Materials and Instrumentation:

- Hypercarb 2.1 x 50 mm, 3 µm column, Thermo Scientific, Cat. No. 35003-052130
- Pyridine nucleotide and adenosine phosphate standards and heavy isotope-labeled internal standards
- 3kDa molecular weight cutoff filter plate (350-µL well volume, 96 wells), Pall Corp., Cat. No. 8033
- Agilent 1290 Infinity HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer
    - Software: MassHunter Acquisition (B.04.01); MassHunter Quantitative Analysis (B.05.00)

Sample Preparation:

1. Retrieve study samples (-80°C freezer) and calibrator/internal standard solutions (-80°C freezer) and allow them to thaw on ice.
    - If not done previously, aliquot bulk study samples at 100 µL into fresh tubes

2. Obtain ten 1.7-mL Eppendorf tubes and place in a plastic tube rack on ice; label them for the calibration samples, C_1-C_{10}; note that C_{10} will be your high calibrator. Transfer 90 µL of 0.5 M perchloric acid to each tube.
    - Tissue study samples should be prepared as homogenate in 0.5 M perchloric acid
    - Pyridine nucleotides and adenosine phosphates are not currently assayed in biological fluids due to low abundance

3. Once thawed, vortex the calibrator and IS solutions retrieved from the freezer. Stock calibrator solutions are labeled as “Cals” from Cal 1 to Cal 10, with each Cal corresponding to a single calibration sample (See table below). Transfer 10 µL of a Cal solution to the corresponding calibration tube, and tap lightly to mix.

The stock calibrator "Cals" are concentrated combinations of all organic acids in 0.1% formic acid. Once diluted as prescribed below, organic acids are present at the following concentrations:

Cal	Volume Spiked into Cal Tubes (uL)	Calibrator	Conc'n of "A" Metabolites present (uM)	Conc'n of "B" Metabolites present (uM)	Conc'n of "C" Metabolites present (uM)	Conc'n of "C" Metabolites present (uM)
10	10	C_{10}	500	200	20	2
9	10	C_9	250	100	10	1
8	10	C_8	125	50	5	0.5
7	10	C_7	62.5	25	2.5	0.25
6	10	C_6	25	10	1	0.1
5	10	C_5	12.5	5	0.5	0.05
4	10	C_4	6.25	2.5	0.25	0.025
3	10	C_3	2.5	1	0.1	0.01
2	10	C_2	1.25	0.5	0.05	0.005
1	10	C_1	0.625	0.25	0.025	0.0025

"A" Metabolites: ATP, ADP, AMP

"B" Metabolite: NAD

"C" Metabolite: NADP

"D" Metabolite: NMN

4. All tubes (both calibrator and study sample) should now contain 100 µL. Vortex the thawed internal standard solution (labeled NAD/ATP IS Mix) and transfer 10 µL to all tubes.

The stock Internal Standard Mix (NAD/ATP IS Mix) solution is a concentrated mix of select "heavy" pyridine nucleotides (NMN and NAD) and ATP in 0.1% formic acid. Once diluted as prescribed below, organic acid ISs are present at the following concentrations:

Solution	Volume Spiked into All Tubes (uL)	Conc'n of NAD IS present (uM)	Conc'n of NMN IS present (uM)	Conc'n of ATP IS present (uM)	Conc'n of AMP IS present (uM)
NAD/ATP Mix	10	0.05	0.00025	0.25	0.25

5. To all tubes, add 100 µL of 1 M ammonium formate. Close the tubes, vortex, and centrifuge at 18,000 x g/5 min/10°C.

6. After centrifugation, transfer the total volume of supernatant liquid (~200 µL) to a 3kDa molecular weight cutoff filter plate according to the following scheme:

	1	2	3	4	5	6	7	8	9	10	11	12
A	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}		
B	1	2	3	4	5	6	7	8	9	10	11	12
C	13	14	15	16	17	18	19	20	21	22	23	24
D	25	26	27	28	29	30	31	32	33	34	35	36
E	37	38	39	40	41	42	43	44	45	46	47	48
F	49	50	51	52	53	54	55	56	57	58	59	60
G	61	62	63	64	65	66	67	68	69	70	71	72
H	73	74	75	76	77	78	79	80	81	82	83	84

*Note: Calibrators should progress from C_1 (low cal) to C_{10} (high cal)

7. Fix the molecular weight cutoff filter plate over a 96-well plate (Corning 1-mL) and filter the extract at 1500 x g at 10°C for at least 30 minutes, or until the volume of filtrate is suitable for LC/MS/MS analysis.

Calibrator Preparation:

Below is a list of pyridine nucleotide and adenosine phosphate standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Metabolite	Carrier	Cat. No.	Stock Conc'n (mM)	Diluent
NMN	Sigma	N3501	0.125	0.1% formic acid
NAD	Sigma	N1636	12.5	0.1% formic acid
NADP	Sigma	N0505	1.25	0.1% formic acid
AMP	Sigma	09130	50	0.1% formic acid
ADP	Sigma	A2754	50	0.1% formic acid
ATP	Sigma	A2383	50	0.1% formic acid

Individual stock solutions are then combined to prepare the highest calibrator standard stock solution. The millimolar concentration of each amino acid in the combined mixture is given below:

Organic Acid	Initial Individual Stock Conc'n (mM)	Final Combined Stock Conc'n* (mM)
NMN	0.125	0.02
NAD	12.5	2
NADP	1.25	0.2
AMP	50	5
ADP	50	5
ATP	50	5

*Combined stock prepared in 0.1% formic acid

This is the stock solution for the highest calibrator, C_{10}. Serial dilution of C_{10} yields the stock solutions for all ten calibrator samples, C_1-C_{10}. For simplicity, the table below only shows the serial dilution scheme in terms of NAD's concentration:

Calibrator Stock Sol'n	Conc'n of NAD in Solution* (mM)
C_{10}	2
C_9	1
C_8	0.5
C_7	0.25
C_6	0.1
C_5	0.05
C_4	0.025
C_3	0.01
C_2	0.005
C_1	0.0025

*All dilutions made in 0.1% formic acid

Based on the protocol above, the calibrator curve ultimately generated during prep will have the following range. Note that there are four "groups" of metabolites, each with its own range in the curve:

Calibrator	Conc'n of "A" Metabolites present (uM)	Conc'n of "B" Metabolites present (uM)	Conc'n of "C" Metabolites present (uM)	Conc'n of "C" Metabolites present (uM)
C_{10}*	500	200	20	2
C_9	250	100	10	1
C_8	125	50	5	0.5
C_7	62.5	25	2.5	0.25
C_6	25	10	1	0.1
C_5	12.5	5	0.5	0.05
C_4	6.25	2.5	0.25	0.025
C_3	2.5	1	0.1	0.01
C_2	1.25	0.5	0.05	0.005
C_1	0.625	0.25	0.025	0.0025

"A" Metabolites: ATP, ADP, AMP

"B" Metabolites: NAD

"C" Metabolites: NADP

"D" Metabolites: NMN

*All dilutions made in 0.1% formic acid

Below is a list of heavy-isotope-labeled amino acid internal standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Metabolite-Int. Std.	Carrier	Cat. No.	Stock Conc'n (mM)	Diluent
ATP	Sigma	710695	100	0.1% formic acid
AMP	Sigma	650676	10	0.1% formic acid
NAD	Synthesized In-House*		50	0.1% formic acid
NMN	Synthesized In-House*		50	0.1% formic acid

*NAD and NMN internal standards ($^{18}O_2$-labeled) were synthesized in-house by the SBMRI Medicinal Chemistry Core

Below is a list of heavy-isotope-labeled amino acid internal standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Metabolite-Int. Std.	**Initial Individual Stock Conc'n (mM)**	**Final Combined Stock Conc'n (mM)**
ATP	100	2.5
AMP	10	2.5
NAD	50	0.5
NMN	50	0.0025

Our limits of quantitation are set by the high and low points of our calibrator curves.

Assay Conditions:

- Autosampler
  - Temperature: 10°C
  - Injection Volume: 1 µL
  - Needle Wash Solution: 80/20 Methanol/Water
- Column
  - Temperature: 30°C
  - Maximum Pressure: 900 bar
- Binary Pump
  - Flow Rate: 0.65 mL/min
  - Solvent A: 10 mM ammonium acetate, pH 9.5
  - Solvent B: Acetonitrile
  - Gradient Conditions:

Segment	Time (min)	% B	Flow Rate (mL/min)
0 (Start)	0.00	5.0	0.65
1	1.00	9.0	0.65
2	2.00	60.0	0.65
3	2.10	95.0	0.80
4	2.70	95.0	0.80
5	2.80	5.0	0.70
6	3.90	5.0	0.70
7	4.00	5.0	0.65

- Mass Spectrometer
  - Gas Temperature: 300˚ C
  - Gas Flow: 15 L/min.
  - Nebulizer: 45 psi
  - Sheath Gas Temperature: 325˚ C

- Sheath Gas Flow: 8 L/min.
- Capillary Voltage: 3500 V
- Nozzle Voltage: 500 V
- Electrospray ionization: Positive

- MRM Transitions

Metabolite	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	RF Lens (V)
NMN	335.2	123.1	23	43
NAD	664.2	136.1	46	85
NADP	744.2	136.1	46	80
AMP	348.1	136.1	22	58
ADP	428.0	136.1	25	68
ATP	508.1	136.1	27	79

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Revision Number	Name	Reason for Revision	Effective Date
01			
02			