NAD+/NADH Colorimetric Assay Kit (384-well) (#BN01118)

(Catalog # BN01118; 400 assays; Store at -20°C)

I. Introduction:

Nicotinamide Adenine Dinucleotide (NAD) is a co-enzyme indispensible for the activity of multiple catalytic proteins. NAD^+ and NADH, its reduced form, participate in many biological redox reactions. NAD^+ is mostly associated with redox enzymes required for catabolic reaction and energy production. Assay Genie's NADH/NAD Quantification Kit provides a convenient tool for a sensitive detection of NADH, NAD and their ratio. There is no requirement to purify NADH or NAD from samples. The NADH selective developer develops color while reacting with NADH and can be detected at OD 450 nm. The total NAD can be easily quantified by comparing with the provided NADH standard. The method is quantitative, rapid, simple, sensitive, and designed for high throughput format. The kit can detect as low as 0.04 pmol of NADH in a 384 well assay plate. The reaction specifically detects NADH and NAD, but not NADP nor NADPH.

II. Application:

- Measurement of NAD,NADH or their ratio in various tissues/cells extracts
- Analysis of metabolism in various cells
- Measurement of NAD, NADH or their ratio in biological fluids

III. Sample Type:

- Animal tissues: liver, kidney etc.
- Cell culture: adherent or suspension cells
- Biological Fluids: Serum and Urine

IV. Kit Contents:

Components	BN01118	Cap Code	Part Number
NADH/NAD Extraction Buffer	50 ml	NM	BN01118-1
NAD Cycling Buffer	15 ml	NM	BN01118-2
NAD Cycling Enzyme Mix	1 vial	Green	BN01118-3
NADH Developer	1 vial	Purple	BN01118-4
Stop Solution	1.2 ml	Red	BN01118-5
NADH Standard	1 vial	Yellow	BN01118-6

V. User Supplied Reagents and Equipment:

- 384-well clear plate with flat bottom
- Multi-well spectrophotometer with 384-well plate reading capability

VI. Storage and Handling:

Store kit at –20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

VII. Reagent Reconstitution and General Consideration:

- **NADH/NAD Extraction Buffer and NAD Cycling Buffer:** Warm both buffers to room temperature before use. Store at -20° C.
- **NAD Cycling Enzyme Mix:** Reconstitute with 220 µl NAD Cycling Buffer. Aliquot the reconstituted Cyclic Enzyme in eppendorf tubes and freeze immediately at -70° C for future use. The enzyme is stable for up to 2 months at -70° C after reconstitution. Keep the aliquoted enzyme vial in ice when setting the assay-experiment.
- **NADH Developer:** Reconstitute NADH developer with 1.2 ml of ddH_2O. Pipette up and down several times to completely dissolve the pellet into solution (don't vortex). Store at -20° C, protected from light. Use within 2 months.
- **NADH Standard:** Reconstitute with 200 µl pure DMSO to generate 1 nmol/µl NADH Standard. Store at -20° C. Use within 2 months.

VIII. NAD/NADH Assay Protocol:

1. **Sample Preparation:**

   For cells: wash cells with cold PBS. Pellet 2 X 10^5 cells for each assay in a micro-centrifuge tube (2000 rpm, 5 min, 4 °C) & extract 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 by homogenization. Centrifuge at 4 °C for 18000 x g, 10 min. Transfer the extracted NADH/NAD supernatant into a new tube.

   For tissues, weigh ~20 mg tissue & wash with cold PBS. Homogenize in 200 µl of NADH/NAD Extraction Buffer in new tube. Centrifuge at 4 °C 18000 x g, 10 min. Transfer the supernatant into a fresh tube.

   For serum and urine, centrifuge the samples at 4 °C for 18000 x g, 10 min to remove any particles. Collect supernatant.

   To detect total NAD (NADt: NADH and NAD): transfer 5 µl of samples into a 384-well clear plate. Make the final volume to 10 µl with NADH/NAD extraction buffer.

   To detect NADH: NAD needs to be decomposed before reacting with NAD Cycling Enzyme Mix. To decompose NAD keeping NADH intact, aliquot 100 µl of extracted samples into eppendorf tubes and incubate (60° C; 30 min). All NAD will decompose, while NADH will still be intact. Cool samples on ice. Do a quick spin of the samples at 4 °C for 18000 x g, 1 min., transfer 5 µl of NAD-decomposed samples into a 384-well clear plate. Make the final volume to 10 µl with NADH/NAD extraction buffer

   Notes:

   a. Cell or tissue lysates and biological fluid samples may contain enzymes that consume NADH rapidly. We suggest removing these enzymes by filtering the samples through 10 kDa molecular weight cut off filters before performing the assay.

b. For unknown samples, we suggest performing a pilot experiment & testing different sample dilutions with the extraction buffer to ensure the readings are within the Standard Curve range.

c. For samples having high background, prepare parallel well(s) containing same amount of sample as in the test well. Adjust the volume to 10 µl/well with NADH/NAD Extraction Buffer.

d. Endogenous compounds may interfere with the reaction. To ensure accurate determination of NADH in the test samples, we recommend spiking samples with 10 pmol of Standard.

e. Instrument reader settings need to be adjusted according to the chosen 384-well plate clear plate. (The right dimension of the used 384-well plate may be available in the manual provided by the plate-manufacturer).

2. **Standard Curve Preparation:** Dilute 2.5 µl of 1 nmol/µl NADH standard with 997.5 µl NADH/NAD Extraction Buffer to generate 2.5 pmol/µl standard NADH. Add 0, 2, 4, 6, 8 and 10 µl of the diluted NADH standard into 384-well plate to generate 0, 5, 10, 15, 20 and 25 pmol/well standards. Make the final volume to 10 µl with NADH/NAD extraction buffer.

   Note: Diluted NADH solution is unstable, must be used within 4 hours.

3. **Reaction Mix:** Prepare a Reaction Mix with NAD Cycling Buffer, NAD Enzyme Mix and NADH Developer. For each reaction:

	Reaction Mix	***Background Control Mix**
NAD Cycling Buffer	20.0 µl	20.5 µl
NAD Cycling Enzyme Mix	0.5 µl	----
NADH Developer	2.5 µl	2.5 µl

   Mix well and add 23 µl of the mix into each well of NADH Standard and samples.
   * For samples having high background, add 23 µl of Background Control Mix to sample background control well(s).

4. **Measurement:** Let the reaction cycling at room temperature for 1 to 4 hrs or longer depending on the absorbance reading at 450 nm. The plate can be read multiple times while the color is being developed. The reactions can be stopped by adding 2 µl of Stop Solution into each well. After addition of Stop Solution the color should be stable for 48 h. in a sealed plate.

5. **Calculation:** Subtract 0 Standard reading from all Standard readings, plot NADH Standard Curve. If sample background control reading is significant then subtract the sample background control reading from sample reading. Plot the NADH Standard Curve. Apply the corrected OD to the NADH Standard Curve to get B pmol of NADH in the sample well.

Sample NADH concentration (C) = B/V X D pmol/µl

Where: **B** is the amount of NADH in the sample well (pmol)
V is the sample volume added into the reaction well (µl)
D is the sample dilution factor

NADH Molecular Weight: 663.4

Note: For spiked samples, correct for any sample interference by subtracting the sample reading from spiked sample reading.

For spiked samples, NADt or NADH amount in well = $\left(\frac{(\mathrm{OD}_{\text{sample (corrected)}})}{(\mathrm{OD}_{\text{sample + NADH Std(corrected)}})-(\mathrm{OD}_{\text{sample(corrected)}})}\right) * NADH\ Spike\ (\mathrm{pmol})$

NAD/NADH Ratio is calculated as: (NADt – NADH)/ (NADH)

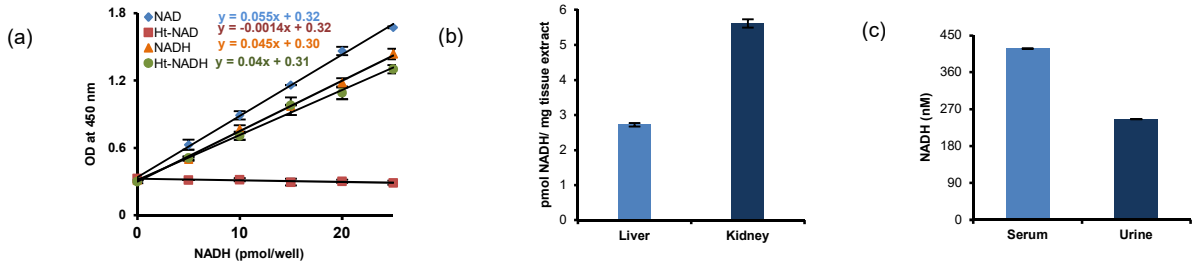

Figure: a) Standard Curve for NADH, NAD and heated NADH (Ht-NADH) and NAD (Ht-NAD); both heated 30 min at 60 °C; (b) Quantitation of NADH in freshly processed rat liver and kidney tissue. Tissue homogenates were prepared following kit's protocol and deproteinized by 10 kDa spin column (12000 x g, 10 min, 4°C). 5 µl filtrates were assayed according to the kit's protocol. (c) Quantitation of NADH in human serum and urine. Samples were centrifuged and supernatant was collected and deproteinized by 10 kDa spin column. Filtrate was spiked with 10 pmol of NADH Standard, assayed following kit's protocol. All standards and samples absorbance readings were taken after 60 minutes cycling reaction at room temperature.

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