

NAD⁺/NADH Quantification Kit

(Catalog #K337-100; 100 reactions; Store kit at -70°C)

I. Introduction:

Assay of nicotinamide nucleotides is of continual interest in the studies of energy transforming and redox state of cells or tissues. AMSBIO's NADH /NAD Quantification Kit provides a convenient tool for sensitive detection of the intracellular nucleotides: NADH, NAD and their ratio. The NAD Cycling Enzyme Mix in the kit specifically recognizes NADH/NAD in an enzyme cycling reaction. There is no requirement to purify NADH/NAD from samples. The reaction specifically detects NADH and NAD, but not NADP nor NADPH. The enzyme cycling reaction significantly increases the detection sensitivity and specificity. NADt (NAD and NADH) or NADH can be easily quantified by comparing with standard NADH.

II. Kit Contents:

Components	K337-100	Cap Code	Part No.
NADH/NAD Extraction Buffer	50 ml	NM	K337-100-1
NAD Cycling Buffer	15 ml	NM	K337-100-2
NAD Cycling Enzyme Mix	1 vial	Green	K337-100-3
NADH Developer	1 vial	Purple	K337-100-4
Stop Solution	1.2 ml	Red	K337-100-5
NADH Standard (MW:763)	152.6 µg	Yellow	K337-100-6

III. NAD/NADH Assay Protocol:

A. Reagent Reconstitution and General Consideration:

- Reconstitute NAD Cycling Enzyme Mix with 220 µl NAD Cycling Buffer. Reconstitute NADH developer with 1.2 ml of ddH₂O. Pipette up and down several times to completely dissolve the pellet into solution (don't vortex). 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 at -70°C after reconstitution.
- Reconstitute NADH standard with 200 µl pure DMSO to generate 1 nmol/µl NADH standard solution.
- Ensure that the NAD Cycling Buffer is at room temperature before use. Keep other enzymes on ice during the assay and protect from light.

B. Sample Preparation:

- For cell samples*, wash cells with cold PBS. Pellet 2 X 10⁵ cells for each assay in a micro-centrifuge tube (2000 rpm for 5 min). Extract the cells with 400 µl of NADH/NAD Extraction Buffer by freeze/thaw two cycles (20 min on dry-ice, then 10 min at room temperature), or homogenization. Vortex the extraction for 10 sec. Spin the sample at 14000 rpm for 5 min. Transfer the extracted NADH/NAD supernatant into a labeled tube.
- For tissue samples*, weight ~20 mg tissue, wash with cold PBS, homogenize with 400 µl of NADH/NAD Extraction Buffer in a micro-centrifuge tube. Spin the sample at 14000 rpm for 5 min. Transfer the extracted NADH/NAD supernatant into a new tube.

Note: Cell or tissue lysates may contain enzymes that consume NADH rapidly. We suggest to remove these enzymes by filtering the samples through 10 Kd molecular weight cut off filters (AMSBIO, Cat # 1997-25) before performing the assay.

C. NADH/NAD Assay Protocol:

- Standard Curve:** Dilute 10 µl of the 1 nmol/µl NADH standard with 990 µl NADH/NAD Extraction Buffer to generate 10 pmol/µl standard NADH (Note: diluted NADH solution is unstable, must be used within 4 hours). Add 0, 2, 4, 6, 8, 10 µl of the diluted NADH standard into labeled 96-well plate in duplicate to generate 0, 20, 40, 60, 80, 100 pmol/well standard. Make the final volume to 50 µl with NADH/NAD extraction buffer.

Samples: To detect total NADt (NADH and NAD), transfer 50 µl of extracted samples into labeled 96-well plate in duplicates. (Note: We recommend performing several different sample dilutions with the Extraction Buffer to ensure the readings fall in the standard curve range).

To detect NADH, NAD needs to be decomposed before the reaction. To decompose NAD, aliquot 200 µl the extracted samples into eppendorf tubes. Heat to 60°C for 30 min in a water bath or a heating block. Under the condition, all NAD will be decomposed, while NADH will still be intact. Cool samples on ice. Quick spin the samples to remove precipitates if precipitation occurs.

Transfer 50 µl of NAD decomposed samples into labeled 96-well plate in duplicates (Note: We recommend performing several different sample dilutions with the Extraction Buffer to ensure the readings fall in the standard curve range).

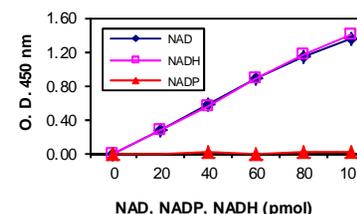
- Prepare a NAD Cycling Mix for each reaction:

NAD Cycling Buffer: 100 µl
NAD Cycling Enzyme Mix: 2 µl

Mix well and add 100 µl of the mix into each well of NADH standard and samples.

- Mix, incubate the plate at room temperature for 5 min to convert NAD to NADH.
- Add 10 µl NADH developer into each well. Let the reaction cycling at room temperature for 1 to 4 hours or longer depend on the reading of OD_{450nm}.
- Read the plate at OD450 nm. The plate can be read multiple times while the color is in developing. The reactions can be stopped by adding 10 µl of Stop Solution into each well and mix well. The color should be stable within 48 hours in a sealed plate after addition of Stop Solution.
- Calculation: Apply the sample readings to NADH standard curve. The amount of NADt or NADH in the sample wells can be calculated, then divide the NADt or NADH amount by the sample amount (e.g. cell number or extract protein amount) you added into the sample wells. The concentration of NADt or NADH can be expressed in pmol/10⁶ cells or ng/mg protein (NADH molecular weight 664.4).

NAD/NADH Ratio is calculated as: $\frac{\text{NADt} - \text{NADH}}{\text{NADH}}$



GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	<ul style="list-style-type: none"> • Use of ice-cold buffer • Omission of a step in the protocol • Plate read at incorrect wavelength • Use of a different 96-well plate 	<ul style="list-style-type: none"> • Buffers must be at room temperature • Refer and follow the data sheet precisely • Check the wavelength in the data sheet and the filter settings of the instrument • Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	<ul style="list-style-type: none"> • Use of an incompatible sample type • Samples prepared in a different buffer • Samples were not deproteinized (if indicated in datasheet) • Cell/ tissue samples were not completely homogenized • Samples used after multiple free-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples 	<ul style="list-style-type: none"> • Refer data sheet for details about incompatible samples • Use the buffer provided in the kit or refer data sheet for instructions • Use the 10 kDa spin cut-off filter or PCA precipitation as indicated • Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope • Aliquot and freeze samples if needed to use multiple times • Troubleshoot if needed, deproteinize samples • Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	<ul style="list-style-type: none"> • Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used 	<ul style="list-style-type: none"> • Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer datasheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	<ul style="list-style-type: none"> • Use of partially thawed components • Pipetting errors in the standard • Pipetting errors in the reaction mix • Air bubbles formed in well • Standard stock is at an incorrect concentration • Calculation errors • Substituting reagents from older kits/ lots 	<ul style="list-style-type: none"> • Thaw and resuspend all components before preparing the reaction mix • Avoid pipetting small volumes • Prepare a master reaction mix whenever possible • Pipette gently against the wall of the tubes • Always refer the dilutions in the data sheet • Recheck calculations after referring the data sheet • Use fresh components from the same kit
Unanticipated results	<ul style="list-style-type: none"> • Measured at incorrect wavelength • Samples contain interfering substances • Use of incompatible sample type • Sample readings above/below the linear range 	<ul style="list-style-type: none"> • Check the equipment and the filter setting • Troubleshoot if it interferes with the kit • Refer data sheet to check if sample is compatible with the kit or optimization is needed • Concentrate/ Dilute sample so as to be in the linear range
<p>Note# The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.</p>		

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