Application Note: HepG2 Labeling and Cell Growth Protocol

HepG2 Labeling and Cell Growth Protocol for IROA Phenotypic Metabolic Profiling

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Introduction

The IROA protocol for metabolic profiling utilizes full metabolic labeling to distinguish between biological compounds (compounds that arise from metabolic networks found in control and experimental samples) and noise and chemical artifacts such as plasticizers, airborne and waterborne contaminants.

Where it is not possible to label the biological sample, the "Phenotypic" IROA Protocol (Figure 1) is applied. Here the sample is collected at natural abundance and mixed with a fully predefined "Standard" that has been isotopically labeled using IROA "13C media" in which all of the carbon components are randomly labeled at 95% ^{13}C. An ideal Standard would be one that represented the entire metabolome of the sample under study.

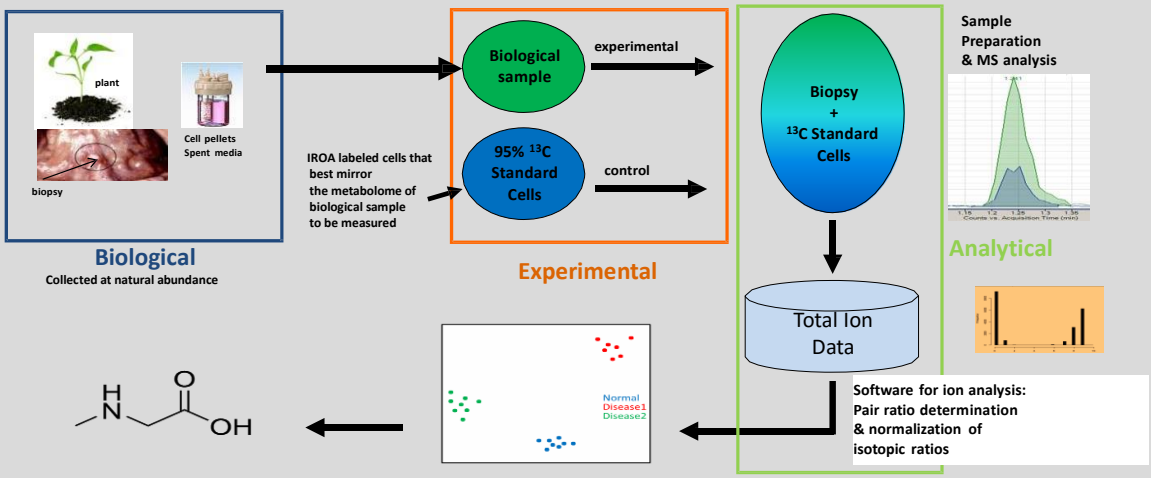


Figure 1: The Phenotypic IROA Protocol

All peaks of the IROA-labeled Standard may be easily identified according to their characteristic M-1 peak (Figure 2).

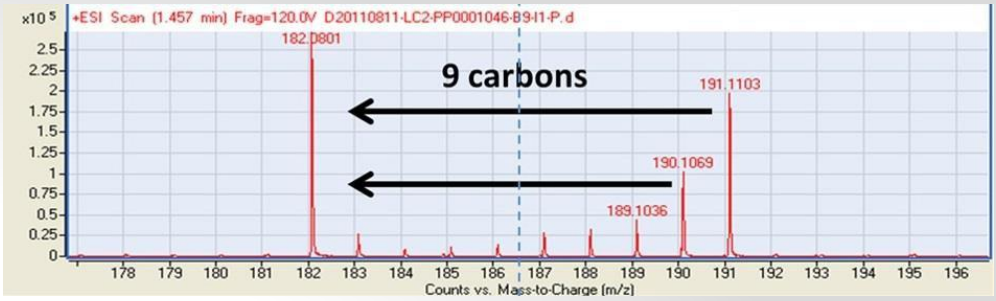


Figure 2: A typical Phenotypic IROA signal for a 9 carbon molecule.
The Phenotypic IROA signal is made up of two halves. The C13 side coming from the Standard tells you were to find the corresponding unlabeled, natural abundance C12 peak from the experimental sample. The pairing makes the identity of all peaks measured unambiguous.

The natural abundance metabolite peaks (paired to each Standard peak) may be readily identified as their exact mass and position are established relative to the Standard. Compounds present in a Standard can be well characterized, produced in sufficient quantities, stored and used to compare samples across multiple experiments. Artifacts have no match in the Standard and can be discarded. Whereas in a basic IROA dataset the ratio of the peak areas represents the relative deviation of the metabolic pool sizes brought about by the experimental condition, in a Phenotypic IROA experiment the overall pattern of deviations from the Standard will define phenotype by difference from the Standard. For further information, please see http://sftp.iroatech.com/page/The%20IROA%20Phenotypic%20Experiment.

Preparation of 95% 13C IROA Media

Materials: IROA 300 C13 Biochemical Labeling Medium (#C13-300-250), IROA 300 C13 labeled component mix (#C13 LC300-250), Dialyzed Fetal Bovine Serum, vacuum filter

1. To the *IROA 300 C13 Biochemical Labeling Medium*, add the *IROA 300 C13 labeled component mix* and ensure that the mixture is completely dissolved.
2. Add dialyzed fetal bovine serum to the IROA media mixture.
3. Vacuum filter media through 0.22 µm PES Hydrophilic BPE 2250 filter.
4. Place media in warm water bath (35°C) for at least one hour.

Media Integrity Check for 95% 13C-Labeling

Materials: 1.5 mL Eppendorf tube, P1000 micropipette, 1000 µL pipette tips, N_2 dryer, centrifuge, ACE Excel 2 C18-PFP column (100 x 2.1, mm) 2.0 µm particle size,
Reconstitution Solution: H_2O with 0.1% Formic Acid LC/MS grade
***Mobile Phases*:** H_2O with 0.1% Formic Acid LC/MS grade and Acetonitrile LC/MS grade

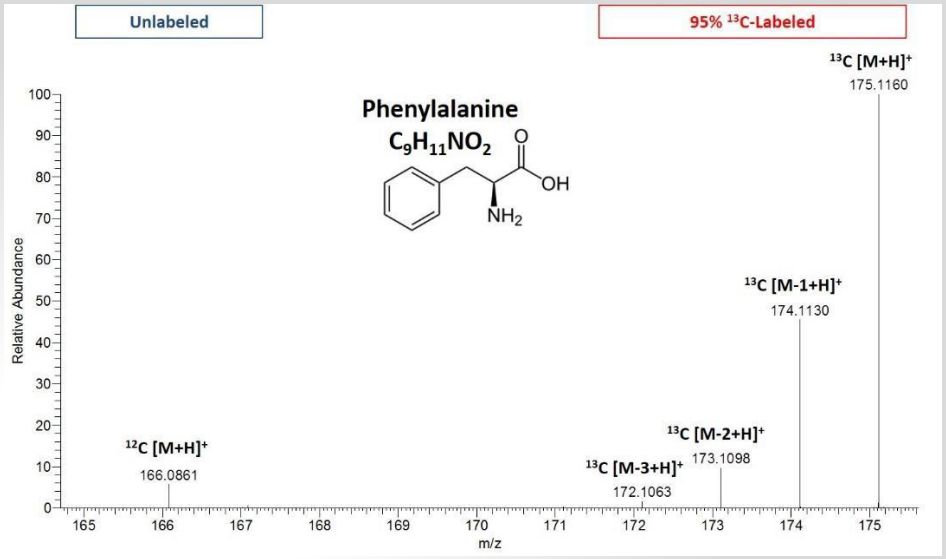
1. Add 0.5 mL of filtered cell media to a clean Eppendorf tube using a P1000 micropipette.
2. Centrifuge media at 2000 rpm for 5 min at 5°C.
3. Transfer 400 µL of supernatant to new, labeled tube.
4. Dry liquid sample using Nitrogen gas in Organomation Associates MultiVap.
5. Reconstitute sample by adding 100 µL H_2O with 0.1% Formic Acid. Vortex sample.
6. Place on an ice bath for 10-15 minutes. Centrifuge again.
7. Transfer supernatant to labeled, glass LC vial with glass insert.

Liquid Chromatography Gradient Information

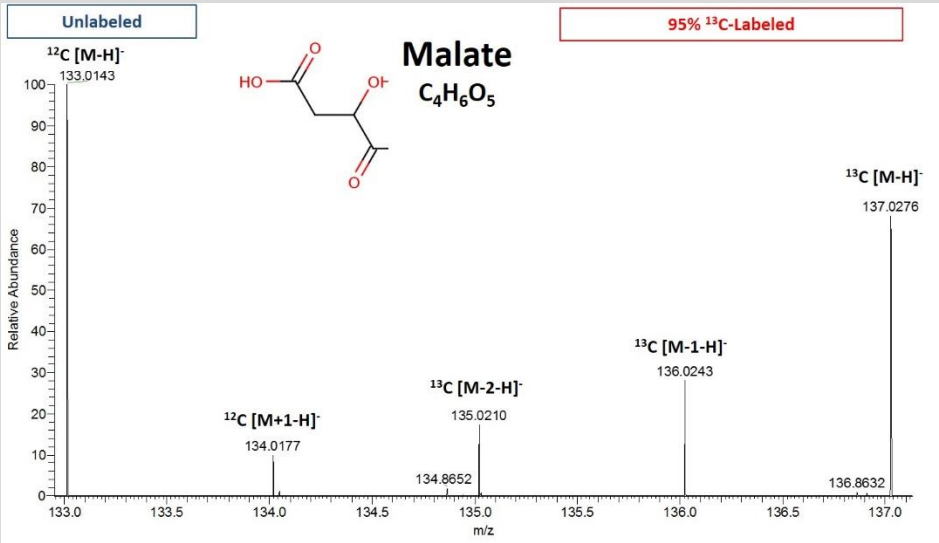
8. Equilibrate ACE Excel 2 C18-PFP column
9. The gradient program[1] consisted of mobile phase A [0.1% formic acid in water] and mobile phase B [ACN modified with 0.1% formic acid] at 0-1 min hold at 0% B, a linear ramp to 65% B at 11 min, a hold at 65% B until 13 min, a linear ramp to 95% B at 18 min, and a hold at 95% B until 20 min.
10. Injection volume: 5 µL/min
11. Flow Rate: 350 µL/min

Thermo Scientific Q Exactive Instrument Parameters (Polarity Switching)		
HESI Probe	Positive (+)	Negative (-)
Probe Temperature	350°C	350°C
Spray Voltage	3500 V	3500 V
Capillary Temperature	320°C	320°C
Sheath Gas	40	45
Auxillary Gas	10	10
Spare gas	1	1
Mass resolution 35,000 @ m/z 200		

12. Confirmation of labeled compounds in media
    a. Positive Ion Mode: ^{13}C $[M+H]^+$ phenylalanine, *m/z* 175.1164, RT: 5.4 min



b. Negative Ion Mode: ^{13}C $[M-H]^-$ malate, *m/z* 137.0276, RT: 1.0 min



Stabilize Cell Growth on EMEM Media *(reported in accordance with procedures outlined by ATCC)*[2]

***Materials*:** HepG2 cells (HB-8065), EMEM Media, liquid nitrogen, warm water bath (37 °C), vial O-ring, 70% ethanol, incubator, centrifuge, 15 mL conical tube

Cell Line Handling

1. Purchase HepG2 cells (HB-8065) as a frozen cell culture from ATCC (Manassas, VA).
2. Remove frozen cells from dry ice packaging and store cell line under liquid N_2 (-130 °C) until use.

Cell Culture Initiation

3. Warm EMEM media with 10% dialyzed fetal bovine serum (prepared according to manufacturer's recommendations) in warm water bath for 20-30 min.
4. Thaw cryopreserved cell line using an O-ring in warm water bath for 1-2 min.
5. Remove the vial from the warm water bath and spray vial with 70% ethanol.
6. Transfer 9 mL of EMEM media into a 15 mL conical tube. Add 1 mL of the cryopreservation vial contents to the conical tube. Gently pipette to mix.
7. Centrifuge the conical tube at 1200 rpm for 5 minutes at 5 °C.
8. Remove the supernatant and resuspend the cell pellet in 10 mL of EMEM media.
9. Transfer the 10 mL cell/media mixture to a labeled 100 mm culture dish.
10. Incubate the culture in an incubator set at 37 °C and 5% CO_2.
11. Grow cell line until 80-90% confluent.

Cell Culture Subculture/Stabilization

12. Remove culture medium from culture dish.
13. Harvest/detach the cells using 2-3 mL of 0.25% (w/w) Trypsin-0.53 mM EDTA.
14. Place culture dish in incubator for 10-15 min.

15. Ensure with visual inspection that cells are fully detached from culture dish. *If not fully detached, either incubate for a longer period or add 1-2 mL of trypsin solution.*
16. Add fresh EMEM media (6-8 mL) to culture dish and gently pipette up and down to aspirate cells.
17. Add an appropriate aliquot of the cell suspension to a new 100 mm culture dish at a subcultivation ratio of 1:5.
18. Incubate culture and grow cell line until 80-90% confluent.
19. Repeat subculture procedure for a total of 5 times to stabilize growth.

Cell Growth on 95% ^{13}C IROA Media

1. Transfer detached cells (using 2-3 mL of 0.25% (w/w) Trypsin-0.53 mM EDTA) to a 150 cm^2 ($1.5x10^7$ average cell yield) or 175 cm^2 flask ($1.75x10^7$ average cell yield).
2. Add 30 or 40 mL of IROA media, respectively to culture flask.
3. Incubate culture and grow cell line until 80-90% confluent.
4. Repeat the following procedure and subculture until the total number of cells or passages desired is obtained (**Figure 1**).

Cell Washing and Storage[1]

1. Harvest/detach the cells during each collection step using 0.25% (w/w) Trypsin-0.53 mM EDTA.
2. Transfer the detached cells to a conical tube.
3. Rinse cells by adding 1 mL of 40 mM ammonium formate to the cell pellet. Centrifuge cells at 1500 rpm for 5 min at 5 °C. Remove the supernatant.
4. Repeat Step #3 two additional times.
5. Store washed cell pellet at -80 °C until analysis.

**Optional: To obtain a larger cell pellet, pool cell pellets from separate passages, centrifuge, remove the supernatant, and store at -80 °C until analysis.*

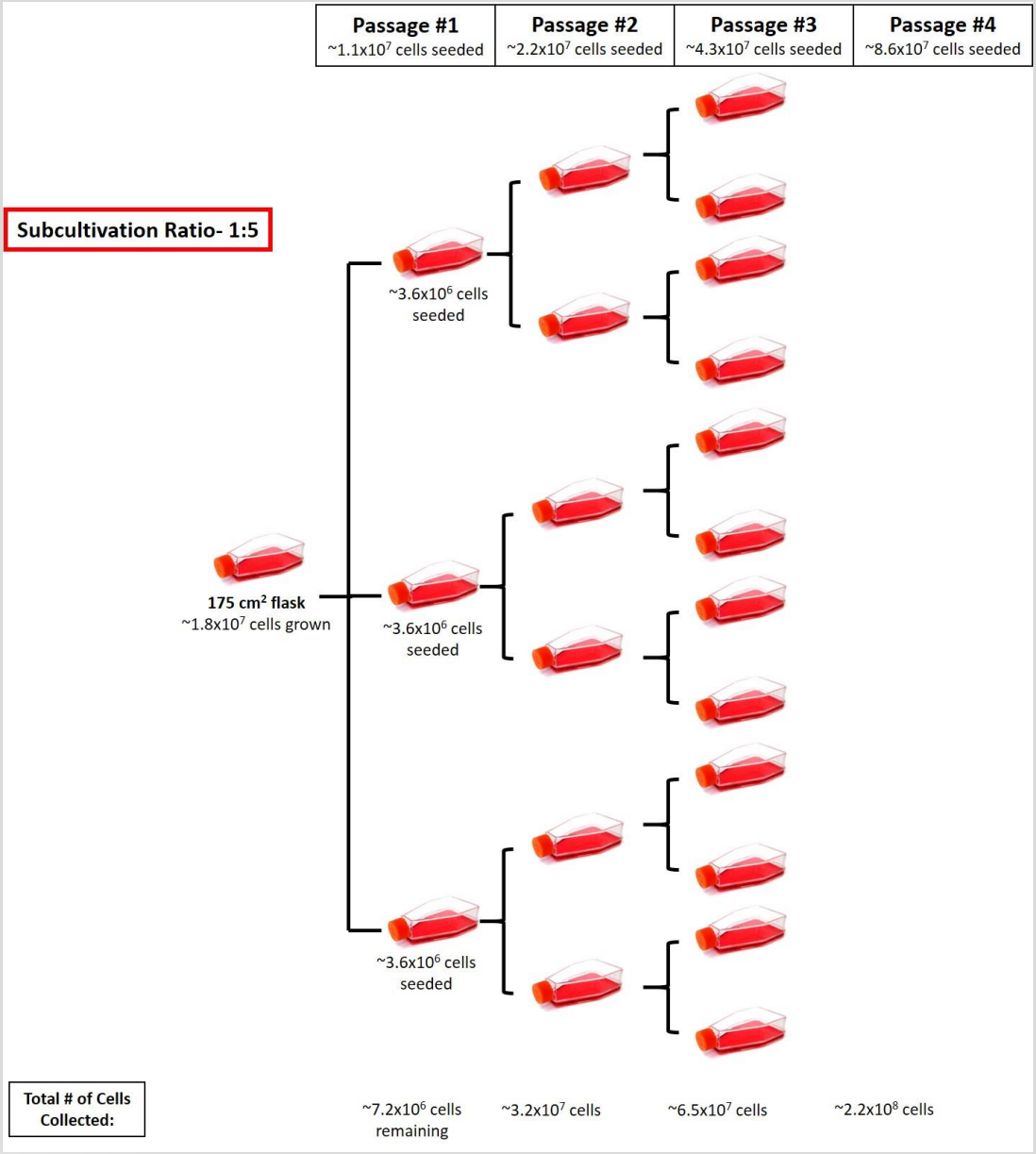


Figure 3: Example Subculture Growth Diagram for Cell Lines on 95% 13C-Labeled IROA Media using 175 cm^2 flasks

Acknowledgements

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References:

(1) Ulmer, C. Z.; Yost, R. A.; Chen, J.; Mathews, C. E.; Garrett, T. J. *J.Proteomics Bioinform.* **2015**, *8*, 126-132.

(2) American Type Culture Collection. Product Sheet: Hep G2 [HEPG2] (ATCC® HB 8065™). **2015**, 1-3.