Technical Bulletin

NAD/NADH Assay Kit

Catalogue Number MAK468

Product Description

Pyridine nucleotides play an important role in metabolism, so there is interest in monitoring their concentration levels in metabolic systems. Quantitative determination of NAD^+/NADH has applications in research pertaining to energy transformation and the redox state of cells or tissue.

Simple, direct and automation-ready procedures for measuring NAD^+/NADH concentration are useful. The NAD/NADH Assay Kit is based on a lactate dehydrogenase cycling reaction, in which the formed NADH reduces a formazan (MTT) reagent. The intensity of the reduced product color, measured at 565 nm, is proportional to the NAD^+/NADH concentration in the sample. This assay is highly specific for NAD^+/NADH with minimal interference (<1%) by $NADP^+$/NADPH. The assay is a convenient method to measure NAD, NADH, and their ratio.

The linear detection range of the kit is .05–10micromolar (µM). The kit is suitable for NAD^+/NADH concentration and ratio determination in cell or tissue extracts.

Components

The kit is sufficient for 100 colorimetric assays in 96-well plates.

- Assay Buffer 10 mL
  Catalogue Number MAK468A
- NAD Extraction Buffer 12 mL
  Catalogue Number MAK468B
- NADH Extraction Buffer 12 mL
  Catalogue Number MAK468C
- Enzyme A 120 µL
  Catalogue Number MAK468D
- Enzyme B 120 µL
  Catalogue Number MAK468E
- Lactate 1.5 mL
  Catalogue Number MAK468F
- MTT Solution 1.5 mL
  Catalogue Number MAK468G
- NAD Standard (1 mM) 0.5 mL
  Catalogue Number MAK468H

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (such as, multichannel pipettor)
- Spectrophotometric multiwell plate reader
- Clear flat-bottom 96-well plates. Cell culture or tissue culture treated plates are not recommended.
- 1.5 mL microcentrifuge tubes
- Dounce tissue grinder set
  (Catalogue Number D9063 or equivalent)
- Microcentrifuge capable of RCF ≥ 14,000 × g
- Phosphate Buffered Saline (PBS)
  (Catalogue Number P3813 or equivalent)

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C.

Preparation Instructions

Briefly centrifuge small vials prior to opening. Equilibrate reagents to room temperature prior to use.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Notes:

a) The following substances interfere and should be avoided in sample preparation: EDTA (>0.5 mM), ascorbic acid, SDS (>0.2%), sodium azide, NP-40 (>1%) and Tween®20(>1%).
b) For samples containing higher than 100 µM pyruvate, the use of an internal standard is recommended.
c) Determination of both NAD and NADH concentrations requires extractions from two separate samples.

1. For tissue samples, weigh ~20 mg of tissue for each sample, wash with cold PBS.
2. For cell samples, wash cells with cold PBS. Pellet~10^5 cells by centrifugation for 10 minutes at 125×g at room temperature.
3. Homogenize samples (either tissue or cells) in a 1.5 mL Eppendorf tube with either 100µL of NAD Extraction Buffer for NAD determination or 100 µL of NADH Extraction Buffer for NADH determination.
4. Heat extract at 60 °C for 5 minutes.
5. Add 20 µL of Assay Buffer and 100 µL of the opposite extraction buffer to neutralize the extracts.
6. Briefly vortex and then centrifuge the samples at 14,000×g for 5 minutes at room temperature.
7. Transfer 40 µL of each supernatant into separate wells of a clear bottom 96-well plate for NAD/NADH assays.

Standard Curve Preparation

At these concentrations, the standard curves for NAD and NADH are identical. Since NADH in solution is unstable, NAD only is provided as the standard.

1. Prepare a 10 µM NAD Standard by mixing 5 µL of 1 mM NAD Standard and 495 µL of purified water.
2. Prepare NAD standards in 1.5 mL microcentrifuge tubes according to Table 1.

Table 1
Preparation of NAD Standards

Well	10 µM NAD	Purified Water	NAD (µM)
1	100 µL	0 µL	10
2	60 µL	40 µL	6
3	30 µL	70 µL	3
4	0 µL	100 µL	0

3. Mix well and transfer 40 µL of each Standard into separate wells of a clear 96-well plate.

Working Reagent Preparation

Note: This assay is based on an enzyme-catalyzed kinetic reaction. To ensure identical incubation time, addition of Working Reagent should be quick, and mixing should be brief but thorough. Use of a multi-channel pipettor is recommended.

1. Mix enough reagents for the number of assays to be performed. For each well, prepare 90 µL of Working Reagent according to Table 2. Fresh reconstitution is recommended.

Table 2.
Preparation of Working Reagent

Reagent	Working Reagent
Assay Buffer	60 µL
Enzyme A	1 µL
Enzyme B	1 µL
Lactate	14 µL
MTT	14 µL

2. Quickly add 80 µL of Working Reagent to all wells. Tap plate to mix briefly and thoroughly.

Measurement

1. Immediately read optical density at 565 nm for time "zero" (OD_0).
2. Incubate the plate for 15 minutes at room temperature.
3. At the 15-minute incubation point, read the plate again at 565 nm (OD_{15}). Alternatively, monitor the optical density of the plate for 15 minutes in kinetic mode at room temperature at 565 nm.

Results

1. Calculate the ΔOD values of each Standard and Sample well by subtracting OD_0 from OD_{15}.
2. Use the ΔOD values for the Standards and respective concentration to plot the standard curve and determine slope.
3. Calculate the NAD and/or NADH concentration of the Sample using the below formula.

$$\text{NAD(H) } (\mu M) = \frac{\Delta OD_{Sample} - \Delta OD_{Blank}}{\text{Slope } (\mu M^{-1})} \times DF$$

where:

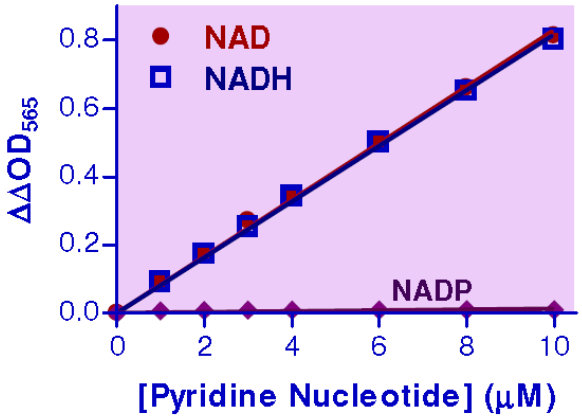
ΔOD_{Sample} = Change in OD values of Sample between zero minutes and 15minutes.

ΔOD_{Blank} = Change in OD values of Blank (Standard #4) between zero minutes and 15 minutes.

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

If the Sample ΔOD values are higher than the ΔOD value for the 10 µM Standard, dilute sample in purified water and repeat the assay. Multiply the results by the dilution factor.

Typical NAD/NADH Standard Curve in 96-well plate assay. The y-axis represents the delta OD of the sample minus the delta OD of the blank, therefore delta, delta OD. Note that NADP was not detected using the protocol.



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