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Product Information

NADP$^+$/NADPH Assay Kit

Catalog Number **MAK312**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Pyridine nucleotides play an important role in metabolism and, thus, there is continual interest in monitoring their concentration levels. Quantitative determination of NADP$^+$/NADPH has applications in research pertaining to energy transformation and the redox state of cells or tissue.

Simple, direct, and automation-ready procedures for measuring NADP$^+$/NADPH concentration are very desirable. This NADP$^+$/NADPH assay kit is based on a glucose dehydrogenase cycling reaction, in which the formed NADPH 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 NADP$^+$/NADPH concentration in the sample. This assay is highly specific for NADP$^+$/NADPH and with minimal interference (<1%) by NAD$^+$/NADH. This assay is a convenient, direct method to measure NADP$^+$/NADPH concentrations and ratio in cell or tissue extracts.

Key Features
Sensitive and accurate – Detection limit of 0.01 μM and linearity up to 1 μM NADP$^+$/NADPH in 96 well plate assay.

Convenient – The procedure involves adding a single working reagent, and reading the fluorescence at time zero and 30 minutes. Room temperature assay.

High-throughput – Can be readily automated as a high-throughput 96 well plate assay for thousands of samples per day.

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

Assay Buffer Catalog Number MAK312A	10 mL
Glucose (1 M) Catalog Number MAK312B	1.5 mL
Probe Catalog Number MAK312C	750 μL
Enzyme A Catalog Number MAK312D	120 μL
Enzyme B Catalog Number MAK312E	120 μL
NADP Standard Catalog Number MAK312F	0.5 mL
NAD(P) Extraction Buffer Catalog Number MAK312G	12 mL
NAD(P)H Extraction Buffer Catalog Number MAK312H	12 mL

Reagents and Equipment Required but Not Provided.
- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorescence assays.
- Fluorescence multiwell plate reader

Precautions and Disclaimer
This product is 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.

Preparation Instructions
Briefly centrifuge vials before opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Storage/Stability
The kit is shipped on ice. Store all components at –20 °C upon receiving.

Procedure
All samples and standards should be run in duplicate. Use ultrapure water for the preparation of standards.

Assay Reaction

1. Sample Preparation – For tissues weigh ~20 mg of tissue for each sample, wash with cold PBS. For cell samples, wash cells with cold PBS and pellet ~10^5 cells for each sample. Homogenize samples (either tissue or cells) in a 1.5 mL tube with either 100 µL of $NADP^+$ extraction buffer for $NADP^+$ determination or 100 µL of NADPH extraction buffer for NADPH determination. Heat extract at 60 °C for 5 minutes and then add 20 µL of Assay Buffer and 100 µL of the opposite extraction buffer to neutralize the extracts. Briefly vortex and spin the samples down at 14,000 rpm for 5 minutes. Use supernatant for $NADP^+$/NADPH assays. Determination of both $NADP^+$ and NADPH concentrations requires extractions from two separate samples.

2. Calibration Curve – Prepare 5,000 µL of 1 µM $NADP^+$ Premix by mixing 5 µL of 1 mM Standard and 4995 µL of ultrapure water. Dilute standard as shown in Table 1.

   Table 1.
   Preparation of $NADP^+$ Standards

#	Premix + Water	NADP (µM)
1	100 µL + 0 µL	1.0
2	60 µL + 40 µL	0.6
3	30 µL + 70 µL	0.3
4	0 µL + 100 µL	0

   Transfer 50 µL of standards into wells of a black flat-bottom 96 well plate.

3. Add 50 µL of sample per well in separate wells.

4. Reagent Preparation – For each reaction well, prepare Working Reagent by mixing 40 µL of Assay Buffer, 1 µL of Enzyme A, 1 µL of Enzyme B, 10 µL of Glucose and 5 µL of Probe. Prepare the Working Reagent just prior to adding to reaction wells.

5. Reaction – Quickly add 50 µL of Working Reagent to each well. Tap plate to mix.

6. Read fluorescence at λ_{ex} = 530 nm/λ_{em} = 585 nm for time "zero" (F_0) and after a 30 minute (F_{30}) incubation at room temperature. Protect plate from light during this incubation.

Notes:
At these concentrations, the standard curves for $NADP^+$ and NADPH are identical. Since NADPH in solution is unstable, only NADP is provided as the standard.

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 multichannel pipettor is recommended.

The following substances interfere with the assay 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%).

Results
Calculation
First compute the ΔF for each standard and sample by subtracting F_0 from F_{30}. Plot the ΔF for each standard and determine the slope. The $NADP^+$(H) concentration of the sample is computed as follows:

$$[\text{NADP(H)}, (\mu\text{M})] = \frac{\Delta F_{SAMPLE} - \Delta F_{BLANK}}{\text{Slope } (\mu\text{M}^{-1})} \times n$$

Where:
ΔF_{SAMPLE} and ΔF_{BLANK} are the change in fluorescence intensity values of the Sample and Blank (STD 4), respectively. Slope is the slope of the standard curve and *n* is the dilution factor (if necessary).

Note: If the sample ΔF values are higher than the ΔF value for the 1 µM standard, dilute sample in ultrapure water and repeat this assay. Multiply the results by the dilution factor.

References

1. Zhao, Z. et al., Comparison of Tissue Preparation Methods for Assay of Nicotinamide Coenzymes. Plant Physiol., **84**, 987-988 (1987).
2. Matsumura, H., and Miyachi, S., Cycling assay for nicotinamide adenine dinucleotides. Methods Enzymol., **69**, 465-470 (1980).
3. Vilcheze, C. et al., Altered NADH/NAD+ Ratio Mediates Coresistance to Isoniazid and Ethionamide in Mycobacteria. Antimicrobial Agents and Chemotherapy, **49**, 708-720 (2005).

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For fluorometric assays, use black plates with clear bottoms
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Reaction Mix before use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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