CTMedChem

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NAD Kit (Cat # NAD001)

Introduction

Nicotinamide adenine dinucleotide (NAD^+), and reduced nicotinamide adenine dinucleotide (NADH) are fundamental common mediators of various biological processes, which are essential for life. These dinucleotides and reduced dinucleotides play critical roles as coenzymes in redox reactions, for instance in the TCA cycle in mitochondria, and therefore mediate energy metabolism and mitochondrial functions. NAD^+ is also a substrate of NAD^+ consuming enzymes including poly(ADP-ribose) polymerases (PARPs), bifunctional ADP-ribosyl cyclases/cyclic ADP-ribose hydrolases, histone deacetylases (e.g. Sir2 family proteins), mono (ADP-ribosyl) transferases (e.g. CD38/157 ectoenzymes), and thus regulates the activities of these enzymes, which are central to life. Furthermore, the level of NAD^+ is considered directly correlated to the passage of time (age), and as a marker of mitochondrial functions and nutrition status, and an indicator of pathological conditions as reduced NAD^+ level has been observed under several pathological conditions such as obesity, diabetes, and etc. Thus knowing the levels of these dinucleotides is especially important. We have developed an NAD^+/NADH kit that can measure both NAD^+ and NADH with three short steps. This kit is easy to use and provides accurate measurements of these dinucleotides.

Materials supplied

Component	Catalogue Number	Store Temperature	Volume (mL)
Extraction Buffer A	NAD01	Room Temp	10
Extraction Buffer B	NAD02	Room Temp	8
Extraction Buffer C	NAD03	Room Temp	1
Standard Buffer A	NAD07	Room Temp	0.15
15 µM NAD^+ Standard	NAD13	-20 °C	0.1
Assay Buffer	NAD08	4 °C	15
Assay Component A	NAD09	4 °C	0.03
Assay Component B	NAD10	4 °C	0.1
Assay Component C	NAD11	4°C	0.2
Reaction Developer	NAD12	-20 °C	2.5

Instrument and supplies required, but not included in this kit

1) Hemocytometry.
2) Vortex Mixer.
3) Sonicator (e.g., UltraSonic Cleanser).
4) pH paper.
5) Black flat bottom 96-well assay plate for fluorometry.
6) Multichannel pipette.

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Procedures

1. Extraction of NAD^+

1) For cell samples, count cells in 10^6 by hemocytometry. For animal tissue samples, grind tissue at low temperature (in liquid nitrogen) and weigh in mg (20–70 mg) before next step. For mitochondria, isolate mitochondria, and measure protein concentration. Total protein content in mitochondria should be at least 100 µg to provide solid measurements. Save lysate from last pelleting step for additional protein and NAD^+ measurements.
2) Treat cell pellets, tissue samples or isolated mitochondria with ice-cold 50–100 µL of Extraction Buffer A.
3) Vortex for 30 s, sonicate for 5 min in ice bath. Then vortex again for 30 s, sonicate for 5 min. (Repeat for total of four cycles).
4) Spin down acid-treated samples to remove insolubles. Collect supernatants and transfer to new tubes.
5) Neutralize the supernatants with Extraction Buffer B followed by Extraction Buffer C (pH = 9) to pH = 7. Use pH paper to determine the pH of samples during neutralization. Caution: add Extraction Buffer B in small volume to avoid overshoot pH. Extraction Buffer C is used to fine tune pH to 7.0. Repellet samples.
6) Neutralized samples can be immediately used for the assay to determine NAD^+ concentration. *Samples can also be stored at -20 or -80 °C for assay at later time.*

2. Assay

Attention: Get plate reader ready before loading samples to the plate, because the assay is very fast and readings should be taken immediately after loading.

1) Prepare complete assay buffer.
   a) Take 14 mL Assay Buffer (Catalogue #: NAD08).
   b) Add 17.5 µL Component A, 87.5 µL Component B and 140 µL Component C.
   c) Mix well.

2) Prepare and load standard
   a) Take 40 µL NAD^+ standard at 15 µM and dilute it to 7.5 µM.
   b) To each well add Standard Buffer (A) and/or NAD+ standard. According to the following table to make the final concentrations of NAD^+ standard in 150 µL as 0, 50, 100, 250, 500, 750 and 1000 nM. Duplicate is recommended

Final [NAD^+] (nM)	Volume of Standard Buffer (A) (µL)	Volume of NAD^+ Standard at 7.5 µM (µL)	Volume of NAD^+ Standard at 15 µM (µL)
0	10	0	
50	9	1	
100	8	2	
250	5	5	
500	0	10	
750	2.5		7.5
1000	0		10

3) Load samples to the plate.
   To each well load 10 µL of sample. Duplicate is recommended.
4) Add 120 µL of complete assay buffer made in Step 1.
5) Add 20 µL of Reaction Developer.
6) Read plate.
   Immediately put the 96-well plate onto a plate reader to record fluorescence at 530 nm excitation and 580 nm emission. Record readings every 25 s for 15 min.

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5. Data Analysis

1) Plot the fluorescence at each time point to obtain slopes, fluorescence versus time (Fig. 1A).
2) Make a NAD^+ standard curve by plotting NAD^+ slope value versus NAD^+ concentration (Fig. 1B) and generate regression, y = 0.1226x + 1.8239.
3) For samples, obtain the slope value of each well and calculate [NAD^+] using the regression equation, for example, $[NAD^+]_{(sample)}$ = ($Slope_{(sample)}$ – 1.8239)/0.1226.
4) Then convert the NAD^+ concentration in each well to the original NAD^+ concentration in samples by correction for dilutions. For example, if 10 µL of sample is used for each well reaction of 150 µL, then the original sample NAD^+ concentration (in pmol/µL) is: NAD^+ concentration in well * 150/10 = 15 * NAD^+ concentration obtained in step 4.

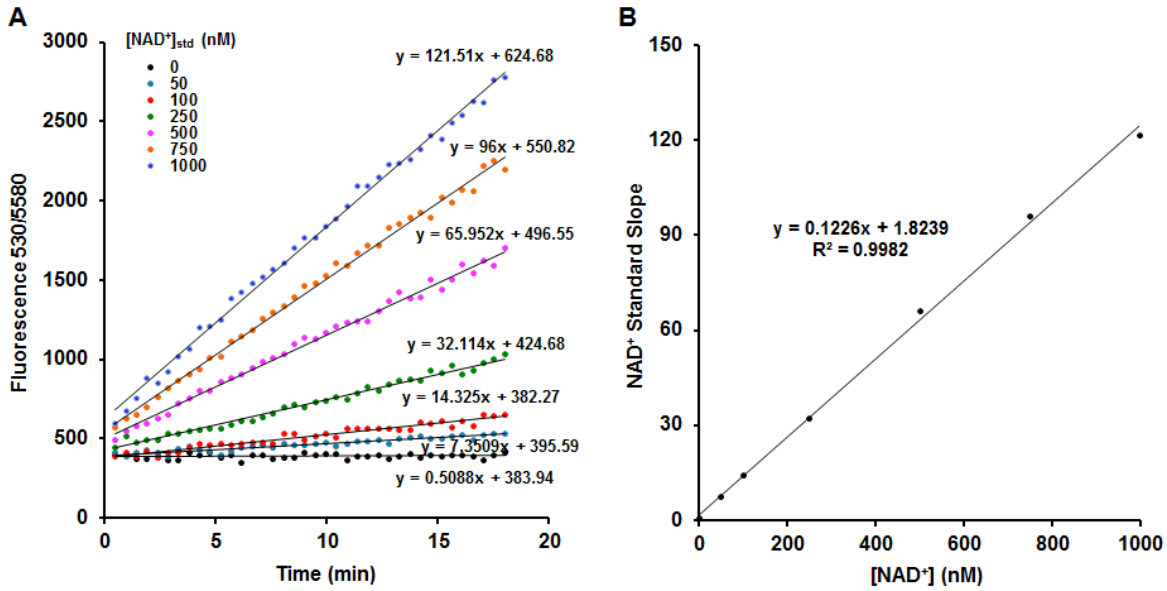

Fig. 1. NAD^+ standard plots

Technical tips

1. For animal tissue samples, tissues need to be finely ground in liquid nitrogen before any treatment.
2. As this assay is a time-sensitive assay, Reaction Developer ideally should be added to each well at the same time to initiate reaction.
3. All the sample values should be in the range of the standard curve. If it is out of range, adjust the sample amount by diluting or adding more samples.