CTMedChem

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NADH Kit (Cat # NADH 001)

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 D	NAD04	Room Temp	10
Extraction Buffer E	NAD05	Room Temp	8
Extraction Buffer F	NAD06	Room Temp	1
Standard Buffer A	NAD07	Room Temp	0.15
NADH Standard	NAD14	-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 NADH

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 **µ**g to provide solid measurements. Save lysate from last pelleting step for additional protein and NADH measurements.
2) Treat cell pellets, tissue samples or isolated mitochondria with ice-cold 50–100 µL of Extraction buffer D (50 mM NaOH/1 mM EDTA).
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 three cycles).
4) Incubate at 60 °C for 30 min, cool on ice for 5 min. Spin down samples to remove insolubles. Collect supernatants and transfer to new tubes
6) Neutralize the supernatants with Extraction Buffer E and Extraction Buffer F to pH 7.0.
7) Neutralized samples can be used for the following cycling assay to determine NADH 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 10 µL NADH standard at concentration of 7.5 µM and dilute it to 1.5 µM.
   b) To each well add Standard Buffer (A) and/or NADH standard. According to the following table to make the final concentrations of NADH standard in 150 µL as 0, 10, 20, 50, 100, 250 and 500 nM. Duplicate is recommended

Final [NADH] (nM)	Volume of Standard Buffer (A) (µL)	Volume of NADH Standard at 1.5 µM (µL)	Volume of NADH Standard at 7.5 µM (µL)
0	10	0	
10	9	1	
20	8	2	
50	5	5	
100	8		2
250	5		5
500	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 NADH standard curve by plotting NADH slope value versus NADH concentration (Fig. 1B) and generate regression, y = 0.2497x + 1.7417.
3) For samples, obtain the slope value of each well and calculate [NADH] using the regression equation, for example, $[NADH]_{(sample)} = (Slope_{(sample)} – 1.7417)/0.2497$.
4) Then convert the NADH concentration in each well to the original NADH 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 NADH concentration (in pmol/µL) is: NADH concentration in well * 150/10 = 15 * NADH concentration obtained in step 4.

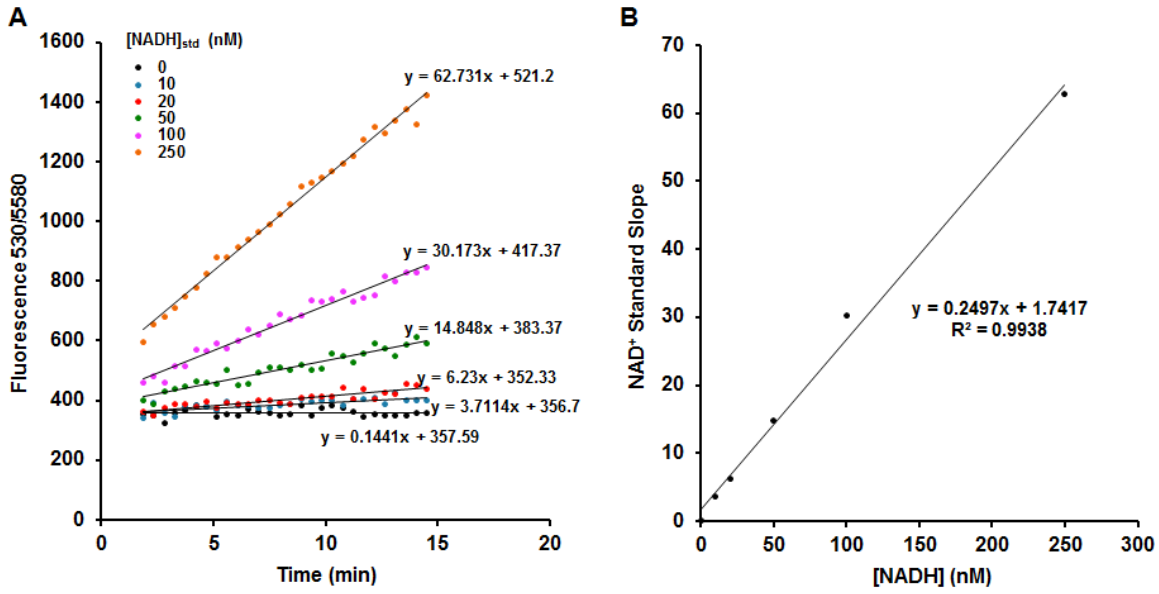

Fig. 1. NADH standard plots

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.