EZ-NAD/NADH Assay Kit

Metabolism Assay Kit

(Colorimetric)

Cat. No. DG-NAD100

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

▪ Product Description

Nicotinamide Adenine Dinucleotide (NAD) is a cofactor found in all living cells and exists in two forms: the oxidized form (NAD^+) and the reduced form (NADH).

In this assay, ethanol is converted to acetaldehyde via an Alcohol Dehydrogenase (ADH)-catalyzed reaction, during which NAD^+ is reduced to NADH. The hydrogen atoms generated upon oxidation of NADH react with WST-8 to form WST-8 formazan. The amount of NADH produced is directly proportional (1:1 molar ratio) to the amount of substrate consumed in the reaction.

The EZ-NAD/NADH Assay Kit enables simple and independent measurement of total NAD (the sum of NAD^+ and NADH) and NADH levels. The amount of NAD^+ can then be conveniently determined by subtracting the NADH value from the total NAD value.

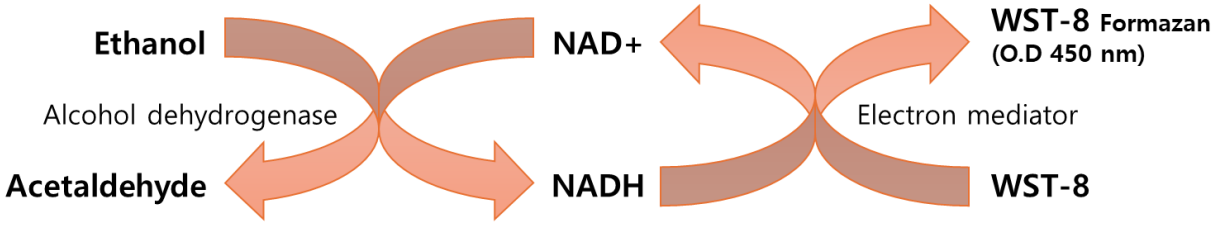

Fig 1. Detection mechanism with EZ-NAD/NADH Assay Kit

▪ Contents and Storage Conditions

Component	100 assays	Storage
NAD/NADH Extraction Buffer	25 ㎖ X 2	-20 ℃
NAD Assay Buffer	8 ㎖ X 2	
NAD Enzyme Mix	1 vial	
NAD/NADH Probe	1 ㎖	
Stop Solution	1 ㎖	
NADH Standard (1 mM)	1 vial	

* Aliquot only the required volume of NAD Enzyme Mix and NADH Standard prior to use.
* Allow Extraction Buffer and Assay Buffer to fully dissolve at room temperature before use.
* This product is intended for research use only and should not be used for human or diagnostic purposes.

▪ Preparation of Reagent

Component	Preparation	Storage and Stability
NAD/NADH Extraction Buffer	Add protease inhibitor to a final concentration of 1X immediately before use.	Store at -20°C after addition of protease inhibitor.
NAD Enzyme Mix	Add 220 µL of distilled water and mix gently by inverting.	Upon opening, aliquot and store at -20°C. Use within 2 months.
NADH Standard (1 mM)	Add 200 µL of NAD Assay Buffer and mix gently by inverting.	

* This protocol is optimized for experiments and measurements using a 96-well plate.

▪ General Protocol

Sample preparation

Note

- For unknown samples, a preliminary experiment is recommended prior to the main assay to confirm that the sample values fall within the range of the standard curve.
- Samples should be used immediately whenever possible. If immediate analysis is not feasible, store samples at -20°C after completing the sample preparation steps, or snap-freeze in liquid nitrogen immediately after extraction and store at -80°C.
- NADH in serum, tissue, and cell samples may be consumed by endogenous enzymes. Filter samples through a 10 kDa spin column to remove enzymes prior to analysis.
- When thawing samples, thaw slowly on ice before use.

- **Serum**

① Pass the serum sample through a 10 kDa Spin Column to remove endogenous enzymes.

➢ Centrifuge the sample at maximum speed at 4°C for 10 minutes and collect the filtrate.

② Use the filtrate immediately for analysis

- **Tissue**

① Wash tissue with cold PBS, then prepare 20 ~ 40 mg of tissue per sample.

② Add 400 µL of NAD/NADH Extraction Buffer and homogenize using a Dounce homogenizer (30 ~ 50 strokes).

③ Centrifuge at maximum speed at 4°C for 5 minutes to remove insoluble material.

④ Transfer the supernatant to a new tube and keep on ice. (NADt sample)

- **Cell lysate**

NADt Sample Preparation

① Wash cells with cold PBS.

② Transfer cells to a microtube and centrifuge at 200 × g for 5 minutes, then discard the supernatant.

- The optimal cell number may vary depending on the cell line. Use the recommended range (2×10^5 ~ 1×10^6) as a reference and optimize accordingly for your cell type.

③ Add 400 µL of NAD/NADH Extraction Buffer and perform 2 freeze-thaw cycles.

- Per cycle: freeze at -80°C (deep freezer) for 20 minutes, then thaw at room temperature for 10 minutes.

④ Vortex for 10 seconds, then centrifuge at maximum speed at 4°C for 5 minutes.

⑤ Transfer the supernatant to a new microtube and keep on ice. Use as the NAD total sample. (NADt sample = NAD^+ + NADH)

- If measuring NAD total only, skip the steps below.

NADH Sample Preparation

① To measure NADH alone, transfer 200 µL of the NADt sample to a new microtube and incubate at 60°C using a heat block or water bath for 30 minutes.

- If samples are not to be used immediately, store as NAD total sample in a deep freezer and prepare the NADH sample on the day of the experiment.
- NADH generated after heat treatment is susceptible to freeze-thaw cycles; therefore, prepare the NADH sample on the day of the experiment.

② Immediately cool on ice, then centrifuge at maximum speed at 4°C for 1–2 minutes.

③ Transfer the supernatant to a new microtube and keep on ice. (NADH sample)

NADH standard preparation

: Prepare a 10 pmol/µL standard solution (10 µM NADH) by mixing 10 µL of NADH Standard (1 mM) with 990 µL of Extraction Buffer in a microtube. Then prepare the standard dilution series as shown in the table below.

Standard Tube No.	10 µM NADH standard solution (µL)	NAD/NADH Extraction Buffer (µL)	Final volume (µL/well)	Final NADH Amount (pmol/well)
1	-	125	125	0
2	5	120	125	20
3	10	115	125	40
4	15	110	125	60
5	20	105	125	80
6	25	100	125	100

* It is recommended to measure the standard alongside each experiment.

* As samples and enzymes are sensitive to heat, keep them on ice throughout the experiment.

NAD/NADH Assay

① Prepare 50 µL of each NADH standard concentration in duplicate.

② Prepare NAD total and NADH samples (2–50 µL each) in duplicate and bring the final volume to 50 µL with Extraction Buffer.

③ Prepare the Reaction Mix according to the table below, scaling for the number of wells + 1.

Component	Reaction Mix (µℓ)	Background Reaction Mix (µℓ)
NAD Assay Buffer	98	100
NAD Enzyme Mix	2	-

④ Mix the Reaction Mix by inverting, then dispense 100 µL into each NADH standard and sample well.

⑤ Mix by tapping the plate or using a plate shaker to convert NAD^+ to NADH, then incubate at room temperature for 5 minutes.

⑥ Add 10 µL of NAD Probe to each well and incubate at room temperature for 0–2 hours, then measure absorbance at 450 nm using a microplate reader.

- It is recommended to take measurements over multiple time points or use kinetic mode.
- Incubation time may vary depending on the O.D. value. Once the desired O.D. value is reached, the reaction can be stopped by adding 10 µL of Stop Solution to each well.

▪ Calculation

NADH concentration of sample (pmol/µL = µM)

1. Calculate the average of the duplicate measurements for the standard, sample, and sample background.
2. If the sample background value is high, subtract the background value from the sample measurement.
3. Subtract the Blank absorbance from all measurements to obtain the corrected absorbance.

   * Blank = $O.D_{450}$nm from NADH Standard No. 1 (0 pmol NADH)
4. Plot the standard curve using NADH standard concentrations (X-axis) against O.D. values (Y-axis) and derive the linear equation.
5. Substitute the sample measurement values into the standard curve equation to calculate the amount of NADH.
6. Calculate the NADt or NADH concentration of the sample as follows.

$$\textbf{NADt or NADH Concentration} = \frac{\text{NADH}}{\text{V}} \times \text{D} = \text{pmol}/\mu\ell = \mu\text{M}$$

NADH = Amount of NADH measured in the well (pmol)

V = Volume of sample added to the well (µL)

D = Dilution factor of the sample

7. Calculate the NAD^+/NADH ratio as follows.

$$\textbf{NAD/NADH ratio} = \frac{\text{NADt} - \text{NADH}}{\text{NADH}}$$

Fig 2. Nitrite standard curve. Assay was performed following the kit protocol.

Spike sample

: Due to matrix effects from components within the sample, NADH values may be measured higher or lower than the actual concentration. To correct for this error and determine the accurate concentration, a spiking method is used, in which a known amount of standard is added to the sample. (e.g., 60 pmol; 6 µL of 10 pmol/µL NADH standard).

$$\textbf{NADt or NADH} = \frac{\text{Sample O.D}}{\text{Spiked Sample O.D} - \text{Sample O.D}} \times \text{amount of NADH spike } (pmol)$$

After determining the NADt and NADH concentrations from the spiked sample, the NAD^+/NADH ratio can be calculated using the method described in step 7 above.

▪ Related Product

	Products	Catalog No.	Assay
Oxidative Stress Assay Kit	EZ-Superoxide Dismutase (SOD) Assay Kit (Colorimetric)	DG-SOD400	400 Assay
	EZ-Glutathione Assay Kit (Colorimetric)	DG-GLU200	200 Assay
	EZ-Catalase Assay Kit (Fluorometric/Colorimetric)	DG-CAT400	400 Assay
	EZ-Hydrogen peroxide/Peroxidase Assay Kit (Fluorometric/Colorimetric)	DG-PER500	500 Assay
	EZ-Lipid Peroxidation (TBARS) Assay Kit (Colorimetric)	DG-TBA200	200 Assay
	EZ-Total Antioxidant Capacity (TAC) Assay Kit (Colorimetric)	DG-TAC200	200 Assay
	EZ-DPPH Antioxidant Assay Kit (Colorimetric)	DG-DPH400	400 Assay
	EZ-ABTS Antioxidant Assay Kit (Colorimetric)	DG-ABT400	400 Assay
	EZ-Glutathione peroxidase (GPx) Assay Kit (Colorimetric)	DG-GPX100	100 Assay
Metabolism Assay Kit	EZ-Lactate Assay Kit (Fluorometric/Colorimetric)	DG-LAC100	100 Assay
	EZ-Acetylcholinesterase Assay Kit (Colorimetric)	DG-ACE100	100 Assay
	EZ-Ascorbic Acid Assay Kit (Colorimetric)	DG-ASC100	100 Assay
	EZ-ATP Assay Kit (Fluorometric/Colorimetric)	DG-ATP100	100 Assay
	EZ-Free Fatty Acid Assay Kit (Fluorometric/Colorimetric)	DG-FFA100	100 Assay
	EZ-Free Glycerol Assay Kit (Fluorometric/Colorimetric)	DG-FGC100	100 Assay
	EZ-Glucose Assay Kit (Fluorometric/Colorimetric)	DG-GCS100	100 Assay
	EZ-HDL, LDL/VDL Assay Kit (Fluorometric/Colorimetric)	DG-CHO100	100 Assay
	EZ-Total Cholesterol Assay Kit (Fluorometric/Colorimetric)	DG-TSC100	100 Assay
	EZ-Triglyceride Quantification Assay Kit (Fluorometric/Colorimetric)	DG-TGC100	100 Assay
	EZ-Nitric Oxide Assay Kit (Colorimetric)	DG-NO500	500 Assay
	EZ-Total Collagen Assay Kit (Colorimetric)	DG-COL100	100 Assay
	EZ-Ethanol Assay Kit (Colorimetric)	DG-ETH100	100 Assay
	EZ-NAD/NADH Assay Kit (Colorimetric)	DG-NAD100	100 Assay