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Assays for NAD⁺-Dependent Reactions and NAD⁺ Metabolites

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Abstract

Nicotinamide adenine dinucleotide (NAD⁺) is an essential redox cofactor and signaling molecule that controls the activity of enzymes involved in metabolism, DNA repair, and cellular survival, such as the PARPs, CD38, and the sirtuins. Here, we describe three methods for measuring the activity of these enzymes: the etheno-NAD⁺ assay measures NAD⁺ hydrolase activity using an NAD⁺ analog to produce a fluorescent product that is measured in real time; the PNC1 assay converts a native product of NAD⁺ hydrolysis, nicotinamide, into a quantitative fluorescent readout; and liquid chromatography tandem mass spectrometry (LC-MS/MS) is used to characterize the entire NAD⁺ metabolome in a sample. These methods will enable new insights into the roles that NAD⁺ and the enzymes that utilize it play in health and disease.

Keywords

Aging; NAD⁺ nicotinamide; ADP-ribose; PARP; ARTD; CD38; BST1; Sirtuin; Metabolomics; Mass spectrometry; Metabolism; Epigenetics; HDAC

1 Introduction

The oxidized form of nicotinamide adenine dinucleotide (NAD⁺) is an essential coenzyme for redox reactions and co-substrate for multiple families of proteins that control how a cell responds to its environment. Processes regulated by NAD⁺ include energy metabolism, inflammation, DNA repair, gene expression, and aging. Classically, NAD⁺ acts as a redox intermediate, shuttling the energy generated from the breakdown of glucose (during glycolysis and the TCA cycle) to complex I of the electron transport chain. More recently, NAD⁺ has also come to be appreciated as a signaling molecule. Three major classes of enzymes utilize NAD⁺ for non-redox reactions: PARPs/ARTDs, CD38/BST1, and sirtuins. All three hydrolyze NAD⁺ to produce nicotinamide and some form of ADP-ribose. In this chapter, we will focus on the latter two families (although all assays are amenable to any NAD⁺ hydrolase) in describing three assays to measure the hydrolysis of NAD⁺.

CD38 and BST1 are membrane proteins that hydrolyze NAD⁺ to produce (cyclic-)ADP-ribose. CD38 is a transcriptional target of NF- κ B that is upregulated in response to

inflammation [1]. It also regulates insulin sensitivity via consumption of NAD⁺ [2]. BST1, a homolog of CD38, regulates stem cell proliferation in response to energy availability via cADPR signaling [3] and has been associated with neurological disorders [4].

Sirtuins are NAD⁺-dependent lysine de-acylases and ADP-ribosyltransferases. Mammals have seven sirtuins (SIRT1–7), each one with a different spectrum of enzymatic reactions, intracellular localizations, and protein substrates (reviewed in [5]). Notable targets of sirtuin deacylation include histones; transcription factors such as p53 [6], PGC-1 α [7], and NF- κ B [8]; enzymes such as glutamate dehydrogenase [9] and LKB1 [10]; and signal transduction effectors such as Notch [11] and IRS1 [12]. Sirtuins respond to the availability of NAD⁺, which increases during calorie restriction or exercise, thereby enhancing DNA repair, increasing mitochondrial function, and reducing inflammation, among other beneficial effects.

All three families of NAD⁺ hydrolases, including PARPs and ARTDs, have two important similarities. First, they consume NAD⁺, which needs to be resynthesized via biosynthetic pathways starting with NAD⁺ precursors such as nicotinamide riboside or tryptophan. Secondly, the dissociation constants of many of these enzymes for NAD⁺ are relatively high (reviewed in [13]). Taken together, these two properties mean that these enzymes are often in competition for the same pool of NAD⁺ and that increased activity of one can suppress the activity of others [14, 15].

Three assays that measure the hydrolysis of NAD⁺ are described in this chapter. The etheno-NAD⁺ assay uses a modified NAD⁺ molecule which, when hydrolyzed, produces a fluorescent etheno-ADP-ribose product. By tracking the increase in fluorescence in real-time, the NAD⁺ hydrolase activity of a sample can be inferred [16]. This assay is amenable for use with recombinant enzymes and cell or tissue samples.

The PNC1 assay measures the other portion of the hydrolyzed NAD⁺ molecule: nicotinamide. In this assay (Fig. 1), an enzyme such as SIRT1 generates nicotinamide from NAD⁺ in **step 1**. An enzyme from budding yeast, yPnc1, is then utilized to convert the nicotinamide into nicotinic acid and NH₄⁺ (free ammonia) in **step 2**. The ammonia is then reacted with ortho-phthalaldehyde (OPT) and dithiothreitol (DTT) to produce the fluorescent 1-alkylthio-substituted isoindoles [17] in **step 3**. Therefore, the fluorescence detected is proportional to the amount of nicotinamide produced during the NAD⁺ hydrolysis reaction.

The final assay described in this chapter, liquid chromatography tandem mass spectrometry (LC-MS/MS), can be used for the quantification of the entire NAD metabolome. This technique has become a standard in the metabolomics field and can detect NAD⁺ and related metabolites over a wide dynamic range down to low picomolar levels. It displays superior specificity over reverse phase high-performance liquid chromatography (HPLC). Two separation methods are employed to characterize the NAD⁺ metabolome: alkaline separation for all metabolites containing a ribose sugar and acid separation for metabolites without a sugar moiety.

It is anticipated that the methods discussed herein will allow for standardization of assays related to NAD⁺ across different laboratories and to overcome challenges associated with translation of preclinical studies toward clinical practice.

2 Materials

2.1 Etheno-NAD⁺ Assay

1. Assay buffer: 250 mM sucrose and 40 mM Tris-HCl, pH 7.4, filtered.
2. Nicotinamide 1,N⁶-ethenoadenine dinucleotide (Sigma-Aldrich, N2630), dissolved in assay buffer at 60 mM. Store at -20 °C in small aliquots, to avoid repeated freeze-thaw cycles.
3. NADase enzyme inhibitor:
 - For example, apigenin (Sigma-Aldrich, N3145) for inhibition of CD38. Dissolve in DMSO for a stock concentration of 6 mM.
 - 3-Aminobenzamide (Sigma-Aldrich, A0788) or PJ-34 (Abcam, ab120981) are popular PARP inhibitors.
4. 96-well black half-area flat bottom polystyrene plates (Corning, 3694).
5. Plate reader capable of reading fluorescence (Ex/Em = 310/410).
6. If using recombinant enzymes in vitro:
 - Recombinant enzyme, such as CD38 (R&D Systems, 2404-AC-010) or BST1 (R&D Systems, 4736-AC-010).
7. If using with cell or tissue lysate:
 - NETN lysis buffer (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5% (v/v). NP-40) with protease inhibitors (Sigma-Aldrich, 11873580001).
 - BCA protein quantification assay (Thermo Fisher, 23227).

2.2 PNC1 Assay

1. Purified recombinant SIRT1 (or another nicotinamide-producing enzyme) and yPnc1 enzymes produced by previously described protocols [18].
2. Sirtuins require acetylated peptide substrates with acetylated lysine near the middle of the sequence (e.g., Ac-TARK(ac) STG-NH₂) (see **Note 4**-for the convenience of the experimenter, notes are grouped by assay are therefore not ordered by when they appear).
3. Reaction buffer: PBS (pH 7.4) supplemented with 1 mM DTT (Thermo Fisher, R0861). Tris buffer (pH 8.0) can be used if PBS is incompatible with the enzyme.
4. OPT Developer Reagent: Dissolve OPT (Sigma-Aldrich, P0657) in pure ethanol (33.3 mM) and dissolve DTT in PBS (14.28 mM). Mix three volumes of ethanol

and seven volumes of PBS to make a 30% ethanol/70% PBS (pH 7.4) solution supplemented with 10 mM OPT and 10 mM DTT. Protect from light and store at -20°C until use.

5. NAD^{+} (Sigma-Aldrich, N7004) aliquots prepared at 100 mM in water and stored at -20°C .
6. Nicotinamide (Sigma-Aldrich, 72,340) stock solution prepared at 100 mM in water, aliquoted and stored at -20°C .
7. 96-well black polystyrene plates (Corning, 3915) suitable for fluorometry.
8. Plate reader capable of reading fluorescence (Ex/Em = 413/476).
9. 37°C incubator.
10. Orbital shaker

2.3 LM/MS/MS Metabolomics

1. Prepare all solutions and buffers using Milli-Q water.
2. Methanol: HPLC-grade 100% methanol (Sigma-Aldrich).
3. Standard curve of NAD^{+} and its metabolites:
 - Obtain NAD^{+} , NMN, NAMN, ADP, ATP, NADH, NAAD, NADP^{+} , NADPH, NA, NAM, 1-methyl NAM, and NR (Sigma-Aldrich).
 - Prepare a 50 mM solution for each metabolite in water (*see Note 10*).
 - Mix all stock solutions and add water to make a solution containing 200 μM of each metabolite.
 - Perform serial dilutions in water to make standard solutions at the following concentrations: 0, 0.1, 0.2, 0.6, 2, 6, 20, 60, and 200 μM .
4. Fleischmann's active dry yeast extract (Icon Isotopes, Summit, New Jersey) contains ^{13}C isotopes of all metabolites with a sugar moiety.
5. Isotopically enriched internal standards:
 - $^2\text{H}_4$ -NAM, $^{13}\text{C}_5$ -adenosine, adenosine-3',5'-cyclic- $^{13}\text{C}_5$ -monophosphate, and adenosine 5'- $^{13}\text{C}_5$ -monophosphate (Toronto Research Chemicals).
 - $^{15}\text{N}_5$ -ADP and adenosine $^{13}\text{C}_{10}$ $^{15}\text{N}_5$ 5'-triphosphate (Sigma-Aldrich).
6. Speed vacuum.
7. Alkaline separation chromatography reagents:
 - Solvent A1: 7.5 mM ammonium acetate with 0.05% (v/v) ammonium hydroxide.
 - Solvent B1: 0.05% (v/v) ammonium hydroxide in acetonitrile.

- Porous graphitic carbon reversed phase material, Hypercarb LC-MS/MS column (1 mm × 100 mm) (Thermo Fisher) (see **Note 11**).
- 8. Acid separation chromatography reagents (see **Note 12**):
 - Solvent A2: 10 mM ammonium acetate with 0.1% formic acid.
 - Solvent B2: 0.1% formic acid in acetonitrile.
 - Hypercarb LC-MS/MS column (2.1 mm × 100 mm) (Thermo Fischer).
- 9. LC-MS/MS instrument: Thermo Scientific UPLC-MSD assembly consisting of an Accela AS injector, Accela UPLC pump, and a TSQ Vantage bench-top mass spectrometer (Thermo Scientific) equipped with a heated electrospray probe (HESI)
- 10. Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fischer, 23200) or the BCA protein quantification assay (Thermo Fisher, 23227).

3 Methods

3.1 Etheno-NAD⁺ Assay

3.1.1 Reagent Preparation

1. Plan the experimental layout. See Fig. 2 for an example layout to generate a dose-response curve with an inhibitor. Include an etheno-NAD⁺ only control. If using an enzyme inhibitor, also include an etheno-NAD⁺ with inhibitor (no enzyme) control, and a no-inhibitor control.
2. If using recombinant enzymes in vitro:
 - Prepare recombinant enzyme on ice. Dissolve it in assay buffer to 3× the desired concentration for the final reaction. For example, CD38 should be prepared at a concentration of 0.3 ng/μL, for a final reaction concentration of 0.1 ng/μL (see **Note 1** for other enzymes).
 - If using cell or tissue lysates:
 - Collect and homogenize the samples in NETN lysis buffer (or an alternative buffer that will lyse the cells but preserve enzyme activity). The specific method for collection/homogenization will depend on the type of sample.
 - Spin the samples in a refrigerated centrifuge at maximum speed for 10 min to remove insoluble material.
 - Quantify the protein concentration in the supernatants using the BCA protein assay. Normalize the concentration across your samples by adding lysis buffer.
3. Dilute the etheno-NAD⁺ 100-fold in cold reaction buffer to a concentration of 600 μM. (The final reaction concentration will be 200 μM.) Each single reaction needs 10 μL of diluted etheno-NAD⁺.

4. If testing an NADase inhibitor, prepare serial dilutions. Dilutions should be prepared in assay buffer to 3× the desired concentration for the final reaction. For example, if the highest concentration of apigenin to be tested is 100 μM , it should be prepared at a concentration of 300 μM (see **Note 2** if using DMSO).
5. Allow all reagents to briefly warm up to room temperature.

3.1.2 Perform the Assay

1. Add 10 μL recombinant enzyme/sample lysate to the bottom of each well of the 96-well plate to be used. For the etheno-NAD⁺ only control and the etheno-NAD⁺ + inhibitor control wells, instead add 10 μL reaction buffer.
2. Add 10 μL of inhibitor solutions to their respective wells. Add 10 μL of reaction buffer to wells without inhibitor. Wait 10 min to allow the inhibitors to interact with the recombinant enzyme/sample lysate.
3. As quickly as possible, add 10 μL of etheno-NAD⁺ to the bottom of each well (preferably with a multichannel pipette). Pipette up and down once when adding to ensure mixing.
4. Immediately place the 96-well plate into the plate reader. Read fluorescence (Ex/Em = 310/410) frequently, at least once per minute. Continue the reading for up to 1 h.

3.1.3 Analysis

1. To determine the NADase activity, calculate the slope of the curve for each well (AFU/min). Include only data points that are representative of the initial reaction rate, when the curve is linear (see **Note 3**).
2. To remove background, subtract the average slope from the etheno-NAD⁺ only control from all wells. If necessary, subtract the average slope of the etheno-NAD⁺ inhibitor control from all wells with inhibitor present (scaled the inhibitor concentration).
3. Use GraphPad Prism (recommended), Microsoft Excel, or similar software to plot the relative NADase activity. If performing a dose response with an inhibitor, GraphPad Prism can be used to calculate the inhibitor's IC₅₀.

3.2 PNC1 Assay

3.2.1 Setup and Reaction

1. Thaw the nicotinamide stock solution on ice and dilute to make 0, 5, 10, 20, 30, 40, and 50 μM standards. Pipette 1 μL of each standard into its respective well of a 96-well plate. (The final nicotinamide concentrations are 100-fold diluted.)
2. Apply 100 μL of the reaction buffer with 1 μg yPNC1 to each standard well and mix by pipetting.
3. Prepare a master-mix on ice. The volume should be enough for assaying two times the number of samples in triplicates, as the extra set will be used to account

for background fluorescence. Each single reaction needs the following: acetylated peptide substrate (for sirtuin reactions) (10–30 μ M), yPnc1 enzyme (1–2 μ g), NAD-consuming enzyme (1–2 μ g for SIRT1) (*see Note 5*) and reaction buffer (100 μ L minus the volume of other components). Mix by gentle vortexing.

4. Divide the master-mix into two tubes. Add NAD^+ (75–200 μ M) to one tube, and add an equal volume of distilled water to another tube (*see Note 6*). Mix again by gentle vortexing.
5. Add 100 μ L of the master-mix to all wells. (All samples should be performed in triplicate.)
6. Test compounds (*see Note 7*), such as enzyme activators or inhibitors, can be added into both with and without NAD^+ wells at this stage. Add equal volume of solvent (e.g., DMSO) into control wells (*see Note 8*). Mix by pipetting.
7. Gently tap the plate to mix the solutions, and cover with a plate sealer. Incubate at 37 °C on a plate shaker with gentle agitation for 1 h (*see Note 9*).

3.2.2 Development

1. Warm OPT Developer Reagent at 42 °C for 15 min (avoid light). Vortex if precipitation of DTT is observed.
2. After the incubation period, quickly add 100 μ L of OPT Developer Reagent into each well under low light with multichannel pipette. Gently tap to mix the solutions, cover with a plate sealer and wrap with aluminum foil. Incubate at room temperature for 1 h on a plate shaker with gentle agitation.
3. Read the fluorescence on a plate reader with filters set to excitation $\sim$ 420 ($\pm$ 10) nm and emission $\sim$ 460 ($\pm$ 20) nm. Both 0.1 and 1 s read times work well.

3.2.3 Analysis

1. Use the nicotinamide standards to plot a standard curve of fluorescence intensity versus nicotinamide concentration.
2. Calculate the net fluorescence for each reaction condition by subtracting the mean fluorescence of the background control reactions (no NAD^+) from the experimental reaction (with NAD^+), $F_{\text{corrected}} = F_{+\text{NAD}} - F_{-\text{NADcontrol}(\text{mean value})}$.
3. Net fluorescence can be converted into amounts of nicotinamide using the linear equation generated from the standard curve. More nicotinamide produced during the reaction represents higher enzyme activity.

3.3 NAD Metabolomics

3.3.1 Extraction from Tissue

1. Collect tissue samples, rinse in ice-cold PBS (*see Note 13*), and remove excess PBS using a Kim wipe. Snap-freeze tissue samples in liquid nitrogen and store at -80 °C until extraction.

2. Weigh tissue samples when commencing metabolite extraction.
3. Add 500 μ L of cold methanol per 10 mg of tissue to a 10 mL Falcon tube on ice.
4. Transfer tissue sample into the tube and keep on ice. Homogenize the frozen tissue sample in methanol using a Polytron homogenizer or TissueLyser (Qiagen) (*see Note 14*).
5. Centrifuge the homogenate at 15,000 rpm at 4 °C for 10 min.
6. Carefully remove the supernatant and transfer to a new sample tube.

3.3.2 Extraction from Cultured Cells

1. Culture cells in correctly supplemented media (*see Note 15*).
2. Aspirate culture media and wash once with ice-cold PBS.
3. Add 500 μ L of prechilled methanol per well for a 6-well plate. (Scale based on surface area for larger plates.)
4. Harvest the cells using a cell scraper and transfer the cell lysate into a 1.5 mL Eppendorf tube on ice (*see Note 13*).
5. Homogenize the cells using a syringe with a 23–26 gauge needle.
6. Centrifuge the homogenate at 15,000 rpm at 4 °C for 10 min.
7. Carefully remove the supernatant and transfer to a new sample tube.

3.3.3 Sample Setup

1. Make a 1:40 dilution of Fleischmann's extract in 10 mM ammonium acetate.
2. Mix each point on the standard curve and all experimental samples 1:1 with diluted Fleischmann's extract.
3. Add 2 μ L of internal standards to each point on the standard curve and all experimental samples.
4. Dry samples and standards using a speed vacuum.
5. Reconstitute samples in 200 μ L of 100 mM ammonium acetate.
6. Transfer reconstituted samples and standards into 200 μ L LC/MS-MS glass vials and capped before analysis.

3.3.4 Liquid Chromatography

1. Alkaline separation for detection of eight metabolites (NMN, NAMN, ADP, ATP, NAD⁺, NADH, NAAD, NADP⁺) (*see Note 16*):
 - Column: 1 mm $\times$ 100 mm Hypercarb.
 - Flow rate: 0.08 mL/min.
 - Column temperature: 60 °C.

- Equilibrate the column for 20 min with solvent A1 before the first injection.
 - After sample injection, apply solvent B1 on a gradient.
2. Acid separation for detection of three metabolites (NA, NAM, and NR) (*see Note 16*):
- Column: 2.1 mm × 100 mm Hypercarb.
 - Flow rate: 0.2 mL/min.
 - Column temperature: 60 °C.
 - Equilibrate the column with solvent A2 before the first injection.
 - After sample injection, apply solvent B2 on a gradient.

3.3.5 Analysis

1. To confirm the identity of the NAD⁺ metabolite peaks, the results from Q1 ion scanning can be compared to MS/MS spectra from online databases Metlin (<http://metlin.scripps.edu/>) and Massbank (<http://www.massbank.jp/?lang=en>). The transitions are shown in Table 1. Peak areas can be integrated using Thermo Scientific Xcalibur™ software (version 2.2, 2011).
2. To quantify metabolites in pmol units, compute the ratio of the integrated peak area for each isotopically labeled metabolite to the peak area of its unlabeled version, for all samples in the standard curve. Interpolate the concentration of each isotopically labeled metabolite (*see Note 17*). By then computing the ratios of the peak areas of the unlabeled vs. labeled metabolites in the experimental samples, pmol concentrations of unlabeled metabolites can be inferred.
3. Normalize the metabolite concentrations to the amount of protein present in the tissue or cell culture sample using the Bradford or BCA protein assays or to the number of cells.

4 Notes

Ethno-NAD⁺ Assay

1. The NADase activity of various enzymes can differ drastically. For example, CD38 has >100 fold the activity of BST1. The enzyme concentration and the length of observation time for the reaction may need to be adjusted depending on this activity.
2. If your inhibitor is first dissolved in DMSO, keep the DMSO concentration the same in all samples. For example, if apigenin is first dissolved at 6 mM in 100% DMSO and then diluted in reaction buffer to 300 μM at 5% DMSO, all serial dilutions should be made in reaction buffer with 5% DMSO. Reaction buffer with 5% DMSO would also be added to the controls without inhibitor.

3. Many plate readers can calculate the slope of the curve for each well. This is recommended, as exporting the raw data and calculating the slope elsewhere can be unwieldy. If this must be done, the use of Pivot tables in Excel or data analysis software such as MATLAB or R is recommended.

PNC1 Assay

4. The peptide length is usually 5–15 amino acids. To reduce background fluorescence, shorter peptides or peptides with fewer aromatic groups are preferred. Also, peptides with hydrophobic groups adjacent to the acetyl-lysine are recommended for STAC-mediated SIRT1 activation [19].
5. For enzymes that have lower activity than SIRT1, the protein amount added should be increased (e.g., SIRT6).
6. NAD⁺ could exhibit fluorescence at high concentrations (>200 μM). If high NAD⁺ concentrations are required, the background reaction formulation can be altered. Instead of not adding NAD⁺, background reactions can include NAD⁺ but exclude the enzyme or use the corresponding non-acetylated peptide.
7. Test compounds should not alter yPnc1 activity or diminish the fluorescence signal. This can be discerned by incubating with a nicotinamide standard curve in this assay.
8. DMSO may inhibit the enzyme activity and lower the signal. Therefore, adding equal amount of DMSO in the same concentration to control wells is important. Typically <4% DMSO in the final reaction is desirable.
9. Longer incubation times may be needed if: (1) the enzyme has lower activity; (2) the activity on the assayed substrate is weak; or (3) the nicotinamide standard curve doses are very high.

NAD⁺ Metabolomics

10. β-Nicotinamide adenine dinucleotide hydrate (Sigma-Aldrich, N1511) should be used to make NAD⁺ standard solution. It may be difficult to dissolve NAD⁺ powder in water, and it may be vortexed rigorously until dissolved. Aliquots of NAD⁺ and its metabolites should be stored in –80 °C freezer. Avoid multiple freeze/thaw cycles with stock solutions. Frozen stock solutions of NAD⁺ metabolites are stable at –80 °C for at least 1 year.
11. Column selection is critical when running LC-MS/MS methodology. The Hypercarb column is suited for measuring NAD⁺ and its metabolites in terms of accuracy, reliability, and reproducibility [20].
12. Formic acid is a very strong and corrosive. It should be handled with care. It is recommended that the solution is prepared in the fume hood and using personnel protective equipment.
13. Tissue/cellular collection should be done as rapidly as possible to prevent degradation of the NAD⁺ metabolites.

14. Use a homogenizer in the fume hood and wear personal protective equipment, including lab coat and safety goggles.
15. Grow cells in 6-well plates or larger for quantification of NAD⁺ metabolites at detectable amounts.
16. The carbohydrate group is absent in NAM, NA, and NR and cannot be labeled by a heavy-labeled glucose. Therefore, NAM, NA, and NR are best quantified using an acidic separation with NAM and NA and ²H₄-NAM as internal standard. This method allows the quantification of eight metabolites using alkaline separation and three metabolites in an acidic separation.
17. Samples are mixed with Fleischmann's extract and internal standards to allow for quantification of each metabolite in pmol units. For instance, if a particular metabolite concentration was between the 20 and 60 μM standards (10 μM and 30 μM after the 1:1 dilution) in the Fleischmann's sample, then in a 2.5 μL injection volume there would be 100 pmol of that metabolite (40 is between 2 and 6; 40 * 2.5 = 100).

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Fig. 1.

Overview of the PNC1 assay, with a sirtuin as the NAD^+ hydrolase

		Highest[inhibitor]--->Lowest [Inhibitor](μM)											
0% activity		100% activity											
	1	2	3	4	5	6	7	8	9	10	11	12	
A	EtNAD only*	EtNAD+Inb**	100	10	1	0.5	0.1	0.01	0***				
B	EtNAD only	EtNAD+Inb	100	10	1	0.5	0.1	0.01	0				
C	EtNAD only	EtNAD+Inb	100	10	1	0.5	0.1	0.01	0				
D	EtNAD only	EtNAD+Inb	100	10	1	0.5	0.1	0.01	0				
E	EtNAD only	EtNAD+Inb	100	10	1	0.5	0.1	0.01	0				
F													
G													
H													

*Etheno-NAD only control (10 μ L etheno-NAD + 20 μ L reaction buffer)

**Etheno NAD + inhibitor (at maximum concentration) control (10 μ L etheno-NAD + 10 μ L inhibitor + 10 μ L reaction buffer)

***No inhibitor control (10 μ L etheno-NAD + 10 μ L enzyme/sample + 10 μ L reaction buffer)

Fig. 2.

Example experimental layout to generate a dose-response curve for an NADase inhibitor (Inb)

Table 1Transitions for NAD₊ metabolites using LC-MS/MS

Analyte	Transition (<i>m/z</i>)
NAM	123
² H ₄ -NAM	127
Adenosine (ADO)	266
¹³ C ₅ -ADO	271
NA	124
NMN	335
NAD	664
NaAD	665
NADH	666
NaMN	336
NADP	744
ADP	426
¹⁵ N ₅ -ADP	431
NADPH	746
ATP	506
¹³ C ₁₀ ¹⁵ N ₅ -ATP	521