Metabolome analysis for cells

Introduction

Metabolome analysis using global metabolic profiling and lipids profiling.

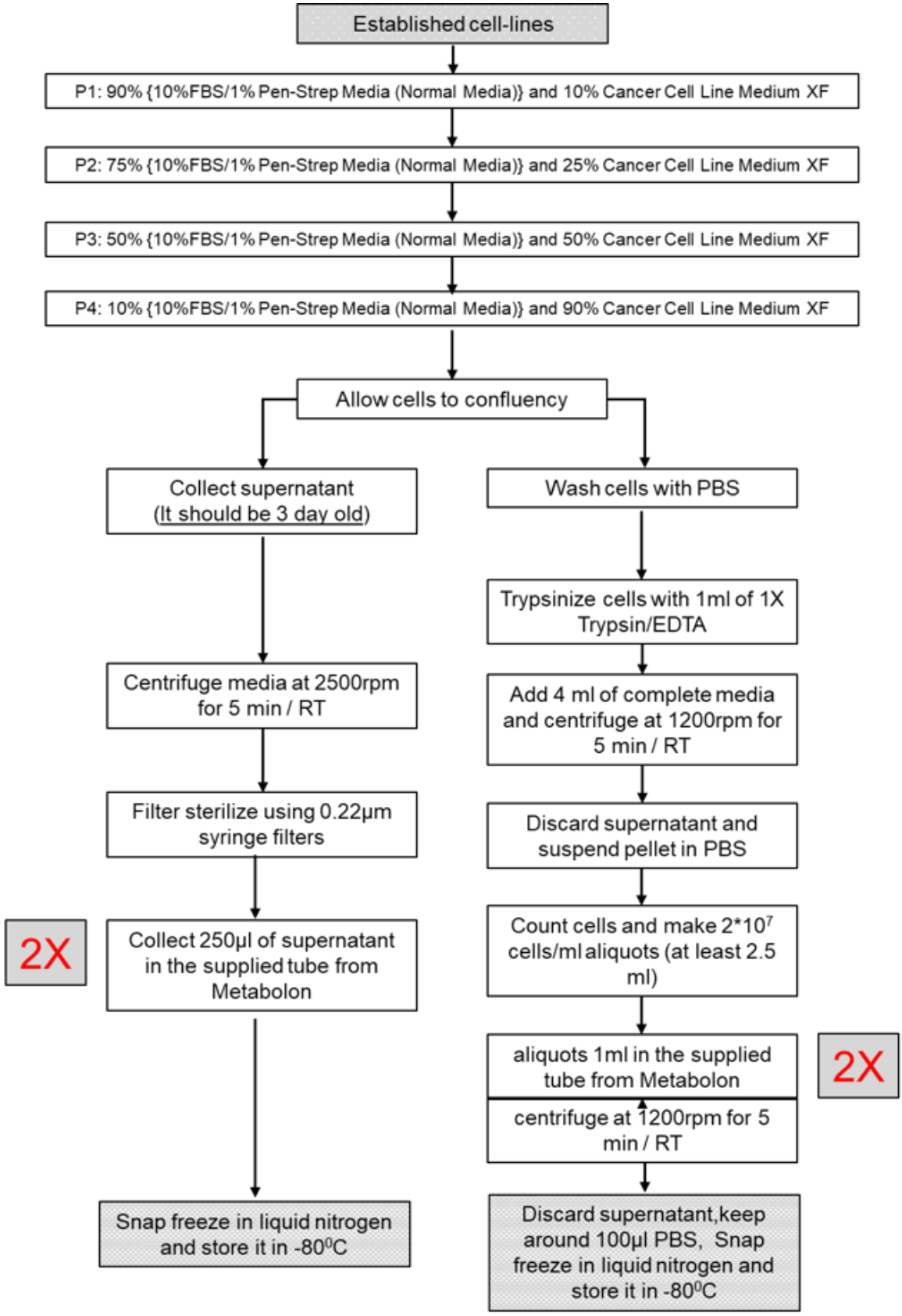
Materials

- Cancer Cell Line Medium XF + **Supplement**
- Fibronectin solution (5ml, 1mg/ml)

Procedure

Transition to serum free media

1. Dilute Fibronectin as concentration of 1ug/ml in PBS.
2. Overlay the culture surface of your tissue culture plates with an amount of the diluted Fibronectin Solution sufficient to effectively coat the complete surface. Be sure that the entire surface is covered.
3. Place flasks on a level surface at RT for 60 min. Aspirate the excess Fibronectin Solution and use immediately or let air-dry the open vessel under a laminar flow bench.
4. follow the chart to gradually transtion to serum free media:


Established cell-lines
P1: 90% {10%FBS/1% Pen-Strep Media (Normal Media)} and 10% Cancer Cell Line Medium XF
P2: 75% {10%FBS/1% Pen-Strep Media (Normal Media)} and 25% Cancer Cell Line Medium XF
P3: 50% {10%FBS/1% Pen-Strep Media (Normal Media)} and 50% Cancer Cell Line Medium XF
P4: 10% {10%FBS/1% Pen-Strep Media (Normal Media)} and 90% Cancer Cell Line Medium XF
Allow cells to confluency
Collect supernatant
(It should be 3 day old)
Centrifuge media at 2500rpm for 5 min / RT
Filter sterilize using 0.22µm syringe filters
2X
Collect 250µl of supernatant in the supplied tube from Metabolon
Snap freeze in liquid nitrogen and store it in -80^{0}C
Wash cells with PBS
Trypsinize cells with 1ml of 1X Trypsin/EDTA
Add 4 ml of complete media and centrifuge at 1200rpm for 5 min / RT
Discard supernatant and suspend pellet in PBS
Count cells and make 2*10^7 cells/ml aliquots (at least 2.5 ml)
aliquots 1ml in the supplied tube from Metabolon
2X
centrifuge at 1200rpm for 5 min / RT
Discard supernatant,keep around 100µl PBS, Snap freeze in liquid nitrogen and store it in -80^{0}C

Global metabolic profiling (Metabolon, Inc. Durham, NC)

5. **Sample Preparation**

a) Automated Sample Preparation:

Use the MicroLab STAR® system from Hamilton Company for automated sample preparation.

b) Addition of Recovery Standards:

Add several recovery standards to each sample prior to the first extraction step for quality control (QC) purposes.

c) **Protein Precipitation:**

- Precipitate proteins by adding methanol to the samples.
- Shake the samples vigorously for 2 minutes using the Glen Mills GenoGrinder 2000.

d) **Centrifugation:**

Centrifuge the samples to separate the protein precipitate from the supernatant.

e) **Fractionation:**

- Divide the resulting supernatant into multiple fractions:
- Two fractions for analysis by reverse phase (RP)/UPLC-MS/MS with positive ion mode electrospray ionization (ESI).
- One fraction for RP/UPLC-MS/MS with negative ion mode ESI.
- One fraction for HILIC/UPLC-MS/MS with negative ion mode ESI.
- Reserve the remaining fractions for backup.

f) **Solvent Removal:**

Use a TurboVap® (Zymark) briefly to remove the organic solvent from the samples.

g) Overnight Storage:

Store the sample extracts overnight under nitrogen gas before proceeding with the analysis.

6. **Quality Assurance/Quality Control (QA/QC)**

a) Control Samples:

- Prepare and analyze several types of control samples alongside experimental samples:
- A pooled matrix sample as a technical replicate.
- Extracted water samples as process blanks.
- A QC standard cocktail added to each sample for instrument performance monitoring and chromatographic alignment.

b) **Instrument and Process Variability:**

- Determine instrument variability by calculating the median relative standard deviation (RSD) for standards added before mass spectrometer injection.
- Determine overall process variability by calculating the median RSD for endogenous metabolites present in 100% of the pooled matrix samples.

c) **Sample Randomization:**

Randomize the experimental samples across the platform run, spacing QC samples evenly among injections.

7. **Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)**

a) **Instrumentation:**

Use a Waters ACQUITY UPLC system coupled with a Thermo Scientific Q-Exactive mass spectrometer, interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer at 35,000 mass resolution.

b) Sample Reconstitution:

Reconstitute dried sample extracts in solvents specific to each of the four methods, each containing standards for consistency.

C) Chromatographic Analysis:

- PosEarly Method: Analyze one aliquot under acidic positive ion conditions, optimized for hydrophilic compounds using a C18 column with water and methanol containing 0.05% PFPA and 0.1% FA.

- PosLate Method: Analyze another aliquot under acidic positive ion conditions, optimized for hydrophobic compounds using the same C18 column with a gradient of methanol, acetonitrile, water, 0.05% PFPA, and 0.01% FA.

- Neg Method: Analyze a third aliquot under basic negative ion conditions using a dedicated C18 column with methanol and water containing 6.5 mM ammonium bicarbonate at pH 8.

- HILIC Method: Analyze the fourth aliquot under negative ion conditions using a HILIC column with water and acetonitrile containing 10 mM ammonium formate, pH 10.8.

d) **Mass Spectrometry:**

- Alternate between MS and data-dependent MSn scans with dynamic exclusion.
- Cover a scan range of 70-1000 m/z.

8. **Bioinformatics**

a) **LIMS:**

Use the Metabolon Laboratory Information Management System (LIMS) for sample accessioning, preparation, analysis, and reporting.

b) **Data Extraction and Compound Identification:**

- Extract raw data, identify peaks, and perform QC using Metabolon's proprietary software.
- Compare identified compounds with a library of purified standards based on retention time, m/z, and MS/MS scores.

c) Compound Quality Control:

Perform curation procedures to ensure accurate identification of chemical entities and remove artifacts.

9. **Metabolite Quantification and Data Normalization**

a) **Peak Quantification:**

Quantify peaks by calculating the area under the curve.

b) Data Normalization:

- For multi-day studies, normalize data to correct for inter-day instrument variability by adjusting compound medians to equal one and normalizing data points proportionately.
- Additional normalization may be performed based on factors such as cell counts or total protein to account for material differences in each sample.

Complex Lipid Platform (Metabolon, Inc. Durham, NC)

10. **Sample Preparation**

a) **Lipid Extraction from Supernatant:**

- Extract lipids from the sample supernatant using a modified version of the Matyash method (PMID: 18281723).
- Use methyl-tert-butyl ether as the extraction solvent, and include deuterated internal standards for quantification.

b) **Lipid Extraction from Cells:**

- Homogenize cell samples in deionized water.
- Reserve a portion of each homogenate for Bradford protein and/or DNA quantification for normalization purposes.

- Subject the remaining homogenate to a modified Bligh-Dyer extraction using methanol, water, and dichloromethane, with internal standards added for quantification.

c) Concentration and Reconstitution:

- Concentrate the lipid extracts under nitrogen gas.
- Reconstitute the concentrated extracts in 0.25 mL of 10 mM ammonium acetate in a dichloromethane (50:50) solution.

d) Sample Preparation for Infusion-MS:

Transfer the reconstituted extracts to inserts and place them into vials suitable for infusion-MS analysis.

11. **Mass Spectrometry Analysis**

a) Instrument Setup:

- Perform infusion-MS analysis using a Shimadzu LC system with nano PEEK tubing.
- Use the Sciex Selexlon-5500 QTRAP mass spectrometer for detection.

b) Electrospray Ionization:

Analyze samples using both positive and negative mode electrospray ionization.

c) **MRM Mode Scan:**

- Conduct the 5500 QTRAP scan in Multiple Reaction Monitoring (MRM) mode.
- Use a total of more than 1,100 MRMs for the analysis.

12. **Data Processing and Quantification**

a) Lipid Quantification:

- Quantify individual lipid species by calculating the peak area ratios of target compounds to their corresponding internal standards.
- Multiply the ratio by the concentration of the internal standard added to the sample to obtain lipid species concentrations.

b) **Background Subtraction:**

Subtract background concentrations using data from process blanks (water extracts).

c) Normalization:

Normalize lipid species concentrations to run-day variations when applicable.

d) **Calculation of Lipid Metrics:**

- Use the background-subtracted, run-day normalized concentrations to calculate:
- Lipid class concentrations.
- Fatty acid total concentrations.
- Mol% composition values for lipid species, lipid classes, and fatty acids.