Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM
Tel 0032 16 58 90 45 info@gentaur.com

NAD+/NADH Quantification Colorimetric Kit

rev. 7/15

(Catalog # K337-100; 100 assays; Store at -20°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. Application:

- Measurement of NADH in various tissues/cells
- Analysis of metabolism in various cells

III. Sample Type:

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

IV. Kit Contents:

Components	K337-100	Cap Code	Part Number
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	1 Vial	Yellow	K337-100-6

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer

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:** Warm NADH/NAD Extraction Buffer to room temperature before use. Store at 4˚C or -20˚C.
- **NAD Cycling Buffer:** Warm NAD Cycling Buffer to room temperature before use. Store at 4˚C or -20˚C.
- **NAD Cycling Enzyme Mix:** Reconstitute with 220 µl 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 at –70˚C after reconstitution.
- **NADH Developer:** 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). 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 solution. Store at –20˚C. Use within 2 months.

  Note: Ensure that the NAD Cycling Buffer is at room temperature before use. Keep other enzymes on ice during the assay and protect from light.

VIII. NAD/NADH Assay Protocol:

1. **Sample Preparation:** Wash cells with cold PBS. Pellet 2 X 10^5 cells for each assay in a micro-centrifuge tube (2000 rpm for 5 min.) & 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. Vortex the extraction for 10 sec. Centrifuge at 14000 rpm for 5 min. Transfer the extracted NADH/NAD supernatant into a labeled tube. For tissues, weigh ~20 mg tissue & wash with cold PBS. Homogenize in 400 µl of NADH/NAD Extraction Buffer in a micro-centrifuge tube. Centrifuge at 14000 rpm for 5 min. Transfer the extracted NADH/NAD supernatant into a new tube. To detect total NADt (NADH and NAD), transfer 50 µl of extracted samples into labeled 96-well plate. To detect NADH, NAD needs to be decomposed before the reaction. To decompose NAD, aliquot 200 µl of extracted samples into eppendorf tubes. Heat to 60˚C for 30 min. in a water bath or a heating block. Under this condition, all NAD will decompose, 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.

   Notes:

   a. Cell or tissue lysates may contain enzymes that consume NADH rapidly. We suggest removing these enzymes by filtering the samples through 10 kDa molecular weight cut off filters (Cat. # 1997) 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 50µl/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 a known amount of Standard (60 pmol).

2. **Standard Curve Preparation:** Dilute 10 µl of 1 nmol/µl NADH standard with 990 µl NADH/NAD Extraction Buffer to generate 10 pmol/µl standard NADH. 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.

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

3. **Reaction Mix:** Prepare a NAD Cycling Mix for each reaction:

	Reaction Mix	*Background Control Mix
NAD Cycling Buffer	98 µl	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.

   * For samples having high background, add 100 µl of Background Control Mix to sample background control well(s).

4. **Measurement:** Add 10 µl NADH developer into each well. Let the reaction cycling at room temperature for 1 to 4 hrs or longer depending on the reading (OD 450 nm). Read the plate at OD 450 nm. The plate can be read multiple times while the color is being developed. The reactions can be stopped by adding 10 µl of Stop Solution into each well and mix well. The color should be stable for 48 hrs in a sealed plate after addition of Stop Solution.

5. **Calculation:** for unspiked samples, apply the sample readings to NADH standard curve. The amount of NADt or NADH in the sample wells can then be calculated. 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/10^6 cells or ng/mg protein (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 sample 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: $\frac{\text{NADt – NADH}}{\text{NADH}}$

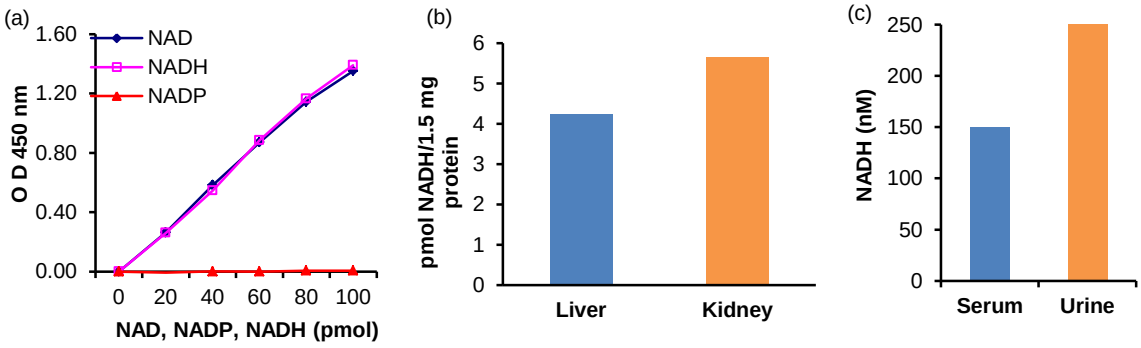

Figure: a) Standard Curve. b) Quantitation of NADH in rat liver (1.5 mg) and kidney (1.5 mg) homogenate. Tissues were washed with cold PBS followed by homogenization using NADH/NAD Extraction Buffer. Homogenates were centrifuged and deproteinized using a 10 kDa spin column (10000 x g, 10 min, 4°C). Filtrates were spiked with NADH (60 pmol) and assayed according to the protocol. (c) Quantitation of NADH in human serum (30 µl) and urine (30 µl).

IX. RELATED PRODUCTS:

NADP/NADPH Quantification Kit (K347)
PicoProbe™ NADPH Quantitation Fluorometric Assay Kit (K349)
Dounce Tissue Homogenizer (1998)
PicoProbe™ NADH Fluorometric Assay Kit (K338)
10K Spin Column (1997)

FOR RESEARCH USE ONLY! Not to be used on humans.