it's working**

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## Abstract

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 analyzed 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>

## Guidelines

- 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>

## Materials

- ✕ Trypsin EDTA **Gibco - Thermo Fisher Scientific Catalog #25-051-CI.**
- ✕ Dulbecco's Modified Eagle's Medium (DMEM) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D5796**
- ✕ Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #11360070**
- ✕ Fetal Bovine Serum **Gibco - Thermo Fisher Scientific 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
- ✕ T25 or T75 Flask
- ✕ 24 Well Bio Assembler Kit **greiner bio-one Catalog #662840**

## Protocol materials

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- ✕ Fetal Bovine Serum **Gibco - Thermo Fisher Scientific Catalog #10270106**
- ✕ T25 or T75 Flask
- ✕ p-Fluoro-DL-phenylalanine **Merck MilliporeSigma (Sigma-Aldrich) Catalog # F525**
- ✕ Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3641**
- ✕ MilliQ water
- ✕ Formic acid, LC-MS grade **Thermo Fisher Scientific Catalog #28905**
- ✕ Acetonitrile

## 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<sub>2</sub> (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<sup>3</sup> 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 the Bio-Assembler™ system (Bio Science 662840, Greiner One Bio, Americana, Brazil) in the n3D Biosciences 24-well configuration (HAISLER et al., 2013).

### Citation

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>

LINK

- 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 .
- 11 Resuspend the cells and fill each well of cell-repellent 24-well plate with an amount of solution necessary to reach  $7.5 \times 10^3$  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<sub>2</sub> (HeraCELL 150) at  37 °C .
- 14 Close the plate and place the levitation drive atop the intermediate lid to levitate the cells.

5m

5h

### Safety information

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

15

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  $\mu\text{L}$  in monolayer to 90% confluence and cell viability (>75%), and incubate in a supplemented medium in repellent hanging drop plate at  37 °C / [M] 5 % (v/v) CO<sub>2</sub>/  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

Using a pipet, take  200  $\mu\text{L}$  of the culture medium of each well and place it into a microtube. Then, add  50  $\mu\text{L}$  of iced isopropanol and keep the microtube at  -20 °C  Overnight . Prepare each sample in triplicate. Additionally, prepare a pooled quality control (QC) sample. For that, an aliquot (  20  $\mu\text{L}$  ) of each sample must be collected and pooled together in a microtube to generate a QC sample containing the chemical composition representative of the sample set. Homogenize this sample and split it into different vials, then prepare them following the same

extraction protocol. Use QC samples for system suitability before batch analysis. Also, inject a QC sample after 10 samples to investigate and filter instrumental variation. Blank samples (N=3) must also be prepared using the culture medium without adding any cell or culture and following the complete protocol for sample preparation. Samples must be organized by starting with QC samples, followed by blank samples, and then the set of test samples randomly organized. A QC sample must be placed every 10 samples. The final sample must be a QC sample.

21 Centrifuge the samples for  12880 x g, 4°C, 00:10:00 .

10m

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

23 Resuspend the dry extracts in  150 µL of a solution composed of

 p-Fluoro-DL-phenylalanine **Merck** **MilliporeSigma** **(Sigma-Aldrich)** **Catalog # F525**

at  200 micromolar (µM) in

 Methanol **Merck** **MilliporeSigma** **(Sigma-Aldrich)** **Catalog #M3641** :

 MilliQ water 1:1 v/v).

## 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 plus  0.1 % (v/v)

 Formic acid, LC-MS grade **Thermo Fisher Scientific** **Catalog #28905** .

Phase B)  Acetonitrile .

25 Set the flow rate to 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 to  30 °C , while the injection volume must be  5 µL for the positive and  2 µL for the negative ionization modes.

## MASS SPECTROMETRY ANALYSES

- 28 Perform the analyses using the XEVO-G2XSQTOF (Waters) instrument equipped with an electrospray ion source.
- 29 Use a  0.5 millimolar (mM) sodium formate solution for the instrument calibration.
- 30 Perform the analyses in the positive (+) and negative (-) ionization modes.
- 31 Optimize the source parameters for better performance. Suggestion of initial parameters:

|  | A                            | B                 | C                 |
|--|------------------------------|-------------------|-------------------|
|  | Parameters                   | Positive Ion Mode | Negative Ion Mode |
|  | Source temperature (°C)      | 140               | 140               |
|  | Desolvation temperature (°C) | 550               | 550               |
|  | Desolvation flow (L/h)       | 900               | 900               |

|  | A                   | B  | C   |
|--|---------------------|----|-----|
|  | Capillary (kV)      | 3  | 2.5 |
|  | Sampling cone (kV)  | 30 | 40  |
|  | Cone gas flow (L/h) | 10 | 50  |

- 32 Acquire the spectra under the acquisition range of 50-1200 Da, using the MS<sup>E</sup> approach (6 V for low-energy, and a 15-30 V ramp for high-energy scanning).
- 33 Use Leucine encephalin [M] 200 Parts per Million (PPM) in an  Acetonitrile :  MilliQ water 1:1 v/v) as the lockmass, infused at 25 µL/min.

## DATA PROCESSING AND POTENTIAL IDENTIFICATION OF COMPOUNDS

- 34 Process the .RAW files obtained after LC-ESI-MS analysis by using the Progenesis QI software 2.4.69.11 (Nonlinear Dynamics, Newcastle, UK)). Choose to use centroid data and the mass resolution of 40,000. Perform peak alignment based on the QC samples. Select the following adduct species: [M+H]<sup>+</sup>, [M+Na]<sup>+</sup>, [M+K]<sup>+</sup>, [M+ACN+Na]<sup>+</sup>, [M+ACN+H]<sup>+</sup>, [M+H-H<sub>2</sub>O]<sup>+</sup>, [M+H-2H<sub>2</sub>O]<sup>+</sup> for the positive ion mode, and [M-H]<sup>-</sup>, [M+FA-H]<sup>-</sup>, [M+Na-2H]<sup>-</sup>, [M-H<sub>2</sub>O-H]<sup>-</sup>, [M+Cl]<sup>-</sup>, for the negative ion mode. Progenesis QI generates a table containing the intensity of the ions listed according to their nominal masses for each sample. This software also generates MS<sup>E</sup>-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.

|  | A        | B           | C       | D       | E     | F                   | G                | H                  | I           | J                            |
|--|----------|-------------|---------|---------|-------|---------------------|------------------|--------------------|-------------|------------------------------|
|  | Compound | Compound ID | Adducts | Formula | Score | Fragmentation Score | Mass Error (ppm) | Isotope Similarity | Description | Annotation Confidence Level* |



|  | A                             | B                   | C                            | D                                                          | E        | F    | G         | H        | I                                                             | J |
|--|-------------------------------|---------------------|------------------------------|------------------------------------------------------------|----------|------|-----------|----------|---------------------------------------------------------------|---|
|  | <b>Mode<br/>Negati<br/>ve</b> |                     |                              |                                                            |          |      |           |          |                                                               |   |
|  | 4.58_2<br>45.092<br>0m/z      | HMD<br>B000<br>4259 | M-<br>H <sub>2</sub> O-<br>H | C <sub>13</sub> H<br>16N <sub>2</sub><br>O <sub>4</sub>    | 38<br>,4 | 0    | -4,2<br>9 | 96,<br>9 | Acetyl-<br>N-<br>formyl-<br>5-<br>methox<br>ykynure<br>namine | 3 |
|  | 8.13_30<br>1.2162<br>m/z      | HMD<br>B0011<br>134 | M-<br>H <sub>2</sub> O-<br>H | C <sub>20</sub><br>H <sub>32</sub><br>O <sub>3</sub>       | 39<br>,9 | 5,12 | -3,3<br>0 | 98,<br>4 | 5-HETE                                                        | 2 |
|  | 9.00_3<br>05.247<br>5m/z      | HMD<br>B000<br>2925 | M-H                          | C <sub>20</sub><br>H <sub>34</sub><br>O <sub>2</sub>       | 38<br>,7 | 0    | -3,6<br>7 | 97,8     | 8,11,14-<br>Eicosatr<br>ienoic<br>acid                        | 3 |
|  | 8.59_3<br>29.247<br>3m/z      | HMD<br>B000<br>1976 | M-H                          | C <sub>22</sub> H<br>34O <sub>2</sub>                      | 38<br>,8 | 0    | -4,0<br>8 | 98,<br>8 | Docosa<br>penta<br>enoic<br>acid<br>(22n-6)                   | 3 |
|  | 8.49_3<br>03.232<br>0m/z      | HMD<br>B000<br>1043 | M-H                          | C <sub>20</sub><br>H <sub>32</sub><br>O <sub>2</sub>       | 40<br>,3 | 5,73 | -3,2<br>7 | 99,<br>6 | Arachid<br>onic<br>acid                                       | 2 |
|  | 0.61_2<br>32.082<br>4m/z      | HMD<br>B001<br>2150 | M+FA<br>-H                   | C <sub>8</sub> H <sub>1</sub><br>3NO<br>4                  | 40       | 11,7 | -1,4<br>6 | 90,<br>2 | 2-Keto-<br>6-<br>acetami<br>docapr<br>oate                    | 2 |
|  | 0.61_13<br>4.0460<br>m/z      | HMD<br>B000<br>0056 | M+FA<br>-H                   | C <sub>3</sub> H <sub>7</sub><br>NO <sub>2</sub>           | 39<br>,8 | 4,66 | 1,51      | 96,<br>0 | beta-<br>Alanine                                              | 2 |
|  | 0.54_8<br>02.669<br>7m/z      | HMD<br>B001<br>3408 | M-H                          | C <sub>46</sub><br>H <sub>94</sub><br>NO <sub>7</sub><br>P | 37,<br>1 | 0    | 0,2<br>9  | 85,<br>9 | PC(o-<br>16:0/22:<br>0)                                       | 3 |
|  | 1.67_29<br>1.0973<br>m/z      | HMD<br>B0011<br>741 | M-<br>H <sub>2</sub> O-<br>H | C <sub>14</sub> H<br>18N <sub>2</sub><br>O <sub>6</sub>    | 38<br>,7 | 0    | -4,2<br>5 | 98,<br>5 | gamma<br>-<br>Glutam<br>yltyrosi<br>ne                        | 3 |
|  | 3.52_2<br>41.1184<br>m/z      | HMD<br>B0011<br>170 | M-<br>H <sub>2</sub> O-<br>H | C <sub>11</sub> H<br>20N <sub>2</sub><br>O <sub>5</sub>    | 41,<br>1 | 13,4 | -3,8<br>3 | 96,<br>4 | gamma<br>-<br>Glutam<br>ylisoleu<br>cine                      | 2 |

|  | A                             | B                   | C                                        | D                   | E        | F     | G         | H        | I                                   | J |
|--|-------------------------------|---------------------|------------------------------------------|---------------------|----------|-------|-----------|----------|-------------------------------------|---|
|  | 0.75_15<br>1.0247<br>m/z      | HMD<br>B000<br>0139 | M+FA<br>-H                               | C3H<br>6O4          | 39<br>,5 | 3,17  | -0,6<br>2 | 95,<br>0 | Glyceri<br>c acid                   | 2 |
|  | <b>Mode<br/>Positiv<br/>e</b> |                     |                                          |                     |          |       |           |          |                                     |   |
|  | 0.56_2<br>51.1008<br>n        | HMD<br>B000<br>0101 | M+N<br>a,<br>M+K,<br>M+H,<br>M+H<br>-H2O | C10H<br>13N5<br>O3  | 39       | 5,71  | -4,3<br>0 | 94,<br>5 | Deoxya<br>denosin<br>e              | 2 |
|  | 0.54_13<br>9.0743<br>n        | HMD<br>B001<br>2234 | M+H<br>-<br>H2O,<br>M+H                  | C6H<br>9N3<br>O     | 39<br>,6 | 10,2  | -2,2<br>0 | 90,<br>2 | Histidin<br>al                      | 2 |
|  | 4.43_4<br>07.1214<br>m/z      | HMD<br>B006<br>2198 | M+A<br>CN+<br>H                          | C12H<br>19N3<br>O8S | 38<br>,9 | 3,93  | -4,5<br>8 | 96,<br>0 | 2-S-<br>glutathi<br>onyl<br>acetate | 2 |
|  | 1.39_2<br>98.097<br>0m/z      | HMD<br>B000<br>1173 | M+H                                      | C11H1<br>5N5O<br>3S | 43<br>,5 | 19    | 0,5<br>9  | 98,<br>9 | 5'-<br>Methylt<br>hioaden<br>osine  | 2 |
|  | 0.77_15<br>3.0402<br>m/z      | HMD<br>B000<br>0292 | M+H                                      | C5H<br>4N4<br>O2    | 39<br>,6 | 4,75  | -3,1<br>3 | 97,<br>2 | Xanthin<br>e                        | 2 |
|  | 0.65_15<br>2.0566<br>m/z      | HMD<br>B000<br>0132 | M+H                                      | C5H5<br>N5O         | 40<br>,6 | 4,18  | -0,4<br>6 | 99,<br>4 | Guanin<br>e                         | 2 |
|  | 0.50_3<br>64.244<br>5m/z      | HMD<br>B0011<br>531 | M+A<br>CN+<br>Na                         | C17H<br>32O4        | 38<br>,8 | 0,505 | -4,3<br>6 | 98,<br>6 | MG(0:0<br>/14:1(9Z<br>)/0:0)        | 2 |

\* Annotation Confidence Level. (1) Reference standard confirmed structure; (2) exact mass, isotopic pattern, retention time, and MS/MS spectrum matched to an in-house spectral database or literature spectra; (3) putative ID assignment based only on elemental formula match with exact mass and isotopic pattern, and (4) unknown compound.

## STATISTICAL ANALYSES

37

The statistical analyses were performed using the Metaboanalyst web platform (version 5.0 - <https://www.metaboanalyst.ca/>). For the negative ionization mode: data



was normalized by the IS (



p-Fluoro-DL-phenylalanine **Merck**  
**Aldrich**) Catalog # F525

**MilliporeSigma**

**(Sigma-**

), log-transformed, and Pareto scaled. The same procedure was used for the positive ionization mode, but the cubic transformation was applied instead of the log.

Perform multiple volcano plots to test between the compared culture conditions and also to blank samples.

38 Rank the relevant molecular features according to the false discovered ratio (FDR) and log<sub>2</sub> fold change (FC) values.

39 For the assignment of cell secretomes, consider only the molecular features that present FDR value < 0.05 and log<sub>2</sub> FC>0 when compared to the secretome of the culture medium (blank sample).

## Citations

### Step 7

Haisler WL, Timm DM, Gage JA, Tseng H, Killian TC, Souza GR. Three-dimensional cell culturing by magnetic levitation.

<https://doi.org/10.1038/nprot.2013.125>