Cell and Tissue Preparation for NAD Metabolomics
Brenner Laboratory
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Mammalian Cell Culture

Materials

a. 200 Proof Ethanol
b. LCMS Grade Water
c. 10 mM HEPES, pH 7.1 (pH adjusted with NaOH)
d. Internal Standard A (^{13}C Yeast Extract)
e. Internal Standard B (^{18}O nicotinamide riboside (NR), ^{18}O nicotinamide (Nam), D_4 nicotinic acid (NA), D_3, ^{18}O MeNam solution (60 µM of heavy NR, Nam, and MeNam and 240 µM of heavy NA)
f. 1.5 mL centrifuge tubes
g. Eppendorf Thermomixer or Heat block
h. Vortex
i. Small table top refrigerated centrifuge
j. P1000 pipette and tips
k. P2 pipette and tips
l. Speed vacuum
m. Thermo Scientific NanoDrop 2000
n. Waters Polypropylene plastic screw-top vials (0.3 mL volume, Catalog number: 186002639).
o. Numbered 1.5 ml snapcap tubes (typically 2 per biological or technical replicate, uniquely numbered with A and B suffixes)

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Determination of Number of Cells

Since values must be within the linear range of a standard curve for proper quantification, the amount of cellular material necessary to measure all desired metabolites should be determined empirically before beginning an experiment. As a general rule of thumb for most cells lines, 1 million cells produces the appropriate signal when extracted and re-suspended in 40 µL of solvent and should be a good starting point for most experiments. The following procedure describes how to empirically determine the number of cells for future experiments:

1. Grow 1 to 2 million cells per analysis. Remember that the standard NAD metabolome requires two analyses: all mononucleotides and dinucleotides plus NAR in the alkaline separation plus NR and the nicotinamide-related metabolites in the acidic separation.
2. There are many ways to prepare cells for metabolomics. The most common way is to trypsinize cells to release them from the bottom of the plate, pellet by centrifugation, resuspend in 4 °C phosphate buffered saline (PBS), and pellet by centrifugation into numbered 1.5 ml conical snap-cap tubes. **If you are shipping samples for analysis, stop here. Ship tubes on dry ice for Tuesday to Friday delivery.**
3. Extract cells using buffered boiled ethanol method (described below).
4. Re-suspend extracts in 40 µL of appropriate solvent (normally using 10 mM ammonium acetate). This solvent is not appropriate for NADH, which should be in either an alkaline (pH 9) solution of a volatile aqueous base (ammonium hydroxide or equivalent) or in a high organic solution (≥ 40% aqueous methanol). Remember to use highest grade solvents for this step (at least HPLC grade).
5. Analyze using LC-MS/MS. Ensure that the metabolites of interest are detected at least S/N of at least 50.

Buffered Boiled Ethanol Extraction Method (the standard Trammell and Brenner NAD metabolome)
You must possess internal standards to do this extraction.

1) Estimate the volume of the pellet in the 1.5 mL centrifuge tube. Using the pellet volume and the number of samples, calculate the amount of buffered ethanol solution to produce based upon the following calculation: (Volume of pellet/ 50 µL) X 300 µL Buffered Ethanol solution X number of samples.
2) Heat the buffered ethanol solution to 80 °C using the Eppendorf Thermomixer or heat block. Cool the centrifuge to 4 °C for later use.
3) Yeast cell walls are more difficult to disrupt if the yeast have been frozen. It is recommended that you prepare yeast samples fresh without storage at -80 °C or flash freezing. If mammalian cells are freshly prepared or frozen, keep on dry ice and perform extraction as quickly as possible. Add either 0.5 µL of IS A or 1 µL of IS B to the cells for alkaline separation and acidic separation, respectively. If an accurate small volume pipette is not available, dilute the internal standards such that 5 – 10 µL of internal standard solution would be added per sample.
4) Add the heated buffered ethanol solution to the cell pellets with 300 µL of extraction solution per 50 µL of cell pellet volume. Place the samples on the pre-heated Eppendorf Thermomixer heated at 80 °C and shake at 1050 rpm for 3 minutes. If using a heat block, vortex the samples for 5 seconds at maximum setting prior to placing the samples on the heat block and follow these steps:
    a. After 1.5 minutes, vortex for 5 seconds, then place back on heat block for another 1.5 minutes.
    b. When 1.5 minutes elapse, place extractions in cooled centrifuge and centrifuge for 10 minutes at 16.1X10^3 x g (maximum speed) and 4 °C.
5) When centrifugation is finished, transfer supernatant (extract) to fresh 1.5 mL centrifuge tube. Dry both the particulate and supernatant using a speed vacuum for 3 -4 hours or overnight.
6) When dry, re-suspend the cells in 40 µL of appropriate buffer and centrifuge (16.1k x g, 10 min, 4 °C). Measure the A260 nm values of the resulting clarified supernatant with 1 µL of extract using a Thermo Scientific Nanodrop. Dilute all extracts to an A260 nm value of 7.
7) Transfer samples to fresh Waters Polypropylene plastic screw-top vials (0.3 mL volume, Catalog number: 186002639).
8) Analyze using LC-MS/MS.

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Harvest of Mammalian Tissue: ideally by freeze-clamp at 2 pm

Metabolomics requires precise control over the animal dissection protocol as well as the tissue sample preparation. NAD abundance and presumably other metabolites of interest are controlled by circadian rhythms. Therefore, animal sacrifices should be performed at the same time of day and as quickly as possible. The largest Brenner experiments to date were performed with 2 pm sacrifices on *ad libitim*-fed animals on standard day-light cycles. In addition, the part of the organ influences metabolite concentrations, and so, when possible, the same lobe of liver should be dissected. For heart, brain and kidney, harvest the whole organ. If preparing kidney, the membrane around the kidney should be removed upon dissection.

In all cases, the speed of dissection of all organs should be as quick as possible. Enzymatic activity is not quenched by dissection and will noticeably change the abundance of the analytes of interest. For example, NAD is quickly degraded to Nam if samples are not dissected and frozen immediately. If the Nam concentration is high (greater than 40% of the NAD concentration) in all samples, degradation from improper quenching may have occurred at the time of dissection and in following steps. Ideally, the organ should be freeze-clamped with tongs cooled in liquid nitrogen or be immediately be snap-frozen to prevent alteration of the metabolome. Washing with cold PBS to remove

blood is generally discouraged as the time taken to perform the procedure may lead to differences in the NAD metabolome. Given that NAD and related metabolites are not highly abundant in blood, traces of blood should not greatly confound analysis. All in all, if one organ is most desired during a dissection, that organ should be removed first followed by any auxiliary organs. Though expensive, use of multiple mice for different tissues is advisable. **If you are shipping samples, stop at freeze-clamping the liver, heart, skeletal muscle or kidney or snap-freezing the brain. Ship uniquely numbered tubes on dry ice for Tuesday to Friday delivery.**

Extraction of Mammalian Tissue
You must possess internal standards to do this extraction.

Tissue preparation often requires mechanical disruption to lyse open cells and maximize the surface area exposed to extraction solvent. The most common methods of disruption include pulverization, homogenization, ultra-sonication, bead beating, or specialized tissue disruptors. The method of mechanical disruption depends upon the consistency of the tissue. Liver is fairly soft and porous and does not require extensive mechanical disruption, whereas skeletal muscle requires sonication in extraction buffer. Before beginning any experiment with mammalian tissue, ensure that the lysis procedure chosen is appropriate for the analytes of interest and that it properly disrupts the tissue. Bead beating has been shown in our lab to be deleterious to NAD and related metabolites and should be avoided. Sonication and homogenizer appears to work well for most tissues and does not adversely affect NAD and related metabolites as long as the samples are kept cold. In the following pages, the pulverization and extraction of liver, heart, and skeletal muscle is described.

Materials

a. Bessman Pulverizer (Spectrum Labs, Catalog 988-14500 (Small) and 988-14512 (Large))
b. Hammer that comes with the Bessman Pulverizer
c. Curved Tweezers
d. Long Forceps
e. 200 Proof Ethanol
f. LCMS Grade Water
g. LCMS Grade Methanol
h. 10 mM HEPES, pH 7.1 (pH adjusted with NaOH)
i. IS A (^{13}C Yeast Extract)
j. IS B (^{18}O nicotinamide riboside (NR), ^{18}O nicotinamide (Nam), D_4 nicotinic acid (NA), D_3, ^{18}O MeNam solution (60 µM of heavy NR, Nam, and MeNam and 240 µM of heavy NA))
k. 1.5 mL and 2 mL centrifuge tubes
l. Eppendorf Thermomixer or Heat block
m. Vortex
n. Small table top refrigerated centrifuge
o. P1000 pipette and appropriate pipette tips
p. P2 pipette and appropriate pipette tips
q. BD 1 mL syringe (REF 309659)
r. BD PrecisionGlide™ Needle 25 gauge x 5/8
s. Acetone (does not need to be high grade)
t. 1 L Beaker
u. 30 – 50 mL Beaker
v. 15 mL conicals
w. Thermometer
x. Branson Sonifier 450
y. Speed vacuum

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z. Waters Polypropylene plastic screw-top vials (0.3 mL volume, Catalog number: 186002639).

Procedure for Tissue Pulverization

1. Place the frozen samples into a Styrofoam container that contains a large amount of liquid nitrogen.
2. Pulverize tissue using a Bessman Pulverizer. The following URL will take you to a youtube page showing general use of the pulverizer: https://www.youtube.com/watch?v=r1mnhq8L3kg. Below is a description of how the device is used in this lab.
    a. Tape down an absorbent pad on your bench top. In the middle of the absorbent pad, tap down four to five sheets of folded rectangular shaped sheets of tin foil.
    b. Get ~4 L of liquid nitrogen if pulverizing 15 – 20 organs.
    c. Place frozen samples and fresh labeled 1.5 mL centrifuge tubes in liquid nitrogen.
    d. Place the disassembled Bessman Pulverizer and curved tweezers (https://www.kentscientific.com/products/productView.asp?productID=6199&Mouse_Rat=Tweezers&Products=Tweezer+%237) in a small Styrofoam container, then pour in liquid nitrogen such that all equipment is submerged. Allow the device to cool down to liquid nitrogen temperature. The liquid nitrogen should stop boiling when this has happened.
    e. Make a glove that we call a claw, or, if you have saved your claw, put the claw on.
        i. *Making a claw*
            1. Put on nitrile or latex gloves.
            2. Put on another nitrile or latex glove on your right hand, if right handed.
            3. Wrap your thumb in tape starting at the top and ending in the base of the thumb. Wrap your Index finger in tape starting at the top and ending at the base. In both cases, use approximately 6 – 8 layers of tape. Remember to cover the tips of your fingers with the tape. The idea is to cover the areas of glove that would be exposed to the liquid nitrogen cooled device.
            4. Congratulations you've made a claw.
    f. Put a cryo glove (http://www.fishersci.com/ecomm/servlet/fsproductdetail_10652_624522__-1_0) on your left hand if right handed.
    g. Open the tube containing the tissue. It is critical that both tubes be cooled to liquid nitrogen temperatures.
    h. Using the long forceps, place the liquid nitrogen cooled pulverizer base on the tin foil. Place the top part over the base.
    i. Using the large tongs, place the tissue aliquot into the opening of the apparatus. If the tissue is stuck to the side of the frozen centrifuge tube, impact the tube onto the rim of the opening with the opening of the tube facing the hole of the apparatus. If this does not work, use the curved tweezers to remove the tissue. If the tissue aliquot is too large to remove with the tweezers, carefully use the hammer to break open the tube and place the pieces in the opening of the pulverizer. When the tissue is placed in the chamber, transfer the liquid nitrogen cooled pin into the opening of the pulverizer. Using the hammer, carefully pound the tissue into a fine powder (~ 10 pounds).
    j. Pick up the pin and top part of the top piece with your right hand and pick up the fresh 1.5 mL centrifuge tube with your left hand. There should be a powder like substance on the bottom. Press the opening of the tube against the center of the apparatus and scrape and press the tube against the bottom to place pulverized material into the tube. Many times there is a disc of tissue at the center that should fall into the tube.

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i. If the disc does not fall into the tube, use the curved tweezers to pick it up and put it into the tube.
ii. If the pulverized material is in the bottom part of the apparatus, use the curved tweezers to gather up the disc and other tissue into the tube.

k. Flip the pulverizer over and knock it against the absorbent pad to remove tissue. Use a chemical wipe to remove any remaining tissue. Make sure to perform this quickly and do not allow ice to form on the device. Use a chemical wipe to remove any tissue from the pin and top. Place all items included curved tweezers back into liquid nitrogen.

l. Repeat steps for all remaining samples.

3. After all samples have been pulverized, 5-20 mg of tissue need to be aliquoted into two fresh, liquid nitrogen cooled 1.5 mL or 2 mL centrifuge tubes (1.5 mL tubes for liver/heart and 2 mL for skeletal muscle). Label these tubes with the sample name followed by a designation for alkaline or acidic separations. Normally, we have used A for alkaline and B for acidic. *Recommended: Another person could weigh the aliquots out while you are pulverizing to save time.* To keep the tissue from thawing, we recommend the following procedure when weighing:
    1. Pour liquid nitrogen into two small Styrofoam containers. Place pulverized samples in one. In the other, place a rack holding the fresh 1.5 mL centrifuge tubes designated for alkaline and acidic separations and a small spatula.
    2. Pick up the tube for the alkaline separation and place directly in liquid nitrogen, then place on scale. Tare the weight of the tube.
    3. Remove the tube, place in liquid nitrogen, place on rack, open tube.
    4. Pick up the fresh centrifuge tube and transfer ~5 – 20 mg of tissue into the tube.
    5. Place on the tared scale and record weight. Immediately transfer back to liquid nitrogen.
    6. Repeat this for the acidic separation tube.
    7. When sample has been aliquoted twice, close the sample tube and move to the next sample.
    8. When all samples have been aliquoted, store at -80 °C or perform extraction on samples after a one hour rest on dry ice.

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Extraction Procedure for Liver and Heart

1. Place frozen, pulverized samples on dry ice.
2. Add internal standard A or internal standard B to the appropriate tube while on dry ice. For internal Standard A, dilute the extract by a half or by a tenth and add 1 or 10 µL to each tube for alkaline separation tube. For internal standard B, add 1 µL of the solution to each tube for acid separation in either a diluted form or as is if you have an accurate P2 pipetman.
3. Make up buffered ethanol solution (75% ethanol/25% 10 mM HEPES, pH 7.1 with NaOH). 100 µL of solution will be added to each sample.
4. Heat buffered ethanol solution to 80 °C using the Eppendorf Thermomixer or a heat block. Allow the solution to set for at least ten minutes to ensure the solution is at 80 °C.
5. Add 100 µL of heated buffered ethanol solution to samples for ~5 seconds.
6. Place the tube on ice.
7. Repeat 5 and 6 for all remaining samples.
8. After all samples have been placed on ice, sonicate tissue using a bath sonicator for 10 seconds, followed by a 15 second rest on ice. Repeat the sonication 2 or 3 times for liver or heart, respectively.
9. Place samples on Eppendorf Thermomixer at 80 °C for 5 minutes with constant shaking at 1050 rpm. If using a heat block, vortex for 5 seconds after the samples have been heated for 2.5 minutes then place back on heat block for the remaining 5 minutes.

10. After five minutes, place tubes in small bench top centrifuge cooled to 4 °C. Centrifuge at 16,100 x g for 10 minutes. This is the maximum speed of the centrifuge. Transfer supernatant into fresh 1.5 mL centrifuge tubes. Dry the extract. In some cases, the pellet could be dried and weighed and used as a normalization factor.
11. When dry, re-suspend the cells in 40 µL of appropriate buffer (10 mM ammonium acetate for alkaline separation and water or acidified water for acidic separation).
12. Analyze using LC-MS/MS.

Extraction Procedure for Skeletal Muscle

1. Place frozen, pulverized samples (20 mg) on dry ice.
2. Heat the Eppendorf Thermomixer to 85 °C.
3. Place a beaker filled with an appropriate amount of LCMS grade methanol on wet ice. 0.2 mL of methanol per sample will be used. Place a 15 mL conical tube(s) containing LCMS grade water and internal standard mixture A or B on the wet ice. Prepare the internal standard solutions in the following manner.
    a. For internal Standard A, dilute the solvent by a tenth and add 10 µL internal standard to 0.29 mL of LCMS grade water. For internal standard B, add 1 µL of the solution to 0.299 mL of LCMS grade water. The amount of aqueous internal standard solution should be 0.3 mL per sample.
4. Place ~450 mL of acetone in a 1 L beaker. Place wet ice in the beaker and allow it to melt. Once you have reached ~850 mL volume, add three large pellets of dry ice and allow them to sublime. Check the temperature using a thermometer and ensure that it is ~ - 4 °C.
5. Place the cold acetone/water bath directly below the Branson Sonifier 450. Set the duty cycle to 40% and the output control to 4. Switch on the sonicator.
6. Wash the probe by sonicating a 50/50 mixture of methanol/water for 20 seconds.
7. Add 0.2 mL of ice cold methanol to an aliquot of tissue using a 1 mL syringe equipped with a 16 gauge (1.6 x 25 mm) needle.
8. Place the sample on wet ice then add 0.3 mL of internal standard solution as described in step 3a.
9. Place the sample in a buoyant holder and place in the acetone/water bath.
10. While holding the lid of the 2 mL centrifuge tube, elevate the beaker such that the probe inserts into the tube. Adjust the elevation such that the probe is half way submerged in extraction buffer.
11. Turn the dial of the machine to begin sonicating. Sonicate for 20 seconds. Open the lid of the next sample while this is occurring. After 20 seconds, place the sample on wet ice.
12. Repeat steps 6-10 for all samples.
13. After all samples have been extracted, place the samples on the 85 °C Eppendorf Thermomixer and incubate with constant shaking at 1050 rpm for 5 minutes. If using a heat block, vortex each sample for 5 seconds after 2.5 minutes on the heat block. Replace the sample on the heat block after vortexing and incubate for another 2.5 minutes.
14. Place the samples on wet ice for 5 minutes. This step appears to be crucial to preserve some analytes and should not be overlooked.
15. Centrifuge (16.1k x g, 10 minutes, 4 °C) the samples. Transfer the supernatant to a fresh 1.5 mL centrifuge tube after centrifugation.
16. Dry the supernatant using a speed vacuum. The pellet can also be dried and later used to normalize data in lieu of wet tissue weight.
17. Re-suspend in 10 mM ammonium acetate, centrifuge (16.1k x g, 3 minutes, 4 °C). Carefully, transfer the supernatant to fresh Waters Polypropylene plastic screw-top vials (0.3 mL volume, Catalog number: 186002639).
18. Analyze using LC-MS/MS.

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NAD(P)H Extraction in Liver—Preparation of C samples for the reduced NAD metabolome

   1. Before beginning, be aware that only 12 samples can be analyzed per queue and that the queue should last no more than 6 hours.
   2. Weigh out 20 mg of pulverized liver sample into fresh, 2 ml centrifuge tubes chilled at liquid nitrogen temperatures.
      a. In addition to your samples, aliquot 25 – 35 mg of any sample of your choosing into three fresh, 2 ml centrifuge tubes chilled at liquid nitrogen temperatures (20 – 35 mg per tube). The sum of the tissue aliquoted should approximately 70 mg or greater. For example, Tube 1: 20 mg, tube 2: 20 mg, tube 3: 30 mg. These tubes will be used to construct a standard curve with standard addition and will be referred to as SAs. A standard addition standard curve is produced by adding known amounts of standard to a sample solution that contains an unknown amount of the analyte of interest. The background is subtracted from each tube and this background subtracted standard curve is used to quantify samples. Standard addition will control for matrix effects on chromatography and ion suppression.

2. Make extraction solvent: methanol/25 mM ammonium acetate pH 9 (80%/20%) and sparge the extraction solvent with nitrogen gas for thirty minutes.
3. Place extraction solvent in -80 C freezer and allow it to cool to -80 C (wait at least an hour).
4. Set the Eppendorf Thermomixer to 60 C.
5. Replace all glass Pasteur pipettes on the nitrogen dryer with fresh pipettes.
6. Once the extraction solvent has reached -80 C, place your samples, SAs, and extraction solvent on dry ice. Surround the extraction solvent in dry ice.
7. Prepare the acetone/water bath as described in the muscle extraction protocol above and remember to monitor temperature throughout extraction.
8. Add 0.5 ml of extraction solvent to the sample or SA and sonicate for 10 seconds (output control = 4, intensity = 40%, 10 seconds). While processing samples, keep the cap on the extraction solvent bottle as much as possible. Place samples and SAs back on dry ice after sonication. Repeat this for all samples and SAs.
9. Place all samples and SAs at 60 C and shake at 1000 rpm for 3 minutes using the Eppindorf Thermomixer.
10. Centrifuge samples (16.1k x g, 4 C, 10 minutes).
11. Transfer supernatants of samples to fresh, dry ice chilled 2 ml centrifuge tubes. Transfer the supernatants of SAs to a fresh, dry ice chilled 15 ml centrifuge tube.
12. Place the pellets back on to dry ice and repeat steps 6 – 9 two more times. Transfer supernatants to the same tubes as used in step 9.
13. After transferring the last supernatant, begin drying the samples with a steady stream of nitrogen at ambient temperature. **Do not dry down the SAs yet.** The samples should take ~1 hour to dry. Check the samples over time to ensure drying.
14. While the samples are drying, construct your standard curve.
    a. Considering NADH and NADPH are very labile in solution, solid standard should be brought up fresh before each experiment.
       i. Weigh out >30 mg of NADH into a 5 ml volumetric flask. Bring the standard up to volume using 25 mM ammonium acetate pH 9 and place on wet ice.
       ii. The NADPH was purchased as 1 mg aliquots. Add 1.199 ml of 25 mM ammonium acetate pH 9 to one vial and place on wet ice.
    b. Check the concentration of each using the Thermo Scientific 2000c Nanodrop. There are methods saved to the computer for both NADH and NADPH. Since the extinction coefficient at 340 nm is the same for both NADH and NADPH, the same method can be used for both. The NADH stock solution should be diluted 1:9 using 25 mM ammonium acetate pH 9 (total volume 0.1 ml). This solution will only be used to check the concentration of the NADH stock solution.

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c. After the concentrations have been determined, dilute both NADH and NADPH into the same solution to a final concentration of 0.5 mM with 25 mM ammonium acetate pH 9 (this solvent will be used to compose the standard curve) and a final volume of 0.2 ml. Keep all standards on wet ice prior to step 14d. Setup the standard curve in the following manner:

Concentration (mM)	
0.375	**75 µl of 0.5 mM stock with 25 µl of solvent**
0.300	80 µl of 0.375 mM stock with 20 µl of solvent
0.250	**50 µl of 0.5 mM stock with 50 µl of solvent**
0.150	60 µl of 0.25 mM stock with 40 µl of solvent
0.100	**20 µl of 0.5 mM stock with 80 µl of solvent**
0.075	75 µl of 0.100 mM stock with 25 µl of solvent
0.045	60 µl of 0.075 mM stock with 40 µl of solvent

d. Determine the concentration of liver tissue to volume in the SAs supernatant. For example, if the final amount of tissue were 70 mg and the final volume is 4.5 ml, the concentration is 15.56 mg/ml. Place nine fresh 2 ml centrifuge tubes at ambient temperature. Determine the amount of extract to add to each tube such that the amount of tissue is equal to 5 mg. For example, 5 mg/15.56 mg/ml = 0.321 ml per tube. Aliquot the calculated amount to each tube.
e. **It is crucial that the extracts be warmer than dry ice temperature when applying the standard. If the tubes are too cold, the standard may partially freeze before transfer, disallowing complete transfer of standard. After the extracts have warmed (5 minutes)**, place 10 µl of standard to one of the aliquots in step 14d. The ninth tube is an extract blank tube. Add 10 µl of 25 mM ammonium acetate pH 9 to this tube.
f. Dry down the standard curve using nitrogen at ambient temperature. The standards should dry down faster than samples as the volume is less. When all standards and samples have been dried, re-suspend the pellet in 0.1 ml of 50 mM ammonium acetate pH 8. Vortex all vigorously, then centrifuge for three minutes at maximum speed at ambient temperature. Normally, the centrifuge in the HRMFS has been used for this purpose.
g. Transfer 0.05 ml of extract to total recovery vials and analyze using LC-MS.
    i. The TQD contains a mass spectrometry method and inlet method labeled Brenner_NAD_P_NADH_P_SIM_080115 and Brenner_080715_TBABEHC18 Modified, respectively.
    ii. Negative ion, single ion monitoring is used for detection of NADH and NADPH.
    iii. Sample injection volume = 2.5 µl, Standard injection volume = 10 µl. **These injections volumes were chosen so that extract equivalent to 5 mg of tissue is loaded onto the column from both sample and standard.**
    iv. Each run lasts 13.5 minutes.

Whole blood

Draw blood samples into EDTA tubes with appropriate circadian thinking. LC-MS analysis requires separate extraction of at least 50 µl and ideally 100 µl of whole blood samples. Assuming standard alkaline and acid separations are to be done, the minimum number of aliquots is 2. However, for human samples that are not limited in volume, please prepare 4 aliquots so that repeats can be done if necessary.

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After mixing blood by inversion in EDTA tubes, pipet aliquots into numbered 1.5 ml snapcap tubes and freeze in liquid nitrogen. Suffixes should be A, B, C and D for each numbered blood sample. **Ship on dry ice for Tuesday-Friday delivery.**

References:

1. Trammell, S. A., & Brenner, C. (2013). Targeted, LCMS-based Metabolomics for Quantitative Measurement of NAD(+) Metabolites. *Computational and structural biotechnology journal*, *4*, e201301012. https://doi.org/10.5936/csbj.201301012
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3. Trammell, S., Weidemann, B., Chadda, A. *et al.* Nicotinamide Riboside Opposes Type 2 Diabetes and Neuropathy in Mice. *Sci Rep* **6,** 26933 (2016). https://doi.org/10.1038/srep26933

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