Supporting Information

Improved Yield for the Enzymatic Synthesis of Radiolabeled Nicotinamide Adenine Dinucleotide

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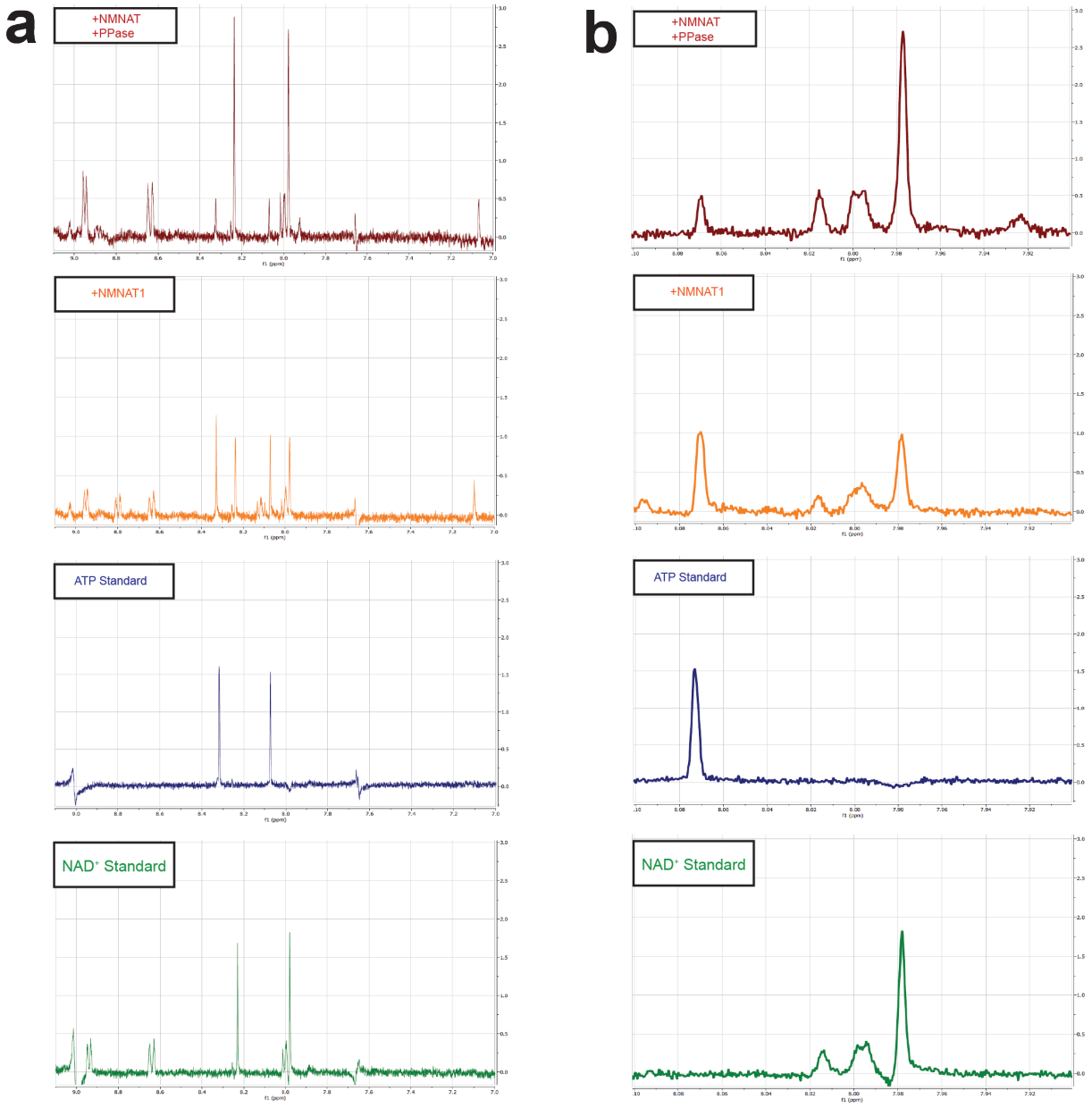
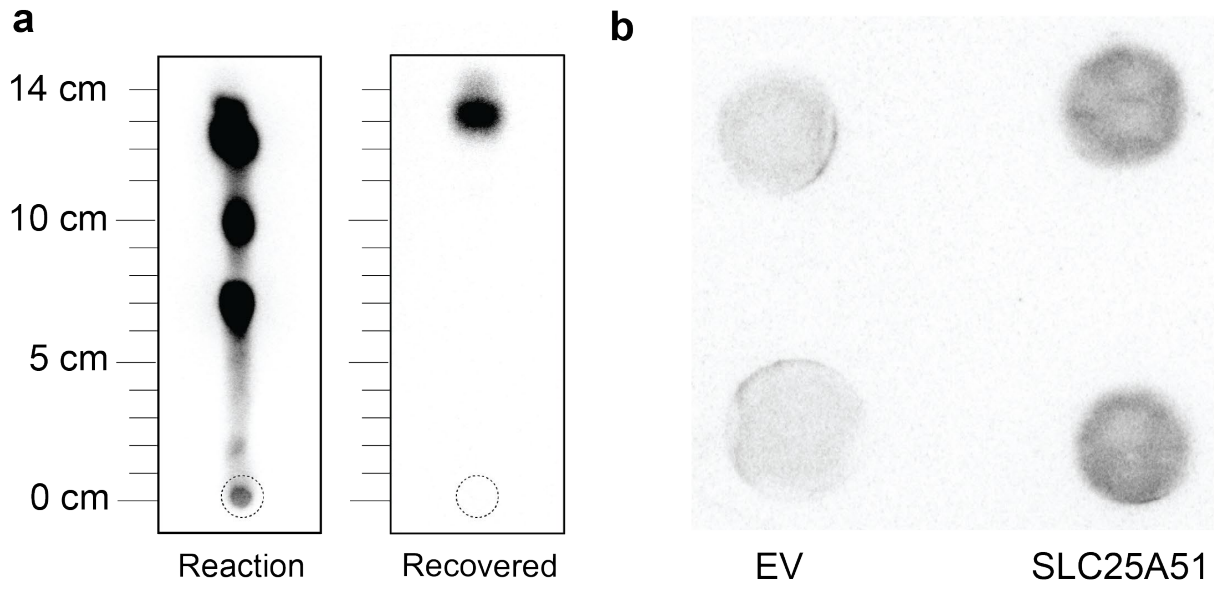


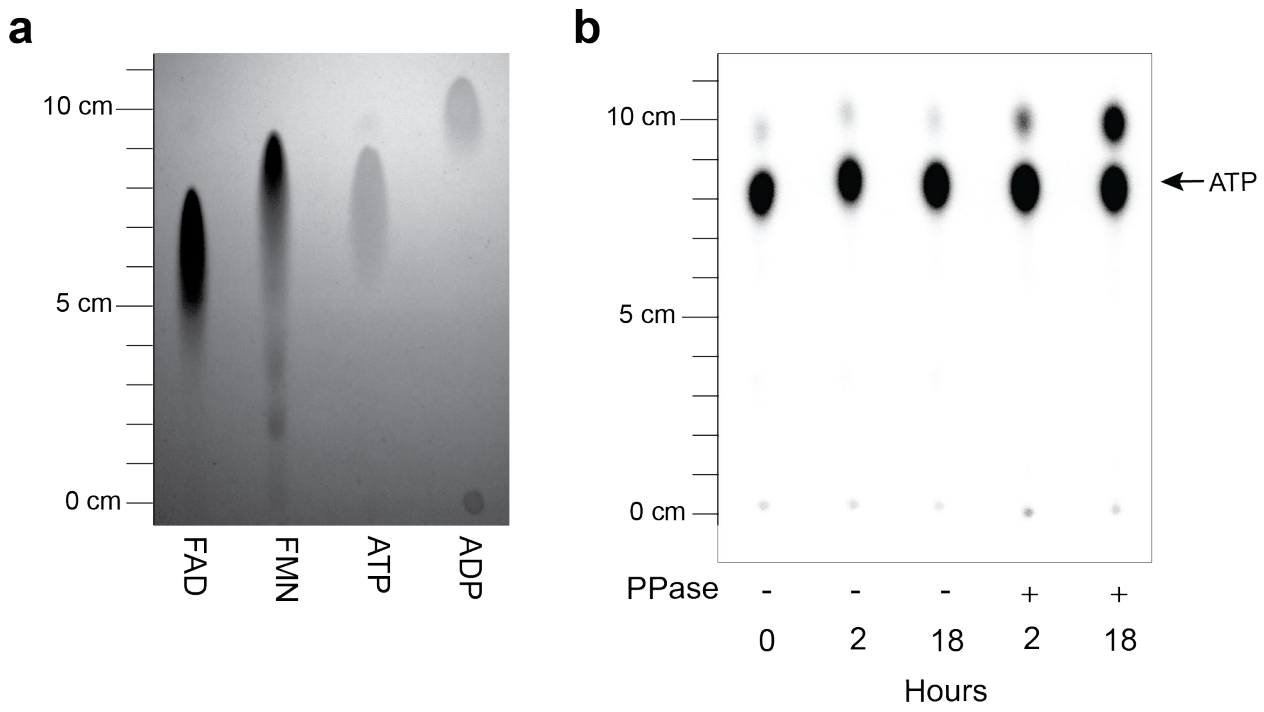
Supporting Figure S1: ^{1}H-NMR of NMNAT1 Reaction with and without PPase.
Aligned ^{1}H-NMR spectra of 1 mM standards for NAD^{+} (green) and ATP (purple) with comparable x- and y-axes. Panel (a) represents a broad view, 7.00 - 9.10 ppm. Panel (b) represents a focused view. 7.90 – 8.10 ppm, to better visualize a representative subset of peaks. In orange the reaction only contained NMNAT1 without PPase and it showed peaks of comparable areas characteristic of NAD^{+} and ATP peaks as determined by the standards. Maroon represents the combined reaction with NMNAT1 and PPase. Here we observed increased areas corresponding to NAD^{+} peaks and decreased areas corresponding to ATP peaks.



Supporting Figure S2: NAD^+ Uptake with Recovered ^{32}P-NAD^+.

(a) Thin layer chromatography (TLC) of the enzymatic NMNAT1 reaction in the absence of PPase show as expected a mixture of ^{32}P-NAD^+, ^{32}P-ATP, and other ^{32}P-species *(Reaction, left)*. 2.5% of total material from the recovered ^{32}P-NAD^+ sample was resolved using TLC (*Recovered, right*).

(b) ^{32}P-dot blots of in vitro NAD^+ import assays using recovered ^{32}P-NAD^+ material and isolated yeast double knockout mitochondria that either expressed empty vector (EV) or human mitochondrial NAD^+ transporter (SLC25A51).



Supporting Figure S3: Attempted FADS enzymatic synthesis of ^{32}P-FAD resulted in aberrant ^{32}P product with PPase inclusion.

(a) FMN, FAD, ATP and ADP standards were resolved with TLC using PEI cellulose F plates and imaged following excitation at 254 nm.

(b) Attempted enzymatic reaction to synthesize radiolabeled ^{32}P-FAD by adenylating FMN with ^{32}P-ATP via recombinant FAD Synthetase. Reactions were setup as indicted in supporting methods, samples obtained at indicated time points and resolved with TLC, and imaged following exposure with a phosphorimaging screen. A ^{32}P-labeled byproduct specifically appeared after incubation with PPase (lanes 4,5), with higher abundance at 18 hours (lane 5).

Detailed Experimental Procedures:

Thin Layer Chromatography of Standards:

Materials:

Standards: ATP, A9062-5X1G, Millipore Sigma, >95%; ADP, A2754-5X1G, Millipore Sigma, >95%; AMP, A1752-5G, Millipore Sigma, >99%; NADH, 606-68-8, Avantor, >95%; NADP, 24292-60-2, Avantor, >95%; NAD^+, N1636-25MG, Millipore Sigma, >99%; FMN, F6750-5G, Millipore Sigma, >73%; FAD, F6625-250MG, Millipore Sigma, >95%

TLC PEI Cellulose F plates: EMD Millipore, Cat#: 1057250001; Thin Layer Chromatography Tank; 254 nm UV Lamp

1. Mark TLC plate with a pencil at 2 cm above edge (origin), 7 cm above edge (5 cm resolve mark) and 17 cm above edge (15 cm resolve mark).
2. 5 µL of 50 µM standards of all nucleotides were spotted onto a TLC PEI Cellulose F plate at 2 cm above the bottom edge of the plate with 2.5 cm between each spot.
3. 50 mL of 18 MΩ H_2O was added to a 30 cm x 27 cm x 10 cm glass chromatography tank such that the liquid was ~1.5 cm deep.
4. The plate was resolved in the tank until the liquid reached 5 cm above origin. The plate was removed and allowed to dry on the bench top.
5. 50 mL of 1.4M LiCl was added to tank and the dry plate was placed back inside. The plate was resolved in the tank until the liquid reached 15 cm above origin.
6. The plate was allowed to dry on bench top and then imaged using a 254 nm UV lamp.

NMNAT1 Synthesis of ^{32}P-NAD^{+} from ^{32}P-ATP:

Materials:

α-^{32}P-ATP: Perkin Elmer Cat #: BLU003H250UC, Batches 07142, 03102, and 12091; Recombinant Human NMNAT1: (MyBioSource Cat#: MBS206313, Lot#: 21320702); Inorganic Pyrophosphatase (PPase): NEB Cat#: M0361S, Lot#: 10117903

1. The reaction mixture was assembled in 20 µL volume total, containing 3 µM α-^{32}P-ATP, 50 µM ATP, 50 µM NMN and 5 µM Recombinant Human NMNAT1 in a final buffer of 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, and 0.1 units (1 µL) inorganic pyrophosphatase as indicated.
2. The reaction was carried out for 2 hours at room temperature, at which time 1 µL was removed and spotted onto a TLC PEI Cellulose F Plate and resolved as described in section "Thin Layer Chromatography of Standards".
3. Upon completion, the reaction was snap-frozen in liquid nitrogen and stored at -20 °C.
4. After drying, the plate was wrapped in clingwrap and placed in a BioRad exposure cassette with a BioRad phosphorimaging screen for 3 minutes, then the screen was imaged on a Typhoon FLA 9500 Imager. The intensity of the dots was quantified using the ImageJ Gel Analyzer feature.

Recovery of NAD^+ from Cellulose TLC Plate

1. To recover NAD^+ from cellulose plate, nucleotide spots were visualized with a 254 nm UV lamp and the NAD^+ spot was circled with a pencil.
2. Cellulose in the circled area was removed with a razorblade and incubated with 500 µL of buffer (120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM HEPES-KOH pH 7.4) for 30 minutes at RT.
3. Buffer and cellulose were filtered through a 0.45 µm cellulose syringe filter to recover ^{32}P-NAD^+.

Analysis and Quantification of Phosphorimaging Data:

1. Import image into Image J. Use the rectangle tool and draw a rectangle around one lane to mark the ROI.
2. Use the copy function to duplicate the ROI for marking additional lanes. Repeat until all lanes are marked. Create plot profiles of each lane.
3. Use the Straight Line to draw a line across the background level on the plot profile to separate individual peaks.
4. Use the Magic Wand tool to highlight each peak. Magic Wand tool will automatically create area under the curve values for each peak selected.
5. Add up the values from each lane to sum the total intensity for that lane. Divide the intensity of any individual peak to get the percent of that species present, i.e. NAD^+ peak over total sum to determine percent NAD^+ of total.
6. Calculate the percent conversion of each species across replicate reactions. Using GraphPad, place replicates into a column data sheet. Compare reaction conditions with a Student's t-test and calculate p-values.

^{1}H-NMR of NMNAT1 Reaction with and without PPase:

1. The reaction mixture was assembled in 100 µL volume total, containing 10 mM ATP, 10 mM NMN and 5 µM Recombinant Human NMNAT1 in a final buffer of 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, and 0.5 units (5 µL) inorganic pyrophosphatase as indicated.
2. Incubate mixture at room temperature for 16 hours.
3. Dilute reaction mixture 1:10 in Milli Q H_2O to get 1 mM final concentration in 1 mL volume.
4. Add 10% D_2O to the reaction and place in glass NMR tube.
5. Measure ^{1}H-NMR using an Agilent 400 MHz NMR Magnet instrument.
6. ^{1}H-NMR spectra analyzed using MestReNova 14.1 software.

NAD^+ Uptake into Isolated Yeast Mitochondria Expressing Human SLC25A51:

Materials:

Buffer A (no NAD^+):

120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM HEPES-KOH pH 7.4

Buffer B (unlabeled NAD^+ only):

120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM HEPES-KOH pH 7.4, 100 μM NAD^+

Uptake Buffer (unlabeled NAD^+ and ^{32}P-tracer NAD^+):

120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM HEPES-KOH pH 7.4, 100 μM NAD^+, 2.9 nM ^{32}P-NAD^+

0.22 μm mixed-cellulose ester filter (Whatman, Cat#: WHA10401706)

KNF Vacuum Pump UN726

Wheaton 25 mm Filtration Assembly (Cat#: 419325)

MitoTracker Red CMXROS (ThermoFisher Cat #: M46752)

Yeast Strains used for isolation of fresh mitochondria: *Δndt1 Δndt2* BY4727 with PRS415-CEN/ARS-TEF-LEU plasmids expressing either Empty Vector (EV) or a yeast codon optimized human SLC25A51 (SLC25A51) sequence.

1. Yeast Mitochondrial Isolation was performed as previously described.[1-2]
2. In short, 1 L of yeast was grown in YP + 2% Raffinose ~16 hours until reaching OD 600 of 3.0. Mitochondria were isolated using zymolyase, dounce homogenization and differential centrifugation to enrich for the mitochondrial fraction.

3. Mitochondria were kept on ice and used within 3 hours of isolation. Mitochondria were confirmed to still be functional using MitoTracker Red to show an intact membrane potential.
4. 1 µL of the reaction was diluted 1:20 (5 µL in 95 µL) into Buffer A to create a working stock of ^{32}P-NAD$^+$ (~147 nM). This ^{32}P-NAD$^+$ stock was further diluted 1:50 (5 µL in 245 µL) into Buffer B to create Uptake Buffer. The final concentration of tracer ^{32}P-NAD$^+$ was ~2.9 nM.
5. 1 mg of isolated mitochondria[1-2] was resuspended in 50 µL Uptake Buffer and incubated for 5 min at room temperature. EV samples were included in parallel as paired negative controls.
6. At 5 minutes, the 50 µL reaction was stopped by adding 950 µL (20X volume) ice-cold Buffer B that only contained unlabeled NAD$^+$.
7. The sample was filtered through a 0.22 µm mixed-cellulose ester filter using the Filtration Assembly attached to the vacuum pump. Filter was washed with an additional 5 mL of ice-cold Buffer A containing no NAD$^+$.
8. The filter was covered in clingwrap and placed in a BioRad exposure cassette with a BioRad phosphorimaging screen for 16 hours, then the screen was imaged and analyzed as described in sections "NMNAT1 Synthesis of ^{32}P-NAD$^+$ from ^{32}P-ATP" and "Analysis and Quantification of Phosphorimaging Data".
9. Signal from EV negative control was averaged and the individual replicates from the EV and SLC25A51 conditions were normalized back to the paired EV average from that experiment.

NAD Kinase Synthesis of ^{32}P-NADP from ^{32}P-NAD^{+}:

Materials:

NADK (Adipogen Cat#:AG-40T-0091-C050, Lot#: A01419)

1. The reaction mixture was created in 20 µL volume with 0.44 µM ^{32}P-NAD^{+}, 100 µM ATP, 50 µM NAD^{+} and 5 µM NADK in a final buffer of 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$).
2. The reaction was carried out for 18 hours at room temperature. At 0.5, 2 and 18 hours, 1 µL was removed and spotted onto a TLC PEI Cellulose F Plate at 2 cm above the bottom edge, with 2.5 cm between each spot, and resolved as described above.
3. After drying, the plate was wrapped in clingwrap and placed in a BioRad exposure cassette with a BioRad phosphorimaging screen for 3 minutes, then the screen was imaged and analyzed as described in sections "NMNAT1 Synthesis of ^{32}P-NAD^{+} from ^{32}P-ATP" and "Analysis and Quantification of Phosphorimaging Data".

Attempted FAD Synthetase Reaction from ^{32}P-ATP:

Materials:

Recombinant human FADS (MyBioSource Cat#: MBS1342413, Lot#: YA05272b1g5)

1. The reaction mixture was assembled in 20 µL volume with 3 µM α-^{32}P-ATP, 50 µM ATP, 50 µM FMN and 5 µM human FADS in a final buffer of 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 12 mM $MgCl_2$ and 1 mM DTT[3], with the addition of 0.1 Units (1 µL) inorganic pyrophosphatase in the proper conditions.
2. The reaction was carried out for 18 hours at room temperature. At 2 and 18 hours, 1 µL was removed and spotted onto a TLC PEI Cellulose F Plate at 2 cm above the bottom edge, with 2.5 cm between each spot, and resolved as described above.
3. After drying, the plate was wrapped in clingwrap and placed in a BioRad exposure cassette with a BioRad phosphorimaging screen for 3 minutes, then the screen was imaged and analyzed as described in sections "NMNAT1 Synthesis of ^{32}P-NAD^+ from ^{32}P-ATP" and "Analysis and Quantification of Phosphorimaging Data".

References:

1. Meisinger C, Pfanner N, Truscott KN. Isolation of yeast mitochondria. *Methods Mol Biol*. **2006,** 313:33-9. DOI: 10.1385/1-59259-958-3:033.
2. Luongo T. S., Eller J. M., Lu M. J., Niere M., Raith F., Perry C., Bornstein M. R., Oliphint P., Wang L., McReynolds M. R., Migaud M. E., Rabinowitz J. D., Johnson F. B., Johnsson K., Ziegler M., Cambronne X. A., Baur J. A. SLC25A51 is a mammalian mitochondrial NAD^+ transporter. *Nature*. **2020**, 588(7836):174-179. DOI: 10.1038/s41586-020-2741-7.
3. Leone P, Tolomeo M, Barile M. Continuous and Discontinuous Approaches to Study FAD Synthesis and Degradation Catalyzed by Purified Recombinant FAD Synthase or Cellular Fractions. *Methods Mol Biol.* **2021**, 2280:87-116. DOI: 10.1007/978-1-0716-1286-6_7.