Gly/TCA/nucleotide and NAD+ related metabolites
Service Code: GTN

Summary: Profile of Central Metabolism, including glycolysis, pentose-phosphate shunt, TCA cycle and nucleotide pools. One step organic solvent extraction of cultured cells or tissues, separated on a 1mm x150mm HILIC column in a 35 min cycle. All analytes and Internal Standards are measured by ESI^- ionization on a LC-QTOF mass spectrometer and reported as uM normalized to wet tissue weight or cell proteins. CV's are generally 15%.

Container: Eppendorf Tube or equivalent
Normal Volume: Plasma (100 ul) Tissue (50-100 mgs); Cells (2E7).
Minimal Volume: Plasma (50 uL)Tissue (30 mg); Cells (~5E6)
Special Handling: If human or primate, note any known presence of infectious agents.
Sample Collection: Snap freeze by liquid nitrogen. For tissues, resect and snap-freeze as soon as practical in tared centrifuge tube. Provide both sample weight and tared vial weight on sample submission

Reference: Matthew A. Lorenz, Charles F. Burant, and Robert T. Kennedy (2011) "Reducing Time and Increasing Sensitivity in Sample Preparation for Adherent Mammalian Cell Metabolomics", *Anal. Chem.* 83(9): 3406–3414.

Table I: Analytes reported. Others on special request:

Analyte	Abbr.	Mol Formula	Rt	LOQ(uM)
Acetyl-CoA	aCoA	$C_{23}H_{38}N_7O_{17}P_3S$	20.70	0.1
Citrate/Isocitrate combined	Cit/i-Cit	$C_6H_8O_7$	19.00	0.1
Succinate	Suc	$C_4H_6O_4$	16.36	0.1
Malate	Mal	$C_4H_6O_5$	16.50	0.1
Glyceraldehyde-3-phosphate	G3P	$C_3H_7O_6P$	18.3	0.1
2-Phosphoglycerate/3-Phosphoglycerate combined	2PG/3PG	$C_3H_7O_7P$	19.30	0.1
Phosphoenolpyruvate	PEP	$C_3H_5O_6P$	19.80	0.1
Adenosine monophosphate	AMP	$C_{10}H_{14}N_5O_7P$	17.20	0.1
Adenosine diphosphate	ADP	$C_{15}H_{23}N_5O_{14}P_2$	20.00	0.1
Adenosine triphosphate	ATP	$C_{10}H_{16}N_5O_{13}P_3$	22.30	0.1
Flavin adenine dinucleotide	FAD	$C_{27}H_{33}N_9O_{15}P_2$	16.61	0.1
Nicotinamide adenine dinucleotide	NAD	$C_{21}H_{28}N_7O_{14}P_2$	13.80	0.1
Nicotinamide adenine dinucleotide,reduced	NADH	$C_{21}H_{29}N_7O_{14}P_2$	16.50	0.1
Nicotinamide adenine dinucleotide phosphate	NADP	$C_{21}H_{29}N_7O_{17}P_3$	19.00	0.1
Nicotinamide adenine dinucleotide phosphate, reduced	NADPH	$C_{21}H_{30}N_7O_{17}P_3$	21.00	0.1
Erythrose 4-phosphate*	E4P	$C_4H_9O_7P$	16.2	1
Ribulose 5-phosphate/Xylulose 5-phosphate/ribose-5-phosphate combined*	R5P/X5P/ Ru5P	$C_5H_{11}O_8P$	15.9	0.1
6-phosphogluconate*	6PG	$C_6H_{13}O_{10}P$	18.80	0.1
Sedoheptulose 7-phosphate*	S7P	$C_7H_{15}O_{10}P$	16.1	0.1
Fructose-6-phosphate + glucose-6-phosphate	F6P/G6P	$C_6H_{13}O_9P$	16.81	0.1

Fructose-bisphosphate	FBP	$C_6H_{14}O_{12}P_2$	20.50	0.1
Nicotinic acid (NA),	NA	$C_6NH_5O_2$		
Nicotinic acid mononucleotide (NaMN)	NaMN	$C_{11}H_{15}N_2O_8P$		
Quinolinic acid (QA),	QA	$C_7H_5NO_4$		
nicotinamide mononucleotide (NMN),	NMN	$C_{11}H_{15}N_2O_8P$		
nicotinamide (NAM),	NAM	$C_6H_6N_2O$		

*Metabolites are low concentrations and below detection limit in some samples.

Table II: Internal standards

Internal Standards	**Source**	**Cat#**	**Metabolites**	Rt
$^{13}C_2$-fumarate	sigma	606073	NAD , Suc, FAD, Sed	16.36
$^{13}C_6$-citrate	sigma	606081	Hexose-6-Phosphate, NADP,6PG, G3P	19.00
$^{13}C_6$-Fructose-bisphosphate	omicron biochem ,	fru-028	FBP,NADPH,PEP,2PG/3PG,	20.5
$^{13}C_{10}$, $^{15}N_5$-ATP	sigma	645702-10MG	ATP, a-CoA, ADP	22.3
$^{13}C_{10}$, $^{15}N_5$-AMP	sigma	650676	AMP, E4P,X5P/R5P, S7P,	17.200
$^{13}C_4$-L-Malic acid	sigma	750484	Mal, NADH	16.5
$^{13}C_6$-Fructose-6-phosphate			Hexose-6-Phosphate	16.80

Materials

1. Agilent 6520 QTOF with 1260 LC unit, chilled autosampler, with standard 54-well autosampler plate
2. Bullet Blender GOLD with appropriate beads and protocol for tissues to be analyzed OR: Branson Sonifier 450 probe sonicator (narrow tip) using 20% duty cycle
3. Vortexer
4. Refrigerated centrifuge, capable of 15,000g with eppendorf tube compatible rotor
5. Eppendorf Vacufuge
6. ice bucket, ice
7. micro-balance
8. prepared internal standard and authentic standards mix solutions.
9. eppendorf tubes (polypropylene)
10. LCMS grade water, acetonitrile, methanol, chloroform, ammonium acetate, acetic acid

Procedure:

Tissue Sample Preparation

1. Weigh frozen tissue samples and transfer to labeled eppendorf tubes, record weight. Homogenize tissues using cooled Bullet Blender Gold or probe sonicator, as appropriate. Keep samples cool while homogenizing.
2. Add appropriate amount of extraction solution to all tubes, then vortex to mix.

3. Incubate 5 minutes on ice water, then vortex again. Incubate 5 more minutes, then vortex again.
4. Centrifuge 10 minutes at 15,000g, 4 °C.
5. Transfer supernatant into a clean, labeled autosampler vial for LC-MS analysis
6. Reserve remaining tissue sample/extract at -80°C until analysis is complete
7. Once analysis is complete, dry and lyophilized extracted tissue, weigh, measure protein content using the Bradford method.

Cell Sample Preparation

1. Put samples in a box with dry ice. Put extraction solvent on dry ice.
2. Working one plate at a time, remove plate from the cooler and place on a surface of regular ice.
3. Clean cell scraper with MeOH and kimwipe.
4. Add 1.5 mL of extraction solvent (750 uL twice) to the plate.
5. Scrape cells with cell scraper, then scrape solvent to one corner of the plate.
6. Transfer supernatant to a labeled 2mL eppendorf vial. Put vial on dry ice.
7. Repeat procedure with all additional eppendorf vials.
8. Centrifuge all vials at 15,000g for 10 minutes at 4 °C
9. Transfer 600 uL of supernatant to clean autosampler vials (no insert). Store samples in refrigerator; store remaining sample at -80 °C.

LC-MS procedure

1. LC column: Phenomenex Luna NH2 column, 1mm x 150mm
2. Mobile phase A: 5mM ammonium acetate in water, pH 9.9
3. Mobile phase B: LCMS-grade Acetonitrile
4. Gradient: 0min, 80%B, 15min, 0%B, 20min, 0%B, 35min, 80%B, flow rate: 75ul/min
5. Autosampler: 4°C, 10 uL injection
6. Agilent 6520 Q-TOF: ESI^-, 350 °C, drying gas 10l/min; ESI: 3500V Method: **M006-1mmNH2-35min_neg.m** or equivalent
7. Collect standard curve data first, then sample data if system is suitable.

Quantification:

Internal standard mixture is spiked in samples and calibration standards. External calibration curve is constructed from calibration standards and it is used to calculate metabolite concentrations in biological samples.