Product Manual

NAD+/NADH Assay Kit (Colorimetric)

Catalog Number

MET-5014 **100 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Nicotinamide adenine dinucleotide (NAD) is a complex organic molecule found in all living cells. NAD consists of two nucleotides, adenine and nicotinamide, connected through phosphate groups. NAD exists as an oxidized form (NAD^+) and a reduced form (NADH). In cells, NAD^+ acts as an electron acceptor, becoming reduced from other molecules to form NADH. The resulting NADH can then act as a reducing agent, donating electrons. An example of this occurs when reduced compounds, such as glucose and fatty acids, become oxidized and transfer energy via electrons to NAD^+ to form NADH. This electron transfer chemistry is observed in glycolysis and the citric acid cycle. In eukaryotic cells, NADH electrons made in the cytoplasm are transported to mitochondrial NAD^+ by mitochondrial shuttles such as the malate-aspartate shuttle. The mitochondrial NADH is then oxidized by the electron transport chain that pumps protons across a membrane, generating ATP through a process known as oxidative phosphorylation.

In addition to electron transfer, NAD^+ and NADH are also used as enzyme substrates to add or remove posttranslational modifications from proteins, as in the process of ADP ribosylation. NAD^+ is also consumed by NAD-dependent deacetylases known as sirtuins. These enzymes can transfer an acetyl group from their substrate protein to the ADP-ribose of NAD, which then cleaves the coenzyme and releases nicotinamide and O-acetyl-ADP-ribose. Non-eukaryotic DNA ligases, which join two DNA ends, also operate in an NAD^+ dependent fashion by using NAD^+ as a substrate to donate an adenosine monophosphate (AMP) moiety to one of the 5' phosphate DNA ends. The resulting intermediate is then attacked by the 3' hydroxyl group of the other DNA end, forming the final phosphodiester bond. NAD^+ has also been identified as an extracellular signaling molecule. NAD^+ is released from neurons in the large intestine, blood vessels, urinary bladder, neurosecretory cells and from brain synaptosomes. NAD^+ is therefore a novel neurotransmitter that transmits a signal from nerves to effector cells.

Cell Biolabs' NAD^+/NADH Assay Kit is a simple colorimetric assay that measures NAD^+ and NADH present in biological samples such as cell lysates or tissue extracts in a 96-well microtiter plate format. The kit is specific for NAD^+, NADH, and their ratio. The kit will not detect $NADP^+$ or NADPH. Each kit provides sufficient reagents to perform up to 100 assays, including blanks, NAD^+ standards and unknown samples. The total NAD^+/NADH concentrations of unknown samples are determined by comparison with a known NAD^+ standard. Determination of both NAD^+ and NADH requires two separate samples for quantification. NAD^+ and NADH do not need to be purified from samples, but rather can be extracted individually with a simple acid or base treatment prior to performing the assay. The kit has a detection sensitivity limit of approximately 4 nM NAD^+.

Assay Principle

Cell Biolabs' NAD^+/NADH Assay Kit is a convenient quantitative tool that measures NAD^+ and NADH within biological samples. The assay is based on an enzymatic cycling reaction in which NAD^+ is reduced to NADH. NADH reacts with a colorimetric probe that produces a colored product which can be measured at 450 nm. The intensity of the product color is proportional to the NAD^+ and NADH within a sample. A simple acid or base treatment will differentiate NADH from NAD^+ within a sample. Samples and standards are incubated for 1-4 hours and then read with a standard 96-well colorimetric plate reader (Figure 1). Samples are compared to a known concentration of NAD^+ standard within the 96-well microtiter plate format.

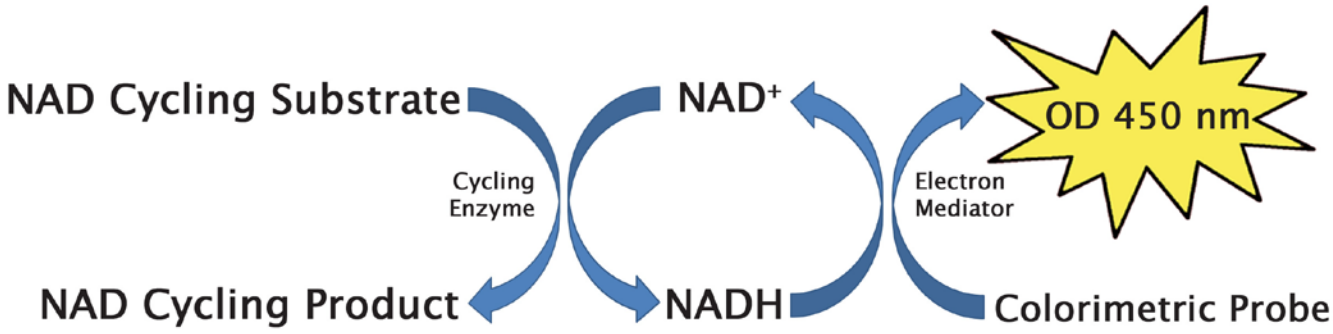

Figure 1. NAD$^+$/NADH Cycling Assay Principle.

Related Products

1. MET-5018: NADP$^+$/NADPH Assay Kit
2. STA-312: Total Glutathione Assay Kit
3. STA-384: Total Cholesterol Assay Kit (Colorimetric)
4. STA-618: Free Fatty Acid Assay Kit (Colorimetric)
5. STA-631: Total Bile Acid Assay Kit (Colorimetric)
6. STA-681: Glucose Assay Kit (Colorimetric)
7. STA-812: Glutathione Reductase Assay Kit
8. XPX-5006: Monoamine Oxidase Assay Kit (Colorimetric)

Kit Components

Box 1 (shipped at room temperature)

1. Colorimetric Probe (Part No. 50181C): One 1.0 mL amber tube
2. NAD Cycling Substrate (Part No. 50142B): One 200 µL tube
3. Assay Buffer (Part No. 50184B): One 25 mL bottle
4. Extraction Buffer (10X) (Part No. 50185B): One 10 mL bottle

Box 2 (shipped on blue ice packs)

1. NAD Cycling Enzyme (Part No. 50144D): One 50 µL amber tube
2. NAD$^+$ Standard (Part No. 50141D): One 50 µL amber tube of a 20 mM NAD$^+$ solution

Materials Not Supplied

1. Distilled or deionized water
2. 0.1 N NaOH

3. 0.1 N HCl
4. 10 kDa molecular weight cutoff (MWCO) centrifuge spin filter (e.g. Amicon Ultra 0.5mL)
5. 1X PBS
6. 0.5 N H_2SO_4
7. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
8. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
9. Standard 96-well clear microtiter plate and/or clear cell culture microplate
10. Multichannel micropipette reservoir
11. Spectrophotometric microplate reader capable of reading in the 450 nm range.

Storage

Upon receipt, store the NAD Cycling Enzyme and NAD^+ Standard at -80°C. Store the Colorimetric Probe at -20°C. Store the remaining components at 4°C.

Preparation of Reagents

- 1X Extraction Buffer: Dilute the stock 10X Extraction Buffer 1:10 with deionized water for a 1X solution. Stir or vortex to homogeneity.
- NAD Cycling Reagent: Prepare an NAD Cycling Reagent for the number of assays being tested and just before use. Prepare by diluting the NAD Cycling Substrate 1:25, NAD Cycling Enzyme 1:100, and Colorimetric Probe 1:5 in 1X Assay Buffer. (eg. For 100 assays, combine 200 µL NAD Cycling Substrate, 50 µL NAD Cycling Enzyme, 1000 µL Colorimetric Probe to 3.750 mL of 1X Assay Buffer for a 5 mL total solution). Use the NAD Cycling Reagent the same day as preparation.

Preparation of Samples

These preparation protocols are intended as a guide for preparing unknown samples. The user may need to adjust the sample treatment accordingly. It is highly recommended that all samples should be assayed immediately upon preparation or stored for up to 1 month at -80°C. A trial assay with a representative test sample should be performed to determine the sample compatibility with the dynamic range of the standard curve. High levels of interfering substances may cause variations in results. Samples may be diluted in deionized water as necessary before testing. Run proper controls and account for any sample dilutions. Always run a standard curve with samples.

- Tissue homogenates: Sonicate or homogenize 100 mg tissue sample in 0.5 mL cold 1X Extraction Buffer. Centrifuge at 14,000 rpm for 5 minutes at 4°C to remove insoluble material. Filter the solution with a 10kDa spin filter to deproteinate the sample. Collect flow through. Perform dilutions in cold deionized water. Sample may be tested immediately for total NAD^+/NADH quantification or extracted with acid or base to separate the cofactors. Store unused samples at -80°C for up to 1 month.

- Cell lysates: Culture cells until confluent and harvest. Centrifuge and wash cell pellet with 1X PBS. Centrifuge to pellet cells and remove wash. Resuspend cells at 1-5 x 10^6 cells/mL in 0.5 mL 1X

Extraction Buffer. Homogenize or sonicate the cells on ice. Centrifuge at 14,000 rpm for 5 minutes 4°C to remove debris. Filter the solution with a 10kDa spin filter to deproteinate the sample. Collect flow through. Perform dilutions in cold deionized water. Sample may be tested immediately for total NAD^+/NADH quantification or extracted with acid or base to separate the cofactors. Store unused samples at -80°C for up to 1 month.

Note: Enzymes in tissue and cell sample lysates may deplete NADH rapidly and affect results. Samples should be deproteinized before extracting the cofactors or using within the assay. A spin filter with a 10 kDa cutoff is recommended for efficient and clean separation.

NADH Extraction Procedure:
To measure NADH and destroy NAD^+, add 25 µL of sample to a microcentrifuge tube. Add 5 µL of 0.1 N NaOH and mix thoroughly. Incubate the tube at 80°C for 60 minutes and protected from light. Centrifuge the tube to pool all sample solution. Add 20 µL of 1X Assay Buffer to shift the pH of the sample back to neutral. Vortex to mix and centrifuge to pool sample. Sample pH should be between 6.0 and 8.0; if not, neutralize accordingly with acid or base. Keep sample on ice until assaying.

NAD^+ Extraction Procedure:
To measure NAD^+ and destroy NADH, add 25 µL of sample to a microcentrifuge tube. Add 5 µL of 0.1 N HCl and mix thoroughly. Incubate the tube at 80°C for 60 minutes and protected from light. Centrifuge the tube to pool all sample solution. Add 20 µL of 1X Assay Buffer to shift the pH of the sample back to neutral. Vortex to mix and centrifuge to pool sample. Sample pH should be between 6.0 and 8.0; if not, neutralize accordingly with acid or base. Keep sample on ice until assaying.

Notes:

- *If testing both total NAD^+/NADH and individual cofactors, dilute the total NAD^+/NADH samples 1:2 with Assay buffer to maintain sample dilution consistency.*
- *Avoid samples containing SH groups like DTT, β-mercaptoethanol, or reduced gluathione.*
- *Samples should be close to neutral pH before harvesting. Samples with extremely high or low pH values could fail to yield reliable results.*

Preparation of Standard Curve

Prepare NAD^+ standards immediately before use. First, dilute the stock 20 mM NAD^+ Standard solution 1:200 in deionized water to yield a 100 µM NAD^+ Solution (e.g. add 5 µL of the stock 20 mM NAD^+ Standard to 995 µL of deionized water). Use the 100 µM NAD^+ solution to prepare a series of NAD^+ standards according to Table 1 below. Do not store standard solutions.

Standard Tubes	100 µM NAD^+ Solution (µL)	1X Extraction Buffer (µL)	NAD^+(µM)	NAD^+(pmol/well)
1	5	495	1	50
2	250 of Tube #1	250	0.5	25
3	250 of Tube #2	250	0.25	12.5
4	250 of Tube #3	250	0.125	6.25
5	250 of Tube #4	250	0.063	3.13
6	250 of Tube #5	250	0.031	1.56
7	250 of Tube #6	250	0.015	0.78
8	250 of Tube #7	250	0.008	0.40
9	250 of Tube #8	250	0.004	0.20
10	0	250	0	0

Table 1. Preparation of NAD^+ Standards.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each sample, including unknowns and standards, should be assayed in duplicate or triplicate.
2. Add 50 µL of each NAD^+ standard or unknown sample into wells of a 96-well microtiter plate.
3. Add 50 µL of NAD Cycling Reagent to each well. Mix the well contents thoroughly and incubate for 1-4 hours at room temperature protected from light.

   Note: This assay is continuous (not terminated) and therefore may be measured at multiple time points to follow the reaction kinetics. The assay may be stopped at a desired time point by adding 50 µL 0.5 N H_2SO_4.
4. Read the plate with a spectrophotometric microplate reader at 450 nm.
5. Calculate the concentration of NAD^+/NADH within samples by comparing the sample OD to the standard curve.

Example of Results

The following figures demonstrate typical NAD^+/NADH Assay Kit results. One should use the data below for reference only. This data should not be used to interpret or calculate actual sample results.

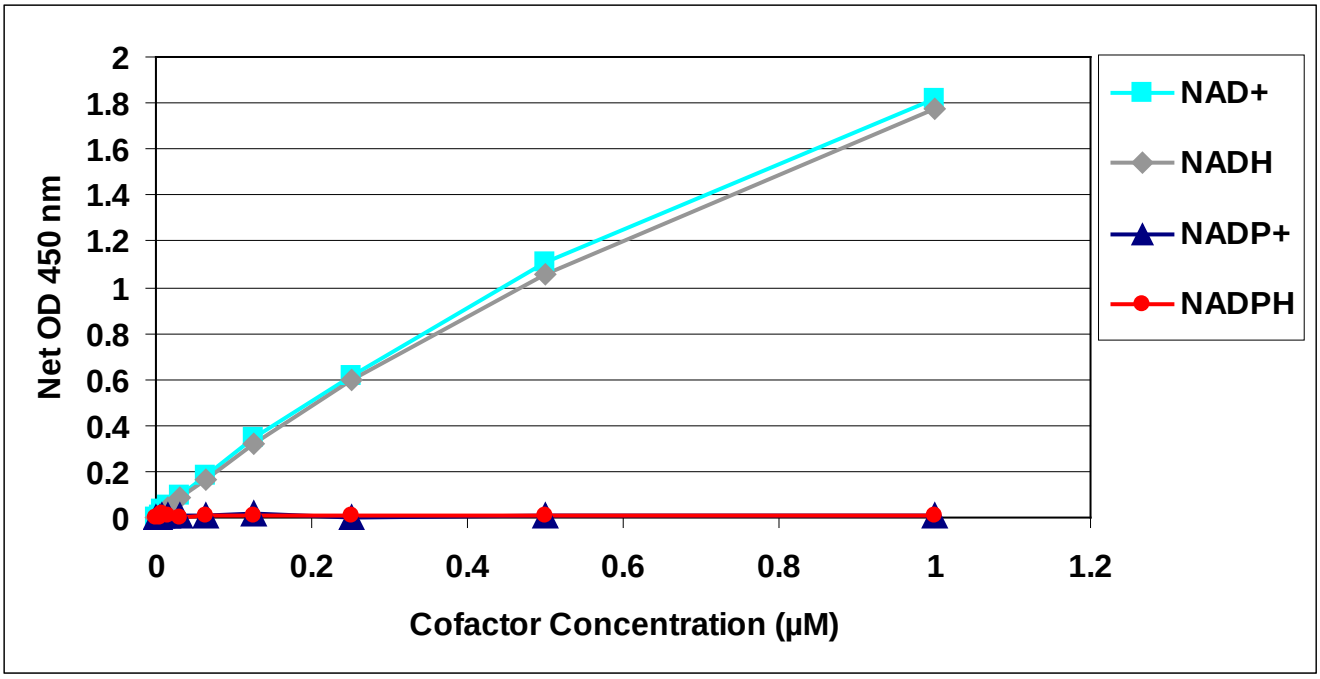

Figure 2: NAD$^+$ Standard Curve and Specificity of Assay for NAD$^+$ and NADH. NAD$^+$, NADH, NADP$^+$, and NADPH were tested in the NAD$^+$/NADH Assay Kit.

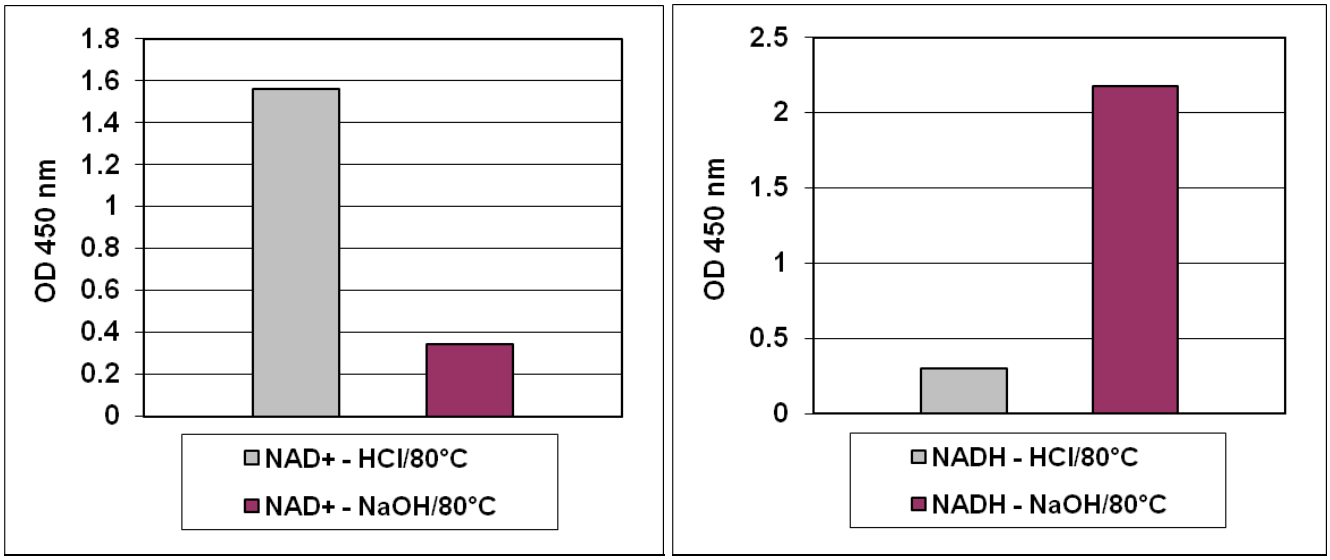

Figure 3: NAD$^+$/NADH Detection. NAD$^+$ and NADH were both tested at 1 µM with the extraction procedure. NAD$^+$ or NADH were incubated for 60 minutes at 80°C with 0.1N HCl or 0.1N NaOH.

Calculation of Results

1. Calculate the average absorbance values for every standard, control, and sample. Subtract the average zero standard value from itself and all standard and sample values. This is the corrected background absorbance. If sample background control value is high, subtract the sample background control value from the sample reading.
2. Plot the corrected absorbance for the NAD^+ standards against the final concentration of the standards from Table 1 to determine the best slope (μM^{-1}). See Figure 2 for an example standard curve.
3. Since all NAD^+ is converted to NADH by the Cycling Reagent, use the standard curve to determine the total NAD^+/NADH concentration in pmoles within the sample. Determine the total concentration of the samples with the equation obtained from the linear regression analysis of the standard curve. Substitute the corrected absorbance values for each sample. Remember to account for dilution factors.

$$\textbf{Total } NAD^+/NADH = \left[\frac{\textbf{Sample corrected absorbance}}{\textbf{Slope}} \right] \textbf{ x Sample dilution}$$

References

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Warranty

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