

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. BioVision'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 2X 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 (BioVision, 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 OD_{450 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 763).

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

