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NAD⁺/NADH Assay Kit (WST-8)

1 Packing list

Components	HY-K0313-100 T
NAD⁺/NADH Extract Solution	50 mL
NADH	0.5 mg
NADH Solution	1 mL
ADH	0.5 mL
Chromogen Solution	1.1 mL
Reaction Buffer	11 mL

2 Introduction

Nicotinamide Adenine Dinucleotide (NAD) serves as a coenzyme in many redox reactions, existing in both oxidized (NAD⁺) and reduced (NADH) forms. MCE NAD⁺/NADH Assay Kit (WST-8) is a colorimetric assay that conveniently detects the oxidized (NAD⁺) and reduced (NADH) forms of coenzyme within cells, tissues, and other samples. This is achieved through colorimetric measurements based on the WST-8 color reaction, eliminating the need for purification of NAD⁺/NADH from the samples.

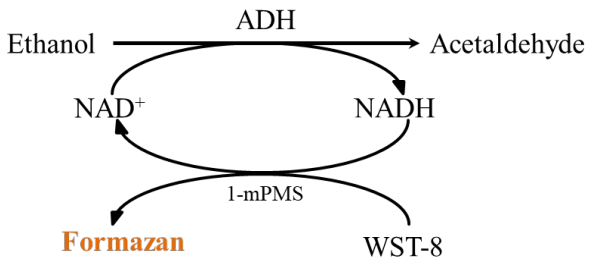
The Detection Principle is as Follows

→ Detection of NAD⁺ and NADH total amounts: Ethanol undergoes oxidation to become acetaldehyde, catalyzed by the enzyme alcohol dehydrogenase (ADH). Simultaneously, NAD⁺ is reduced to NADH. The NADH interacts with the electron-coupled reagent (1-mPMS, 1-Methoxy-5-methylphenazinium Methyl Sulfate), transforms WST-8 into an orange-colored Formazan displaying maximum absorption at 450 nm. In conclusion, there is a linear relationship between the amount of Formazan generated in the system and the total volume of NAD⁺ and NADH.

→ Detection of NADH amount: After the sample is pre-heated at 60°C for 30 minutes, only NADH will remain in the system as NAD⁺ decomposes. The retained NADH then reduces WST-8 to orange Formazan (orange-colored). The quantity of Formazan produced is determined using a colorimetric method and directly corresponds to the NADH amount in the sample.

→ Detection of NAD⁺ amount and NAD⁺ to NADH ratio: Based on the results from the first two steps of detection, we can determine the amount of NAD⁺ in the sample and the ratio of NAD⁺ to NADH.

Schematic of MCE NAD⁺/NADH Assay Kit (WST-8)



3 General Protocol

1. Reaction solution preparation

1) Preparation of NADH standard solution: Dissolve 0.5 mg of NADH in 665μL of NADH Solution to prepare a 1 mM NADH standard solution.

2) Setting up NADH standard curve: Dilute 1 mM NADH with NAD^+/NADH extract solution to the concentrations of 0 μM、0.25 μM、0.5 μM、1 μM、2 μM、4 μM、6 μM、8 μM、10 μM. Add 20 μL of each standard into separate wells of a 96-well plate, and the standard content in each well corresponds to 0 pmol、5 pmol、10 pmol、20 pmol、40 pmol、80 pmol、120 pmol、160 pmol、200 pmol, respectively. The standard concentration of 0 μM serves as the blank control, containing only the NAD^+/NADH extract solution.

3) Preparation of ADH working solution: 90 μL of ADH working solution is required for each standard or sample. Considering the number of experimental samples, take an appropriate amount of ADH and dilute it 20 times with the Reaction Buffer.

Note: a. NADH is unstable; it should be prepared and used immediately. It is recommended to portion NADH solution (1 mM) appropriately and store them at -80°C, and keep away from light.

b. Prepare an adequate amount of the ADH working solution and use it immediately.

2. Sample preparation

1) Cell Sample Preparation

a) For adherent cells: For 1 × 10^6 cells, aspirate the culture medium. Add 200 μL of pre-cooled NAD^+/NADH extract solution to lyse the cells. For suspension cells: For 1 × 10^6 cells, centrifuge at 600 g for 5 min, aspirate the culture medium. Add 200 μL of pre-cooled NAD^+/NADH extract solution to lyse the cells.

b) The lysis process takes approximately 10 min. It can be carried out either at room temperature or on ice, depending on the experiment's specific requirements.

c) Centrifuge at 12,000 g for 5-10 min at 4°C. The supernatant is the sample to be tested.

2) Tissue sample preparation

a) Wash the tissues with pre-cooled PBS, take 10-30 mg of the tissue and cut it with scissors and place it into a homogenizer. Add 400 μL of pre-cooled NAD^+/NADH extract solution, homogenization at room temperature or on ice.

b) Centrifuge at 12,000 g for 5-10 min at 4°C. The supernatant is the sample to be tested.

3. Detection

1) Detection of total NAD^+/NADH in sample: Transfer 20 μL of the sample to be tested into a 96-well plate. It is recommended to set up 3-5 duplicate wells to minimize experimental error.

Detection of NAD^+, NADH or NAD^+/NADH ratio in samples: Transfer 50 μL-100 μL of the sample to centrifuge tube, heat it at 60°C for 30 min to decompose NAD^+.

2) Please refer to the table below to set up the reaction system using a 96-well plate.

	Blank	Standard	Sample
Test Samples/μL	/	20	20
NAD^+/NADH extract solution/μL	20	/	/
ADH working solution/μL	90	90	90

3) Incubate the samples at 37°C for 10 min to reduce NAD^+ to NADH.

4) Mix the Chromogen Solution, add 10 μL of Chromogen Solution to each well and mix thoroughly. Incubate at 37 °Caway from light for 30 min. After the Chromogen Solution is added, the reaction starts, leading to the formation of Formazan. Absorbance at 450 nm can be measured following this reaction.

Note: a. If the total amount of NAD^+ and NADH in the final experiment is found to exceed the standard curve's range, the sample can be appropriately diluted with the NAD^+/NADH extract solution and retested. Conversely, if the total amount quantified is too low, the amount of cells or tissues used should be increased for subsequent assays.

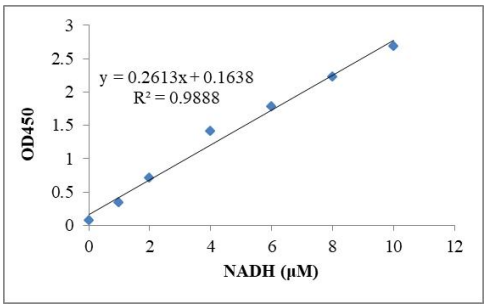
b. ADH working solution must be added gently to avoid air bubbles. If bubbles form, they can be burst with a small blowgun or needle.

c. If the color appears light after adding the Chromogen Solution, the incubation time can be extended to 45-60 min. Prolonging the incubation time will intensify the color.

4. Calculation

1) Calculate the average absorbance for each point in the standard sample group, and subtract the absorbance of the blank control group as the absorbance for each standard.

2) Plotting the standard curve: Use the NADH concentration as the x-axis (horizontal coordinate) and the absorbance as the y-axis (vertical coordinate), and plot the standard curve (illustrated below).



3) Calculate the total concentration of NAD^+ and NADH, or the concentration of NADH in the cell and tissue samples according to the standard curve. The total concentration of NAD^+ and NADH (NAD^+ + NADH) can be calculated for the group that has not been heated in a 60°C, while the concentration of NADH can be calculated for the group that was heated in a 60°C.

4) Calculate the NAD^+ and NADH amount and the total amount of NAD^+/NADH (NAD^+ + NADH), based on the measured concentrations, the volume of the sample, and the formula provided below.

$[NAD^+] = [NAD^+ + NADH] - [NADH]$

$[NAD^+]/[NADH] = ([NAD^+ + NADH] - [NADH])/[NADH]$

Note: a. The amount of NAD^+, NADH, and the total of NAD^+/NADH can be expressed either per unit number of cells or per unit weight of tissue.

b. The protein concentration in the sample can also be measured using the BCA method, for the final conversion of NAD^+, NADH amount or the total NAD^+/NADH amount per unit of protein.

4 Storage

-20°C, 1 year

Keep away from light and avoid repeat freeze-thaw cycles

5 Precautions

1. Thaw and gently mix the frozen components before use.
2. Once NADH has been prepared into a solution, it should be appropriately portioned and stored at -80°C. For NADH is unstable, an unsatisfactory standard curve could suggest degradation of the standard.
3. NAD^+/NADH extract solution is relatively viscous. It's crucial to ensure uniform dilution during the process; otherwise it could lead to substantial fluctuations in the experimental data.
4. During the detection process, it's crucial to avoid generating air bubbles as much as possible, as they might affect the final absorbance measurement.
5. It is recommended to redraw the standard curve for each test to ensure accuracy.
6. NAD^+ and NADH are unstable and tend to degrade easily during freezing. It is recommended to use fresh samples for the assay to ensure accurate results.
7. This product is for R&D use only, not for drug, house hold, or other uses.
8. For your safety and health, please wear a lab coat and disposable gloves to operate.