NAD/NADH Assay Kit

Catalog No.: abx096007

Size: 96 tests

Detection Range: 0.02 µmol/L – 5.0 µmol/L

Sensitivity: 0.02 µmol/L

Storage: Store all components at -20°C in the dark.

Application: For detection and quantification of NAD+ and NADH in tissue and cell samples.

Introduction

NAD (Nicotinamide adenine dinucleotide) is a cofactor for anabolic reactions which requires NADH as a reducing reagent. NAD plays important role in biosynthetic reactions including lipid and nucleic acid synthesis. NADH can be synthesized *de novo* from tryptophan or aspartic acid, or through salvage pathways. In mammals, the main source of NAD is the salvage pathway that recycles nicotinamide.

Abbexa's NAD/NADH Assay Kit is a quick, convenient, and sensitive method for measuring and calculating NAD/NADH concentration. Reduction of NAD to NADH during this reaction and further action of a carrier on NADH transfers electrons to WST-8, which produces a yellow product which has an absorption maximum at 450 nm. The intensity of yellow color is proportional to the total concentration of NAD and NADH, which can then be calculated. For the detection of NADH, heating the sample at 60°C for 30 min degrades NAD, leaving only NADH to reduce WST-8. Measuring the OD value at 450 nm will give the concentration of NADH.

Kit components

1. 96-well microplate
2. Extraction solution: 60 ml x 2 vials
3. Buffer solution: 16 ml
4. Detection reagent: 5 ml
5. Enzyme reagent: 2 vials
6. Standard: 1 vial
7. Plate sealer: 2

Materials Required But Not Provided

1. Microplate reader (450 nm)
2. Ultrapure water
3. PBS (0.01 M, pH 7.4)
4. Pipette and pipette tips
5. Vials/tubes
6. Incubator or Sonicating water bath
7. 10 kDa ultrafiltration tube.

Protocol

A. Preparation of samples and reagents

1. Samples

Isolate the test samples soon after collecting and analyze immediately or aliquot and store at -20°C or -80°C for long term storage. Avoid multiple freeze-thaw cycles.

The following sample preparation methods are intended as a guide and may be adjusted as required depending on the specific samples used.

- **Tissue Homogenates:** Weigh the tissue homogenate. For each 1 g of homogenate, add 9 ml of Extraction Solution. Homogenize by hand, using a mechanical homogenizer, or by ultrasonication. Centrifuge the homogenate at 10,000 x g at 4°C for 10 min. Collect the supernatant, the protein concentration in the supernatant should be determined separately (abx097193). Filter the supernatant through a 10 kDa ultrafiltration tube at 10,000 x g for 10 minutes. Take the filtered supernatant and assay immediately.
- **Cell lysates:** Collect cells and wash with precooled PBS (0.01 M, pH 7.4). Centrifuge at 1000 × g at 4 °C for 10 min. Collect cells (1.5× 10^6 cells) and add Extraction reagent at a ratio of 1 : 0.4. Homogenize by hand, using a mechanical homogenizer, or by ultrasonication. Centrifuge the homogenate at 10,000 x g at 4°C for 10 min. Collect the supernatant, the protein concentration in the supernatant should be determined separately (abx097193). Filter the supernatant through a 10 kDa ultrafiltration tube at 10,000 x g for 10 minutes. Take the filtered supernatant and assay immediately.
- ***Note:*** *Tissue homogenates and cell lysates contain enzymes that can degrade NADH. The filtration step following centrifugation is essential to prevent this.*

Samples should not contain detergents such as SDS Tween-20, NP-40 and Triton X-100, or reducing reagents such as DTT.

It is recommended to carry out a preliminary experiment to determine the optimal dilution factor of samples before carrying out the formal experiment. Dilute samples into different concentrations with Extraction Solution then carry out the assay procedure.

The recommended dilution factor for different samples is as follows (for reference only):

Sample Type	Dilution Factor
HeLa	1
293T cells	1
10% Mouse Kidney tissue homogenate	1
10% Mouse Muscle tissue homogenate	1

Note:

- Fresh samples or recently obtained samples are recommended to prevent degradation that may lead to erroneous results.
- Lysis buffers may interfere with the kit. It is therefore recommended to use mechanical lysis methods at room temperature or on ice for cell lysates and tissue homogenates.
- Filtration of supernatant through a 10 KDa ultrafiltration tube is essential after sample extraction and centrifugation to remove the enzymes.
- This kit shows no significant cross-reactivity with NAD+ and NADH.

2. Reagents

Bring all reagents to room temperature prior to use.

- **Enzyme reagent working solution:** Dissolve a vial of enzyme reagent with 200 µl of ultrapure water. Prepare immediately before carrying out the assay and mix fully. Unused Enzyme reagent working solution can be stored in dark at 4°C for up to 2 days.
- **Reaction working solution:** Dilute Enzyme reagent working solution 1:39 with Buffer solution. Prepare immediately and keep in dark before carrying out the assay and mix fully.
- **250 µmol/L standard solution:** Dissolve a vial of standard with 200 µl of ultrapure water. Prepare immediately before carrying out the assay and mix fully. Unused aliquoted standard solution can be stored in dark at -20°C for up to 2 days.
- **5 µmol/L standard solution:** Dilute 250 µmol/L standard solution 1:49 with Extraction solution. Prepare immediately before carrying out the assay and mix fully.

B. Assay Procedure

1. **Standard curve preparation:** Label 8 tubes with 0, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 µmol/L. Dilute the 5 µmol/L standard solution with Extraction solution to concentrations of 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 µmol/L. The Extraction solution itself serves as the 0 µmol/L (blank) standard.

2. Pretreatment of samples

2.1. NADH only: Heat 0.2 ml of filtered sample supernatant at 60°C for 30 minutes to measure NADH concentration. After heating, allow the samples to cool under running water before carrying out the assay.

2.2. Total NADH and NAD+: Take the filtered sample supernatant directly for assay.

3. Chromogenic Reaction

3.1. Set the Standard and Sample wells.
3.2. Add 20 µl of prepared standards to the Standard wells.
3.3. Add 20 µl of sample supernatant to Sample wells.
3.4. Add 120 µl of reaction working solution to each well.
3.5. Add 40 µl of Detection reagent immediately to each well.
3.6. Mix fully with microplate shaker and incubate at 37°C for 30 minutes.
3.7. Measure the OD values at 450 nm with a microplate reader.

C. Calculation of Results

The standard curve can be plotted as the absolute OD_{450} of each standard solution (*y*) vs. the respective concentration of the standard solution (*x*). A linear fit is recommended for the standard curve ($y = ax + b$). The NAD and NADH concentration of the samples can be interpolated from the standard curve.

1. Total NAD and NADH concentration:

$$[\mathrm{NAD}]_{\mathrm{total}}(\mu\mathrm{mol/g\ protein}) = \frac{\Delta A_1 - \mathrm{b}}{\mathrm{a}} \times \frac{f}{C_P}$$

2. NADH concentration:

$$[\mathrm{NADH}]\ (\mu\mathrm{mol/g\ protein}) = \frac{\Delta A_2 - \mathrm{b}}{\mathrm{a}} \times \frac{f}{C_P}$$

3. NAD concentration

$$[\mathrm{NAD}]\ (\mu\mathrm{mol/g\ protein}) = [\mathrm{NAD}]_{\mathrm{total}} - [\mathrm{NADH}]$$

4. NAD / NADH concentration ratio

$$[\mathrm{NAD}]\ /\ [\mathrm{NADH}]\ (\%) = \frac{[\mathrm{NAD}]_{\mathrm{total}} - [\mathrm{NADH}]}{[\mathrm{NADH}]} \times 100$$

where:

$[\mathrm{NAD}]_{\mathrm{total}}$	Total concentration of NAD and NADH
$[\mathrm{NADH}]$	Concentration of NADH only
$[\mathrm{NAD}]$	Concentration of NAD only
$[\mathrm{NAD}]\ /\ [\mathrm{NADH}]$	Percentage ratio of NAD to NADH
ΔA_1	OD value of the sample for NAD and NADH ($\mathrm{OD_{Sample}} - \mathrm{OD_{Blank}}$)
ΔA_2	OD value of the sample for concentration of NADH ($\mathrm{OD_{Sample}} - \mathrm{OD_{Blankl}}$)
a	gradient of the standard curve (linear fit)
b	y-intercept of the standard curve (linear fit)
f	dilution factor of the sample before carrying out the assay
C_P	concentration of protein in sample supernatant before filtration, g protein/L