# Glycolysis – TCA-nucleotides analysis

## Analysis summary

The assay is intended to generate the profile of central metabolism, including glycolysis, pentose-phosphate shunt, TCA cycle and nucleotide pools. Analysis is performed by liquid chromatography – mass spectroscopy. Absolute quantitation (µM concentrations of analytes) is obtained using appropriate internal standards, data are normalized to original sample weight. Assay coefficient of variation is usually within 15%.

## Analyte extraction and sample preparation

Extraction solvent - methanol : chloroform : water 8 : 1 : 1, 1% v/v of 13C Gly-TCA-nucleotides isotope-labeled internal standards solution (IS, a mixture of all compounds listed in table 1, at 100 µM final concentration each).

Table 1. Glycolysis – TCA – nucleotides isotope-labeled internal standards solution stock (IS)

|  |  |  |  |
| --- | --- | --- | --- |
| Compound | Supplier | Catalog #  | Metabolites |
| 13C2-fumarate | Sigma | 606073 | NAD , Suc, FAD, Sed |
| 13C6-citrate | Sigma | 606081 | Hexose-6-Phosphate, NADP,6PG, G3P |
| 13C6-fructose-bisphosphate | Omicron Biochem | fru-028 | FBP,NADPH,PEP,2PG/3PG |
| 13C10, 15N5-ATP | Sigma | 645702-10MG | ATP, a-CoA, ADP |
| 13C10, 15N5-AMP | Sigma | 650676 | AMP, E4P,X5P/R5P, S7P |
| 13C4-L-malic acid | Sigma | 750484 | Mal, NADH |

Table 2. Glycolysis – TCA – nucleotides internal standards solution stock (STD mix)

| Compound | Abbreviation | Formula  | LOQ (µM) |
| --- | --- | --- | --- |
| Acetyl-CoA | A-CoA | C23H38N7O17P3S | 0.1 |
| Citrate/Isocitrate (combined) | Cit/i-Cit | C6H8O7 | 0.1 |
| Succinate | Suc | C4H6O4 | 0.1 |
| Malate | Mal | C4H6O5 | 0.1 |
| Hexose-6-Phosphate | H6P | C6H13O9P | 0.1 |
| Glyceraldehyde-3-phosphate | G3P | C3H7O6P | 0.1 |
| 2-Phosphoglycerate/3-Phosphoglycerate (combined) | 2PG/3PG | C3H7O7P | 0.1 |
| Phosphoenolpyruvate | PEP | C3H5O6P | 0.1 |
| Adenosine monophosphate | AMP | C10H14N5O7P | 0.1 |
| Adenosine diphosphate | ADT | C15H23N5O14P2 | 0.1 |
| Adenosine triphosphate | ATP | C10H16N5O13P3 | 0.1 |
| Flavin adenine dinucleotide | FAD | C27H33N9O15P2 | 0.1 |
| Nicotinamide adenine dinucleotide | NAD | C21H28N7O14P2 | 0.1 |
| Nicotinamide adenine dinucleotide, reduced | NADH | C21H29N7O14P2 | 0.1 |
| Nicotinamide adenine dinucleotide phosphate | NADP | C21H29N7O17P3 | 0.1 |
| Nicotinamide adenine dinucleotide phosphate, reduced | NADPH | C21H30N7O17P3 | 0.1 |
| Erythrose 4-phosphate\* | E4P | C4H9O7P | 1  |
| Ribulose 5-phosphate/Xylulose 5-phosphate (combined)\* | R5P/X5P | C5H11O8P | 0.1  |
| 6-phosphogluconate\* | 6PG | C6H13O10P | 0.1  |
| Sedoheptulose 7-phosphate\* | S7P | C7H15O10P | 0.1  |
| Sedoheptulose\* | Sed | C7H14O7 | 1  |

\*Low concentrations, may be below detection limit in some samples.

Table 3Glycolysis – TCA – nucleotides calibration standards

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Name | Concentration, µM | methanol, µL | chloroform, µL | Volume of STD mix, µL | Volume of IS, µL |
| STD 0 | 0 | 445 | 50 | 0 | 5 |
| STD 1 | 0.6 | 443.5 | 50 | 1.5 | 5 |
| STD 2 | 2 | 440 | 50 | 5 | 5 |
| STD 3 | 6 | 430 | 50 | 15 | 5 |
| STD 4 | 20 | 395 | 50 | 50 | 5 |

###  Tissue extraction

* Homogenize tissue samples using the liquid nitrogen chilled homogenizer (Bullet Blender Gold) or probe sonicator, depending on tissue type.
* Weigh ~30mg of pulverized tissue in pre-labeled micro-centrifuge tube.
* Add 1mL of extraction solvent, vortex, sonicate for 10-20 seconds using the probe sonicator at 20% output, level 4.
* Incubate for 10min at 4oC, vortex again.
* Centrifuge for 10min at 4oC, 15,000g.
* Transfer 120µL of supernatant to an auto-sampler vial with glass insert, store samples at -20oC until LC-MS.
* Create pooled sample by combining 10µL aliquots of each individual extract.

### Cell culture extraction

* 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.

### 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.

## Gly-TCA-nucleotide calibration curve standards

Calibration curve standards are prepared according to table 3 above. IS stands for isotopically-labeled standards mixture (table 1), STD mix – for mixture of unlabeled standards (table 2, each compound at 200µM final concentration)

## 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 4 below
* Auto-sampler temperature 4°C.
* Injection volume 10 µL.
* Mass-spectrometer parameters
	+ Instrument - Agilent 6520 Q-TOF
	+ Mode – ESI negative, ionization voltage 3.5kV.
	+ Drying gas – 10 L/min at 350oC.

Table 4. 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

[Matthew A. Lorenz](http://pubs.acs.org/action/doSearch?action=search&author=Lorenz%2C+M+A&qsSearchArea=author), [Charles F. Burant](http://pubs.acs.org/action/doSearch?action=search&author=Burant%2C+C+F&qsSearchArea=author), and [Robert T. Kennedy](http://pubs.acs.org/action/doSearch?action=search&author=Kennedy%2C+R+T&qsSearchArea=author) (2011) "Reducing Time and Increasing Sensitivity in Sample Preparation for Adherent Mammalian Cell Metabolomics", Anal. Chem.83(9): 3406–3414.