

DATE: LCMS extraction procedure for GlyTcaNuc and NAD for cell matrix

Samples

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Extraction Solvent

Extraction solvent: _____ mL of 8:1:1 (Methanol: Chloroform: H₂O) + _____ μ L 13C GlyTcaNuc IS (_____ mL total). NOTE: **0.5ml for 6cm plate, 1.5ml for 10cm plate**

Ratio of 811 to 13C Internal standard mix = 50:1, which is same as in calibration standard preparation.

Standard Mix stock (STD) and Internal Standard stock (IS)

- 1) **GLY/TCA STD mix 200 μ M.** (200mM, 25 x 80ul of 12C metabolites: a-kg, oaa, cit, mal, fum, 2pg, g6p, FAD, F16,BP, g3p, g6p, pep, Lac, amp, adp, atp, sed, x5p, 6pg, r5p, e4p) (box F stored in -80°C)
- 2) **NAD/ NADP/NADH STD 200 μ M**
- 3) **NADPH STD 200 μ M**
- 4) **GLY/TCA IS: 13C 100 μ M**
- 5) **NAD STD 200 μ M**

Standard Preparation (STD Mix)--LCMS

First make STD 7: 75 μ L of GlyTcaNuc + 75 μ L of NAD/NADP/NADH + 75 μ L of NADPH + 525 μ L 8:1:1 = final volume of 750 μ L. Prep the following in microtubes

STD ID	[Final] μ M	Vol. of Prior STD (μ L)	Vol. of 8:1:1 (μ L)	Transfer into glass insert (300 μ L)	Vol. (μ L) of IS 100 μ M add to insert
STD 0	0	0	350	294	6
STD 1	0.3125	350 of STD 2	350	294	6
STD 2	0.625	350 of STD 3	350	294	6
STD 3	1.25	350 of STD 4	350	294	6
STD 4	2.5	350 of STD 5	350	294	6
STD 5	5	350 of STD 6	350	294	6
STD 6	10	350 of STD 7	350	294	6
STD 7	20	See above	525	294	6

In a separate microtube add 22.5 μ L of GlyTcaNuc + 22.5 μ L of NAD/NADP/NADH + 22.5 μ L of NADPH + 6 μ L of IS + 226.5 μ L 8:1:1 = 300 μ L [15 μ M]. This is a validation point for curve/ dilution competency. Transfer to autovial containing glass insert and run with curve.

Repeat curve for NAD compounds- excluding GLYTCA STDs.

Notes:

Sample Preparation

- 1) Place all samples on dry ice until extraction procedure has been completed.
- 2) While over wet ice **add Extraction solvent (0.5ml for 6cm plate, 1.5ml for 10cm plate)** containing Internal Standards (IS) to the plate, rock plate back and forth to allow chemical extraction to occur and release cells. Use a scraping device to complete cell release and homogenization (making sure to scrape entire plate).
- 3) **Transfer entire solvent sample** from plate to pre-labeled, clean microtube, vortex.
- 4) Keep eppendorf tubes at **4°C for 10min** to allow complete extraction, remove from 4°C repeat vortex.
- 5) **Centrifuge** all tubes at 14,000RPM for 10min in 4°C.
- 6) **Create a pooled** sample by transferring μL of each sample to an autosampler vial with insert.
- 7) **Transfer 80 μL** of supernatant to an autosampler vial (with insert) for LC-MS analysis. **Transfer 200 μL** of supernatant to an autosampler vial (with no insert) for GC-MS analysis

MS Analysis

Q-ToF method:

Column: Luna Nh2 150x1mm column.

MP A: 5mM Ammonium Acetate in H₂O, pH 9.9, adjust with LC-MS grade Ammonium Hydroxide

MP B: 100% Acetonitrile

QQQ method:

Column: Luna Nh2 150x1mm column.

MP A: 5mM Ammonium Acetate in H₂O, pH 9.9, adjust with LC-MS grade Ammonium Hydroxide

MP B: 100% Acetonitrile

Table I: Analytes reported with quantitative measurement (Additional Gly-TCA metabolites (Lactate, Fumarate, Alpha ketoglutarate) may be obtained for an additional cost by selecting TCA-Supplement in MiCores.)

Analyte	Abbr.	Mol Formula	LOQ(μM)
Acetyl-CoA	aCoA	C ₂₃ H ₃₈ N ₇ O ₁₇ P ₃ S	1
Citrate/Isocitrate combined	Cit/i-Cit	C ₆ H ₈ O ₇	1
Succinate	Suc	C ₄ H ₆ O ₄	1
Malate	Mal	C ₄ H ₆ O ₅	1
2-Phosphoglycerate/3-Phosphoglycerate combined	2PG/3PG	C ₃ H ₇ O ₇ P	1
Phosphoenolpyruvate	PEP	C ₃ H ₅ O ₆ P	1
Adenosine monophosphate	AMP	C ₁₀ H ₁₄ N ₅ O ₇ P	1
Adenosine diphosphate	ADP	C ₁₅ H ₂₃ N ₅ O ₁₄ P ₂	1
Adenosine triphosphate	ATP	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	1
Flavin adenine dinucleotide	FAD	C ₂₇ H ₃₃ N ₉ O ₁₅ P ₂	1
Nicotinamide adenine dinucleotide	NAD	C ₂₁ H ₂₈ N ₇ O ₁₄ P ₂	1
Nicotinamide adenine dinucleotide, reduced	NADH	C ₂₁ H ₂₉ N ₇ O ₁₄ P ₂	1
Nicotinamide adenine dinucleotide phosphate	NADP	C ₂₁ H ₂₉ N ₇ O ₁₇ P ₃	1
Nicotinamide adenine dinucleotide phosphate, reduced	NADPH	C ₂₁ H ₃₀ N ₇ O ₁₇ P ₃	1
Erythrose 4-phosphate	E4P	C ₄ H ₉ O ₇ P	1
Ribulose 5-phosphate/Xylulose 5-phosphate/ribose-5-phosphate combined	R5P/X5P/Ru5P	C ₅ H ₁₁ O ₈ P	1
6-phosphogluconate	6PG	C ₆ H ₁₃ O ₁₀ P	1
Sedoheptulose 7-phosphate	S7P	C ₇ H ₁₅ O ₁₀ P	1
Fructose-6-phosphate + glucose-6-phosphate	F6P/G6P	C ₆ H ₁₃ O ₉ P	1
Fructose-bisphosphate	FBP	C ₆ H ₁₄ O ₁₂ P ₂	1

NOTE: Metabolites in this assay may be below the detection limit in some samples, especially plasma and samples with less than 3 million cells.

Table II: Additional metabolites reported for most cell or tissue samples in the TCA Plus assay as relative abundance

Alanine and Aspartate Metabolism	Alanine
	Asparagine
	Aspartate
Aminosugar Metabolism	N-Acetyl-glucosamine-1-phosphate
Creatine Metabolism	Creatinine*
Food Component/Plant	Gluconate
Glutamate Metabolism	Glutamate
	Glutamine
Glutathione Metabolism	Glutathione, oxidized (GSSG)
	Glutathione, reduced (GSH)
Glycine, Serine and Threonine Metabolism	3-Phospho-serine
Glycine, Serine and Threonine Metabolism Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	Serine
	Threonine
	Hexose (glucose etc.)
Histidine Metabolism	Histidine
Leucine, Isoleucine and Valine Metabolism	Valine
Long Chain Fatty Acid	Oleic acid
Long Chain Fatty Acid Lysine Metabolism	Palmitic acid
	Stearic acid
	Lysine
Methionine, Cysteine, SAM and Taurine Metabolism	Methionine
Methionine, Cysteine, SAM and Taurine Metabolism	Taurine
Nucleotide Sugar	UDP-D-glucose
Nucleotide Sugar Oxidative Phosphorylation	UDP-D-glucuronate
	UDP-N-acetyl-D-glucosamine
	Acetylphosphate*
Pantothenate and CoA Metabolism	Pantothenate*
Phenylalanine and Tyrosine Metabolism Phenylalanine and Tyrosine Metabolism	Phenylalanine
	Tyrosine
Purine Metabolism, (Hypo)Xanthine/Inosine containing	Inosine 5'-monophosphate (IMP)
Purine Metabolism, (Hypo)Xanthine/Inosine containing	Inosine
	Xanthine
Purine Metabolism, Guanine containing	Guanosine diphosphate
Pyrimidine Metabolism, Cytidine containing	Cytidine monophosphate
Pyrimidine Metabolism, Uracil containing	Uridine 5'-diphosphate (UDP)
Pyrimidine Metabolism, Uracil containing Tryptophan Metabolism	Uridine 5'-monophosphate (UMP)
	Uridine 5'-triphosphate (UTP)
	Tryptophan

Table II cont'd:

Glycine, Serine and Threonine Metabolism	3-Phospho-serine
	Serine
	Threonine
Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	Hexose (glucose etc.)
Histidine Metabolism	Histidine
Leucine, Isoleucine and Valine Metabolism	Valine
Long Chain Fatty Acid	Oleic acid
	Palmitic acid
	Stearic acid
Lysine Metabolism	Lysine
Methionine, Cysteine, SAM and Taurine Metabolism	Methionine
	Taurine
Nucleotide Sugar	UDP-D-glucose
	UDP-D-glucuronate
	UDP-N-acetyl-D-glucosamine
Oxidative Phosphorylation	Acetylphosphate*
Pantothenate and CoA Metabolism	Pantothenate*
Phenylalanine and Tyrosine Metabolism	Phenylalanine
	Tyrosine
Purine Metabolism, (Hypo)Xanthine/Inosine containing	Inosine 5'-monophosphate (IMP)
	Inosine
	Xanthine
Purine Metabolism, Guanine containing	Guanosine diphosphate
Pyrimidine Metabolism, Cytidine containing	Cytidine monophosphate
Pyrimidine Metabolism, Uracil containing	Uridine 5'-diphosphate (UDP)
	Uridine 5'-monophosphate (UMP)
	Uridine 5'-triphosphate (UTP)
Tryptophan Metabolism	Tryptophan

NOTE: Metabolites in this assay may be below the detection limit in some samples, especially plasma and samples with less than 3 million cells; Compounds show with * are usually only detected in tissue.