# Fluxomics analysis by LC-MS and GC-MS

## Analysis summary

The assay is intended to evaluate the flux of metabolites through various biochemical pathways by following the faith of stable-isotope labeled precursor compounds in a pulse-chase experiment.

## Analyte extraction and sample preparation

### Cell culture extraction (samples supplied on culture plates)

Extraction solvent – methanol : chloroform 1 : 1.

* Place the sample plates and extraction solvent on dry ice.
* Clean cell scraper with paper tissue soaked in methanol.
* One plate at a time, move each plate on regular ice, add 1.5 mL of extraction solvent, and scrape cells, then scrape cell suspension to the side of the tilted plate.
* Transfer cell suspension to a pre-labeled 2mL micro-centrifuge tube, place the tube on dry ice.
* Centrifuge for 10 min at 4°C, 15,000g.
* Transfer 600µL of supernatant to glass auto-sampler vials, store samples at -20oC until LC-MS.
* Create pooled sample by combining 10µL aliquots of each individual extract.
* Transfer 100µL aliquot of extract to glass auto-sampler vial for GC-MS derivatization.

### Cell culture extraction (samples supplied as precipitated cells in micro-centrifuge tubes)

Extraction solvent - methanol : chloroform : water 8 : 1 : 1

* Add 300µL of extraction solvent to each cell sample, vortex to completely re-suspend the pellet.
* Sonicate at 40% output power, 20% duty cycle for 20 seconds, keep samples on ice throughout the procedure.
* Leave for 5 minutes at 4°C or on ice, vortex.
* Centrifuge for 5 min at 4°C, 14,000rpm.
* Transfer 100µL of supernatant to auto-sampler vial with glass insert for LC-MS analysis.
* Create pooled sample by combining 10µL aliquots of each individual extract.
* Transfer 100µL aliquot of extract to glass auto-sampler vial for GC-MS derivatization.

### Sample derivatization for GC-MS analysis

* Dry sample extracts and standards in a vacuum centrifuge at 45oC.
* While samples are drying, prepare a 20mg/mL solution of methoxyamine hydrochloride in pyridine in a glass vial; use glass syringe or pipette to dispense pyridine, vortex to dissolve.
* Add 50µL of the methoxyamine hydrochloride solution to dried samples, cap the vials and incubate at 37°C for 90min (preferably in a dry box).
* Uncap the vials, add 50µL of MTBSTFA + 1% TBDCMS to all vials using glass syringe or pipette, re-cap vials and incubate at 70°C (sand bath) for 60min; alternatively leave overnight at room temperature.
* Cool the vials to room temperature; if contents is cloudy, centrifuge for 2min; transfer contents to auto-sampler vials with glass inserts using a glass Pasteur pipette, cap the vials, promptly analyze on GC-MS.

## LC-MS

* Chromatographic column - Luna® 3 µm NH2 100 Å, LC Column 150 x 1 mm, Ea (Phenomenex Inc.).
* LC gradient
  + Phase A: 5mM ammonium acetate in water, pH 9.9 (adjusted using LC-MS grade ammonium hydroxide).
  + Phase B: 100% acetonitrile.
  + timetable – listed in table 1 below.
* Auto-sampler temperature 4°C.
* Injection volume 10 µL (may vary between experiments).
* Mass-spectrometer parameters
  + Instrument - Agilent 6520 Q-TOF
  + Mode – ESI negative.

The specific LC-MS method details are provided in supplementary material (QTOF-002-HILIC-35min-1mm\_LC\_PARAMS.xml and QTOF-002-HILIC-35min-1mm\_MS\_PARAMS.xml files).

Table 1. LC gradient timetable

|  |  |  |
| --- | --- | --- |
| Time, min | %B | Flow, ml/min |
| 0 | 80 | 0.075 |
| 15 | 0 | 0.075 |
| 20 | 0 | 0.075 |
| 20.1 | 80 | 0.075 |
| 25 | 80 | 0.075 |
| 30 | 80 | 0.09 |
| 34.9 | 80 | 0.09 |
| 34.99 | 80 | 0.075 |

## GC-MS

Samples are analyzed on DB-5MS, 250µm ID x 30m column from Agilent or equivalent. The specific GC-MS method details are provided in supplementary material (ALPHA KETO ACIDS-FULL.txt file).

## References