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Product Information

NAD/NADH Quantification Kit

Catalog Number **MAK037**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Nicotinamide adenine dinucleotide (NAD) is an enzymatic cofactor involved in many redox reactions. NAD functions as an electron carrier, cycling between the oxidized (NAD) and reduced (NADH) forms. In addition to its role in redox reactions, NAD plays critical roles in ADP-ribosylation reactions and as a substrate for sirtuins.

The NAD/NADH Quantification Kit provides a convenient tool for sensitive detection of the intracellular nucleotides, NAD and NADH, and their ratio without the requirement to purify NAD/NADH from samples. This assay is specific for NAD and NADH, and does not detect NADP nor NADPH. NAD_{total} (NAD and NADH) or NADH are quantified in a colorimetric assay (450 nm).

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

NADH/NAD Extraction Buffer Catalog Number MAK037A	50 mL
NAD Cycling Buffer Catalog Number MAK037B	15 mL
NAD Cycling Enzyme Mix Catalog Number MAK037C	1 vl
NADH Developer Catalog Number MAK037D	1 vl
Stop Solution Catalog Number MAK037E	1.2 mL
NADH Standard, (MW 763 g/mole) Catalog Number MAK037F	152.6 µg

Reagents and Equipment Required but Not Provided.
- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader.
- 10 kDa Molecular Weight Cut-Off (MWCO) Spin Filter
- High-quality DMSO (Catalog Number D2650 or equivalent)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

NAD Cycling Buffer – Allow buffer to come to room temperature before use.

NAD Cycling Enzyme Mix – Reconstitute in 220 µL of NAD Cycling Buffer. Aliquot enough NAD Cycling Enzyme mix (2 µL per assay) for the number of assays to be performed in each experiment and freeze the stock solution immediately at –70 °C for future use. The enzymes are stable for up to 2 months when stored at –70 °C after reconstitution.

NADH Developer – Reconstitute in 1.2 mL of water. Mix well by pipetting, do not vortex.

NADH Standard – Reconstitute in 200 µL of DMSO to generate 1 mM solution. Mix well by pipetting. Store at –20 °C.

Storage/Stability
The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

NADH Standards for Colorimetric Detection
Dilute 10 µL of the 1 mM NADH standard with 990 µL of the NADH/NAD Extraction Buffer to prepare a 10 µM standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 10 µM NADH standard into a 96 well plate, generating 0 (blank), 20, 40, 60, 80, and 100 pmole/well standards. Add NADH/NAD Extraction Buffer to each well to bring the volume to 50 µL.
Note: The 10 µM NADH solution is unstable and must be used within 4 hours.

Sample Preparation
Cell samples – Wash cells with cold PBS. Pellet 2×10^5 cells for each assay in a microcentrifuge tube by centrifuging at 2,000 rpm for 5 minutes. Extract cells with 400 µL of NADH/NAD Extraction Buffer by homogenization or freeze/thawing for 2 cycles of 20 minutes on dry ice followed by 10 minutes at room temperature. Vortex for 10 seconds and then centrifuge the samples at 13,000 × *g* for 10 minutes to remove insoluble material. Transfer extracted NAD/NADH supernatant into a labeled tube.

Tissue samples – Wash 20 mg of tissue with cold PBS. Homogenize with 400 µL of NADH/NAD Extraction Buffer in a microcentrifuge tube. Spin the sample at 14,000 rpm for 5 minutes. Transfer extracted NAD/NADH supernatant into a labeled tube.

Note: Cell or tissue lysates may contain enzymes that consume NADH rapidly. Samples should be deproteinized before use in assay by filtering through a 10 kDa cut-off spin filter.

Bring samples to a final volume of 50 µL with NADH/NAD Extraction Buffer.

Assay Reaction
Detection of total NADH and NAD (NAD_{total})
1a. Transfer up to 50 µL of extracted samples in duplicate into a 96 well plate. Bring samples to a final volume of 50 µL with NADH/NAD Extraction Buffer.

For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range.

Detection of NADH only
1b. To detect NADH, NAD must be decomposed before the reaction. Decompose NAD by aliquoting 200 µL of extracted samples into microcentrifuge tubes and heat to 60 °C for 30 minutes in a water bath or a heating block. Cool samples on ice. Quickly spin samples to remove any precipitates. Under these conditions, NAD will be decomposed leaving NADH only. Transfer up to 50 µL of NAD decomposed samples in duplicate into a 96 well plate. Bring samples to a final volume of 50 µL with NADH/NAD Extraction Buffer.

For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range.

2. Set up the Master Reaction Mix according to the scheme in Table 1. 100 µL of the Master Reaction Mix is required for each reaction (well).

Table 1.
Master Reaction Mix

Reagent	Master Reaction Mix
NAD Cycling Buffer	98 µL
NAD Cycling Enzyme Mix	2 µL

3. Add 100 µL of the Master Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 5 minutes at room temperature to convert NAD to NADH (for NAD_{total} determinations).

4. Add 10 µL of NADH Developer into each well. Incubate at room temperature for 1–4 hours. Final incubation time will be dependent on color development. Measure the absorbance at 450 nm (A_{450}). The plate can be read multiple times while the color is developing.

5. The reactions can be stopped by adding 10 µl of Stop Solution into each well and mixing well. The color is stable within 48 hours in a sealed plate after addition of Stop Solution.

Results

Calculations

The background is the value obtained for the 0 (blank) NADH standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate NADH standards to plot a standard curve.

The amount of NADH or NAD_{total} present in the samples (all NAD in the NAD_{total} reaction will have been converted to NADH by the NAD Cycling Enzyme Mix) may be determined from the standard curve.

Note: A new standard curve must be set up each time the assay is run.

Divide the amount of NAD_{total} or NADH by either the cell number or protein concentration of the extract (ng/mL) added to the sample well. The concentration of NAD_{total} or NADH can be expressed in pmole/10^6 cells or ng/mg.

The ratio of NAD/NADH in a sample may be determined by the following equation:

$$\text{ratio} = \frac{NAD_{total} - NADH}{NADH}$$

NAD_{total} = Amount of total NAD (NAD + NADH) in unknown sample (pmole) from standard curve.
NADH = Amount of NADH in unknown sample (pmole) from standard curve

NADH molecular weight: 664.4 g/mole

Example:
NAD_{total} amount = 87.0 pmole
NADH amount = 43.0 pmole

The ratio of NAD/NADH is:

$$\frac{87.0 - 43.0}{43.0} = 1.02$$

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay Not Working	Assay Buffer Ice Cold	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 fluorescence assays, use black plates with clear bottoms. For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Samples were not deproteinized	Use a 10 kDa MWCO spin filter to deproteinize samples
	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	Prepare fresh Master Reaction Mix before each 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	Always 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 Master 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 that readings are in the linear range