**MANUAL**

# NAD+/NADH Assay Kit (Colorimetric)

Catalog #: MA-NAD

ISO 13485:2016

## Introduction

NAD is a complex organic molecule found in all living cells. It exists in two forms: oxidized (NAD+) and reduced (NADH). In cells, NAD+ acts as an electron acceptor, becoming reduced to NADH by accepting electrons from other molecules. In addition to electron transfer, NAD+ and NADH are also used as enzyme substrates to add or remove posttranslational modifications from proteins, such as in the process of ADP ribosylation. Quantitative determination of NAD+/NADH has applications in research related to energy transformation and redox state of cells or tissues.

NAD+/NADH Assay Kit provides a simple, reproducible, and sensitive tool for measuring NAD+/NADH concentration in cell lysates, serum, plasma and other biological liquid samples. The assay is based on an enzymatic cycling reaction. NAD+ is reduced to NADH by alcohol dehydrogenase during the oxidation of ethanol to acetaldehyde. Then NADH reacts with a colorimetric probe that produces a colored product that can be measured at 450 nm. The intensity of the product color is proportional to the NAD+ and NADH within a sample.

## Storage

The entire kit should be stored below -20 °C for up to 6 months from the date of shipment. For prepared reagent storage, see table below.

| Component | Size / Description | Storage After Preparation |
|---|---|---|
| Microplate | A 96-well (12 strips x 8 wells) plate | Room Temperature |
| Assay Buffer | 15 ml | 2–8 °C, 3 months |
| NADH Standard* | 1 vial (Lyophilized) | Aliquot and store at -20 °C, 3 months |
| NAD Cycling Enzyme Mix | 1 vial (Lyophilized) | Aliquot and store at -20 °C, 3 months |
| Colorimetric Probe | 1 vial (Lyophilized) | Aliquot and store at -20 °C, 3 months |
| Ethanol Solution | 1 vial (250 µL) | Room Temperature, 6 months |
| NAD+ Extraction Solution | 10 ml | 2–8 °C, 3 months |
| NAD+ Neutralization Solution | 10 ml | 2–8 °C, 3 months |
| NADH Extraction Solution | 10 ml | 2–8 °C, 3 months |
| NADH Neutralization Solution | 10 ml | 2–8 °C, 3 months |

**Since NAD+ is converted to NADH in an enzyme cycling reaction and is relatively more labile than NADH, this kit provides only NADH.*

## Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Tubes to prepare sample dilutions
4. Ultrapure water
5. 15 ml conical tubes

## Sample Tips and General Considerations

Numerous extraction methods can be used to isolate NAD+ and NADH. The following protocols for NAD+ and NADH have been shown to work with a number of different mammalian cell lines and are **provided as examples** of suitable methods. If desired, other methods for extraction of NAD+ and NADH may be employed.

**Determination of NAD+ and NADH requires two separate samples: acid extract for NAD+ measurement and alkaline extract for NADH measurement.**

1. For adherent cells, after washing with PBS, trypsinize, harvest, and transfer the cells (1.0-5.0×$10^6$ cells) to microcentrifuge tubes followed by centrifugation at 2,000 rpm for 5 minutes.
   NOTE: Each investigator should optimize the number of cells used per test.

   For non-adherent cells, harvest and transfer the cells (1.0-5.0×$10^6$ cells) to microcentrifuge tubes followed by centrifugation at 2,000 rpm for 5 minutes.
   NOTE: Each investigator should optimize the number of cells used per test.
2. Wash the cells twice with cold PBS by centrifugation at 2,000rpm for 5 minutes.
3. Spin down the cells by centrifugation at 10,000rpm for 1 minute. Remove the supernatant as much as possible by aspiration.

**Acid extract for NAD+ measurement**

1. Vortex the cell pellet gently. Extract cells with 100 µL of **NAD+ Extraction Solution** by vortexing 3-4 times for 1 minute each with equal time intervals or by homogenization using standard techniques (i.e. sonicate 4 times for 5 seconds each on ice). Then, let stand for 30 minutes on ice.
2. Add 100 µL of **NAD+ Neutralization Solution** to the acid extract and mix well by vortexing for neutralization.
3. Centrifuge the neutralized cell extract at 15,000 rpm for 5 minutes at 4°C. Transfer the supernatant to a new microcentrifuge tube. The final pH of the supernatant should be 7.5-8.5 (use pH paper to test 1 µL of sample). Ensure that the pH is within this range. If it is not, adjust to pH 7.5-8.5 using either **NADH Neutralization Solution** or **NAD+ Neutralization Solution**.
4. Keep the tube of the cell extract for NAD+ measurement on ice.

**Alkaline extract for NADH measurement**

1. Vortex the cell pellet gently. Extract cells with 100 µL of **NADH Extraction Solution** by vortexing 3-4 times for 1 minute each with equal time intervals or by homogenization using standard techniques (i.e. sonicate 4 times for 5 seconds each on ice). Then, incubate at 60°C for 30 minutes to reduce the viscosity of the samples.
2. Add 100 µL of **NADH Neutralization Solution** to the alkaline extract and mix well by vortexing for neutralization. Then, let stand for at least 5 minutes on ice.
3. Centrifuge the neutralized cell extract at 15,000rpm for 5 minutes at 4°C. Transfer the supernatant to a new microcentrifuge tube. The final pH of the supernatant should be 7.5-8.5 (use pH paper to test 1 µL of sample). Ensure that the pH is within this range. If it is not, adjust to pH 7.5-8.5 using either **NAD+ Neutralization Solution** or **NADH Neutralization Solution**.
4. Keep the tube of the cell extract for NADH measurement on ice.

**NOTE:**

1. If necessary, the cell extracts can be stored at -80°C. Avoid multiple freeze/thaw cycles.
2. Make sure the final preparations of cell extract are at a neutral pH, pH 7.5-8.5.
3. Avoid samples containing -SH groups like DTT, β-mercaptoethanol, or reduced gluathione.

**Preparation of Serum/Plasma samples:**

1. Plasma samples:

Collect blood with an anticoagulant such as citrate, EDTA or oxalate and mix by inversion. Centrifuge the blood at 1000 x g at 4°C for 10 minutes. Collect plasma supernatant without disturbing the white buffy layer. Sample should be tested immediately or frozen at -80°C for storage. The sample may be assayed directly or diluted as necessary in Assay Buffer.

2. Serum samples:

Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Samples should be tested immediately or frozen at -80°C for storage. The sample may be assayed directly or diluted as necessary in Assay Buffer.

## Reagent Preparation

| REAGENT | PREPARATION |
|---|---|
| Microplate | No preparation |
| Assay Buffer | |
| Ethanol Solution | |
| NAD+ Extraction Solution | |
| NAD+ Neutralization Solution | |
| NADH Extraction Solution | |
| NADH Neutralization Solution | |
| NADH Standard | See “Standard Preparation” section |
| NAD Cycling Enzyme Mix | For each vial, quick spin, add 230 µL Ultrapure water, mix thoroughly, and quick spin again. |
| Colorimetric Probe | For each vial, quick spin, add 230 µL Ultrapure water, mix thoroughly, and quick spin again. |
| **Working Solution** | For a single well, add 2 µL reconstituted NAD Cycling Enzyme Mix, 2 µL reconstituted Colorimetric Probe, and 2 µL Ethanol Solution into 84 µL Assay Buffer to make the **Working Solution**. Prepare enough Working Solution for the number of wells being used, (*see recommended volume below, extra volume added for pipetting error*) and **make immediately before use**. Any unused working solution should be discarded. |

| | | extra volume added for pipetting error | |
|---|---|---|---|
| **Working Solution** | **1 well** | **50 wells** | **100 wells** |
| NAD Cycling Enzyme Mix | 2 µL | 115 µL | 230 µL |
| Colorimetric Probe | 2 µL | 115 µL | 230 µL |
| Ethanol Solution | 2 µL | 115 µL | 230 µL |
| Assay Buffer | 84 µL | 4830 µL | 9660 µL |

## Standard Preparation

To prepare a dilution series of NADH Standard in the concentration range of 0 nM – 1000 nM (see Table below),

1. Label 8 microtubes #1 through 8 with the following concentrations: 1000, 500, 250, 125, 62.5, 31.25, 15.625, 0 nM.
2. Pipette 190 µL Assay Buffer into labeled tube #1, and 100 µL Assay Buffer into labeled tube #2 through #8.
3. Briefly spin down the NADH standard tube provided, add 400 µL Assay Buffer to make a 20 µM stock, and mix well.
4. Pipette 10 µL of the 20 µM Stock into tube #1, mix thoroughly, and quick spin to make a 1000 nM NADH Standard.
5. To make the 500 nM standard, pipette 100 µL of tube #1 into the tube labeled #2. Mix thoroughly and quick spin.
6. Repeat this step with each successive concentration, preparing a dilution series as shown in the Table below. Each time, use 100 µL of the prior concentration until the 15.625 nM is reached. Mix each tube thoroughly before the next transfer. Standards should be prepared fresh and used immediately.

| Labeled Tubes | NADH Standard (µL) | Assay Buffer (µL) | NADH Conc. (nM) |
|---|---|---|---|
| 1 | 10 µL of 20uM Stock | 190 µL | 1000 |
| 2 | 100 µL of Tube #1 | 100 µL | 500 |
| 3 | 100 µL of Tube #2 | 100 µL | 250 |
| 4 | 100 µL of Tube #3 | 100 µL | 125 |
| 5 | 100 µL of Tube #4 | 100 µL | 62.5 |
| 6 | 100 µL of Tube #5 | 100 µL | 31.25 |
| 7 | 100 µL of Tube #6 | 100 µL | 15.625 |
| 8 | 0µL | 100 µL | 0 |

## Assay Procedure

Each NADH Standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.
*Since NAD+ is eventually converted into NADH in an enzyme cycling reaction, we use NADH as a standard for both NAD+ and NADH sample measurement.*

1. Add 10 µL of the diluted NADH Standards or NAD+/NADH samples to the 96-well microtiter plate.
2. Initiate the reaction by adding 90 µL Working Solution (See “Reagent Preparation”) to each well. Carefully, shake the plate for a few seconds to mix.
3. Incubate the plate for 30 minutes at room temperature with gentle mixing in the dark.
4. Measure the absorbance at 450nm using a plate reader.

# Calculation of Results

### Subtract the blanks

Average the absorbance value of the blank wells (Standard 0 nM) and subtract this from the absorbance values of all the other wells. These are the corrected absorbance.

### Plotting the standard curves

Make a plot of corrected absorbance of the standards at 450nm as a function of NADH concentration.

### Determination of sample NAD+/NADH concentration

NAD+ (nM) = $\frac{OD_{NAD+\_Sample} - OD_{Blank}}{Slope} \times DF$

NADH (nM) = $\frac{OD_{NADH\_Sample} - OD_{Blank}}{Slope} \times DF$

NAD+/NADH Ratio = $\frac{NAD+\ concentration}{NADH\ concentration}$

$OD_{NAD+\_Sample}$ = Optical density (OD) reading of the NAD+ Sample

$OD_{NADH\_Sample}$ = Optical density (OD) reading of the NADH Sample

$OD_{Blank}$ = Optical density (OD) reading of the Blank (Standard 0 nM)

Slope is from the plot of NADH concentration vs. Absorbance (as shown in Typical data below).

DF = Sample Dilution factor (DF = 1 for undiluted Samples)

*Note:* If the calculated NAD+/NADH concentration of the sample is higher than 1000 nM, dilute the sample in Assay Buffer and repeat the assay.

## A. Typical Data

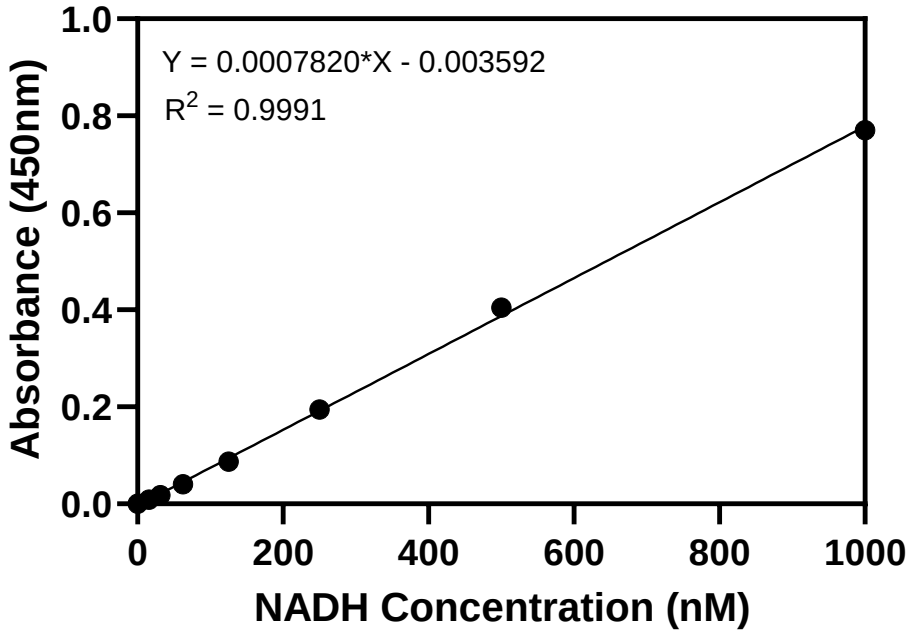


Figure 1. This standard curve is for demonstration only. A standard curve must be run with each assay.

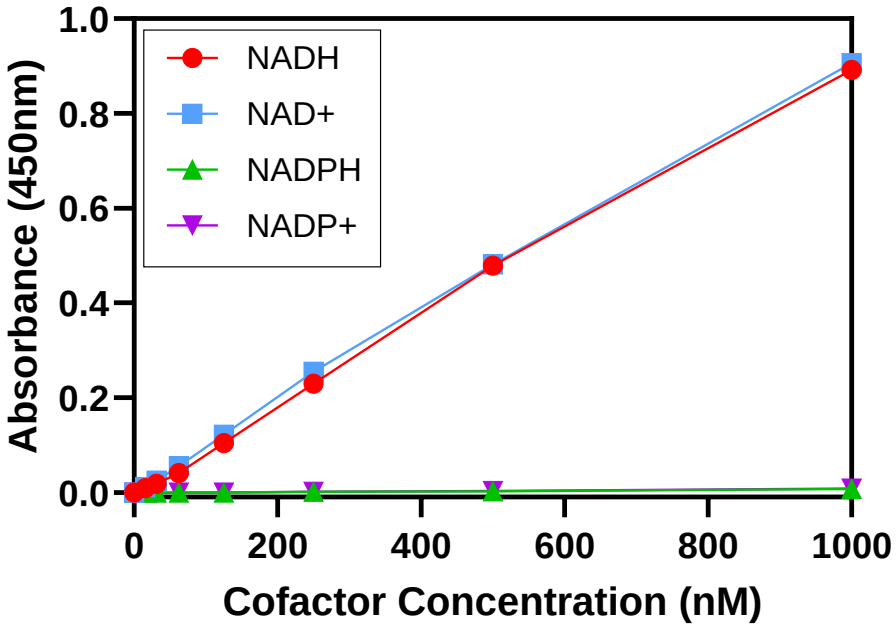


Figure 2. Specificity test. This kit only detects NAD+ and NADH standards. (NAD+, NADP+, and NADPH standards are not provided in this kit)

| | Hela | U251 |
|---|---|---|
| **NAD+ (nM)** | 14205.1 | 7966.9 |
| **NADH (nM)** | 2411.4 | 1317.8 |
| **NAD+/NADH ratio** | 5.89 | 6.05 |

Table 1. Example of NAD+, NADH, NAD+/NADH ratio in cell extracts of Hela and U251 cells ($1.0\times10^6$ cells each).

## B. Sensitivity

The minimum detectable concentration of NAD+/NADH is 5.8 nM.
Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank.

## C. Reproducibility

Intra-assay Precision (Precision within an assay):
To assess intra-assay precision, 16 wells per sample (total of 4 samples) were tested on a single plate. The intra-assay coefficient of variation was found to be 1.0%.

Inter-assay Precision (Precision between assays):
To assess inter-assay precision, 4 samples were tested in separate assays (n=4). The inter-assay coefficient of variation was found to be 4.4%.

This product is for research use only.