Materials and Instrumentation:

- HSS T3 2.1 x 100 mm, 1.8 µm column, Waters Corp., Cat. No. 186003539
- Pyridine nucleotide 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 200 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 190 µL of 50/50 0.1 M NaOH/MeOH to each tube.
    - Tissue study samples should be prepared as homogenate in 50/50 0.1 M NaOH/MeOH
    - Pyridine nucleotides 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 NADH and NADPH in 0.1 M NaOH. Once diluted as prescribed below, NADH and NADPH are present at the following concentrations:

Cal	Volume Spiked into Cal Tubes (uL)	Calibrator	Conc'n of NADH and NADPH Present (uM)
10	10	C_{10}	100
9	10	C_9	50
8	10	C_8	25
7	10	C_7	12.5
6	10	C_6	5
5	10	C_5	2.5
4	10	C_4	1.25
3	10	C_3	0.5
2	10	C_2	0.25
1	10	C_1	0.125

4. All tubes (both calibrator and study sample) should now contain 200 µL. Vortex the thawed internal standard solution (labeled NADH IS) and transfer 10 µL to all tubes.

The stock Internal Standard solution is a concentrated solution of "heavy" NADH in 0.1 M NaOH. Once diluted as prescribed below, the NADH IS is present at the following concentration:

Solution	Volume Spiked into All Tubes (uL)	Conc'n of NADH IS present (uM)
NADH IS	10	2.5

5. 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 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
NADH	Sigma	N8129	12.5	0.1 M NaOH
NADPH	Sigma	N7505	12.5	0.1 M NaOH

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)
NADH	12.5	2
NADPH	12.5	2

*Combined stock prepared in 0.1 M NaOH

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 NADH's concentration:

Calibrator Stock Sol'n	Conc'n of NADH 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 M NaOH

Based on the protocol above, the calibrator curve ultimately generated during prep will have the following range:

Calibrator	Conc'n of NADH and NADPH present (uM)
C_{10}*	100
C_9	50
C_8	25
C_7	12.5
C_6	5
C_5	2.5
C_4	1.25
C_3	0.5
C_2	0.25
C_1	0.125

Below is a table of the heavy-isotope-labeled internal standard used for the construction of the calibration curve, as well as the initial stock concentration prepared:

Metabolite-Int. Std.	Carrier	Cat. No.	Stock Conc'n (mM)	Diluent
NADH	Synthesized In-House*		50	0.1 M NaOH

*The NADH internal standard ($^{18}O_2$-labeled) were synthesized in-house by the SBMRI Medicinal Chemistry Core

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: 40°C
  - Maximum Pressure: 900 bar
- Binary Pump
  - Flow Rate: 0.54 mL/min
  - Solvent A: 5 mM ammonium acetate, pH 6
  - Solvent B: Acetonitrile
  - Gradient Conditions:

Segment	Time (min)	% B	Flow Rate (mL/min)
0 (Start)	0.00	0.0	0.54
1	0.76	0.0	0.54
2	2.10	50.0	0.54
3	2.20	90.0	0.80
4	3.20	90.0	0.80
5	3.30	0.0	0.65
6	5.30	0.0	0.65
7	5.40	0.0	0.54

- 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)
NADH	666.2	649.2	25	85
NADPH	746.2	729.2	25	80

Created By:	Jeffrey A. Culver	Date: October 22, 2015
Reviewed By:	Christopher Petucci	Date: October 22, 2015
Approved By:	Christopher Petucci	Date: October 22, 2015

Revision Number	Name	Reason for Revision	Effective Date
01			
02			