Technical Bulletin

NAD+/NADH Assay Kit

Catalog Number MAK460

Product Description

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

The NAD^+/NADH Assay Kit is based on a lactate dehydrogenase cycling reaction in which the formed NADH reduces a probe into a highly fluorescent product. The fluorescence intensity of this product, measured at λ_{Ex} = 530 nm/λ_{Em} = 585 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 and is a convenient method to measure NAD, NADH, and their ratio. The method has a detection limit of 0.02 µM and is linear up to 1 µM.

The kit is suitable for the determination of NAD and NADH and the evaluation of drug effects on NAD/NADH metabolism in cells, tissue extracts, etc.

Components

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

- Assay Buffer 10 mL
  Catalog Number MAK460A
- Enzyme A 120 µL
  Catalog Number MAK460B
- Lactate 1.5 mL
  Catalog Number MAK460C
- Enzyme B 120 µL
  Catalog Number MAK460D
- Probe 750 µL
  Catalog Number MAK460E
- NAD^+ Standard (1 mM) 0.5 mL
  Catalog Number MAK460F
- NAD^+ Extraction Buffer 12 mL
  Catalog Number MAK460G
- NADH Extraction Buffer 12 mL
  Catalog Number MAK460H

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescent multiwell plate reader
- Black flat-bottom 96-well plates for fluorometric assay. Cell culture or tissue culture treated plates are **not** recommended.
- Microcentrifuge capable of RCF ≥ 14,000 × *g*
- 1.5 mL microcentrifuge tubes
- Phosphate Buffered Saline (PBS) (Catalog 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.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Note: 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%).

For samples containing higher than 100 µM of pyruvate, the use of an internal standard is recommended.

1. For tissues, weigh ~20 mg of tissue for each sample. Wash with cold PBS.
2. For cell samples, wash cells with cold PBS and pellet ~10^5 cells for each sample.
3. Homogenize samples (either tissue or cells) in a 1.5 mL tube with **either** 100 µL of NAD^+ Extraction Buffer for NAD determination **or** 100 µL of NADH Extraction Buffer for NADH determination.
4. Heat extracts at 60 °C for 5 minutes.
5. Add 20 µL of Assay Buffer and 100 µL of the opposite extraction buffer as used in step 3 to neutralize the extracts.
6. Briefly vortex and then centrifuge the samples at 14,000 × *g* for 5 minutes.
7. Use the supernatant for NAD/NADH assays. Determination of both NAD and NADH concentrations requires extractions from two separate samples.
8. Transfer 50 µL of each Sample into separate wells of a black flat-bottom 96-well plate.

Standard Curve Preparation

Note: At these concentrations, the Standard curves for both NAD^+ and NADH are identical. Since NADH in solution is unstable, only NAD^+ Standard is provided.

1. Prepare a 1 µM NAD^+ Standard by mixing 5 µL of the 1 mM NAD^+ Standard with 4995 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	1 µM NAD^+ Standard	Purified Water	NAD^+ (µM)
1	100 µL	-	1.0
2	60 µL	40 µL	0.6
3	30 µL	70 µL	0.3
4	-	100 µL	0

3. Mix well and transfer 50 µL of each Standard into separate wells of the 96-well plate.

Working Reagent

Note: This assay is based on an enzyme-catalyzed kinetic reaction. Addition of Working Reagent should be quick and mixing should be brief but thorough. Use of a multi-channel pipettor is recommended.

Prepare Working Reagent fresh just prior to assay. Mix enough reagents for the number of assays to be performed. For each Standard and Sample well, prepare 57 µL of Working Reagent according to Table 2.

Table 2.
Preparation of Working Reagent

Reagent	Working Reagent
Assay Buffer	40 µL
Enzyme A	1 µL
Enzyme B	1 µL
Lactate	10 µL
Probe	5 µL

Measurement

1. Quickly add 50 µL of Working Reagent to each well and tap plate to mix.
2. Immediately measure the fluorescence intensity at λ_{Ex} = 530 nm/λ_{Em} = 585 nm for time zero (F_0).
3. Protect the plate from light and incubate the plate at room temperature for 10 minutes.
4. Immediately after the 10 minute incubation, measure the fluorescence intensity at λ_{Ex} = 530 nm/λ_{Em} = 585 nm (F_{10}).

Results

1. Calculate the ΔF for each Standard and Sample by subtracting the F_0 value from the F_{10} value.
2. Plot the Standard ΔF values against the Standard concentrations and determine the slope of the Standard curve.
3. Calculate the NAD^+/NADH concentration of the Sample:

NAD^+/NADH (µM) =

$$\frac{\Delta F_{Sample} - \Delta F_{Blank}}{Slope\ (\mu M^{-1})} \times DF$$

where

ΔF_{Sample} = Change in fluorescence intensity values of the Sample

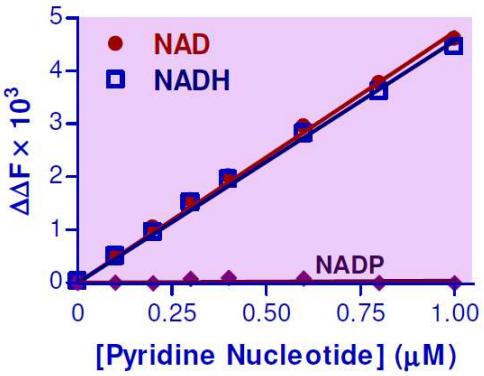
ΔF_{Blank} = Change in fluorescence intensity values of the Blank well (Standard #4)

Slope (μM^{-1}) = Slope of the NAD^+ Standard curve

DF = Dilution factor of the Sample, if required (DF = 1 for undiluted Samples)

Note: If the Sample ΔF values are higher than the ΔF value for the 1 µM standard, dilute the Sample in purified water and repeat the assay. Multiply the result by the dilution factor.

Figure 1.
Typical NAD^+ Standard Curve



References

1. Joe, Y., et al., Cross-talk between CD38 and TTP is essential for resolution of inflammation during microbial sepsis. *Cell Rep.*, **30**, 1063-1076.e5 (2020).
2. Zhang, M., et al., Dysregulated metabolic pathways in age-related macular degeneration. *Sci. Rep.*, **10**, 2464 (2020).
3. Chaurasiya, A., et al. Pathogen induced subversion of NAD^+ metabolism mediating host cell death: a target for development of chemotherapeutics. *Cell Death Discov.*, **7**, 10 (2021).

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