# Technical Manual

# NAD+/NADH Colorimetric Assay Kit (WST-8)

- Catalogue Code: MAES0233
- Size: 96T
- Research Use Only

# 1. Key Features and Sample Types

## Detection method:

Colorimetric method

## Specification:

96T

## Range:

0.02-5.0 umol/L

## Sensitivity:

0.02 umol/L

## Storage:

-20°C for 12 months

## Expiry:

See Kit Label

## Experiment Notes:

This kit is for **research use only.**

Instructions should be strictly followed. Changes of operation may result in unreliable results.

The validity of kit is 12 months.

Do not use components from different batches of kit.

## 2. Background

NAD+ and NADH are coenzymes that transfer electrons during REDOX reactions, and can be used as cofactors of many enzymes to participate in intracellular reactions.

## 3. Intended Use

This kit can be used to measure NAD+, NADH content and their ratio in animal tissue and cell samples.

## 4. Detection Principle

Detect total content of NAD+ and NADH
Ethanol generates acetaldehyde under the action of enzyme. Meanwhile, NAD+ is reduced to NADH, NADH, under the action of hydrogen transmitter, transfer electrons to WST-8 to produce the yellow product, which has a characteristic absorption peak at 450 nm. Therefore, the total content of NAD+ and NADH can be quantified by measure the OD value at 450 nm.

Detect NADH
After treating sample, heat at 60℃ water bath for 30 min. The NAD+ of the sample is decomposed and only NADH remains. NADH reduces WST-8 to form yellow product, and the amount of NADH is determined by measure the OD value at 450 nm.

Detect NAD+ and NAD+/NADH
The content of NAD+ and the ratio of NAD+/NADH in the sample can be obtained according to the total content of NAD+ and NADH obtained of the first two steps as well as the separate content of NADPH. Note: NADP+ and NADPH have no effect on the determination results.

## 5. Kit Components & Storage

| Item | Specification | Storage |
|---|---|---|
| **Extracting Solution** | 60 mL × 2 vials | -20℃, 12 months |
| **Buffer Solution** | 16 mL × 1 vial | -20℃, 12 months |
| **Chromogenic Agent** | 5 mL × 1 vial | -20℃, 12 months, shading light |
| **Enzyme Reagent** | Powder × 2 vials | 20℃, 12 months, shading light |
| **Standard** | Powder × 2 vials | -20℃, 12 months, shading light |
| **Microplate** | 96 wells | No requirement |
| **Plate Sealer** | 2 pieces | |

**Note:** The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other. For a small volume of reagents, please centrifuge before use, so as not to obtain sufficient amount of reagents.

### Materials required but not supplied

- Micropipettor
- 37℃ water bath
- Microplate Reader (450 nm)
- 10 KD filters tube
- Double distilled water
- PBS (0.01 M, pH 7.4)

## 6. Assay Notes:

1. Keep enzyme working solution at 2-8℃ with shading light for 5 h before use. Prepare in advance.

2. The sample must be fresh.

3. Heat the prepared sample at 60℃ water bath for 30 minutes, during this process, the EP tube should be sealed to prevent liquid volatilization. After heating, due to condensation of water vapor, it is necessary to mix thoroughly before proceeding to the next step.

## 7. Reagent Preparation:

1. Equilibrate all reagents to room temperature before use.

2. Preparation of enzyme working solution:
   Dissolve one vial of enzyme reagent with 200 μL of double distilled water, mix well to dissolve. Keep enzyme working solution at 2-8℃ with shading light for 5 h before use. Store at 4°C for 7 days protected from light.

3. Preparation of reaction working solution:
   For each well, prepare 120 μL of reaction working solution (mix well 3 μL of enzyme working solution and 117 μL of buffer solution). The reaction working solution should be prepared on spot and stored protected from light. The prepared solution should be used up within 2 hours.

4. Preparation of 250 μmol/L standard:
   Dissolve one vial of standard with 200 μL of double distilled water, mix well to dissolve. Store at -20°C for 7days protected from light.

5. Preparation of 5 μmol/L standard:
   Before testing, please prepare sufficient 5 μmol/L standard according to the test wells. For example, prepare 1000 μL of 5 μmol/L standard (mix well 20 μL of 250 μmol/L standard and 980 μL of extracting solution). The 5 μmol/L standard should be prepared on spot and stored protected from light. The prepared solution should be used up within 1 day.

6. The preparation of standard curve:
   Always prepare a fresh set of standards. Discard working standard dilutions after use. Dilute 5 μmol/L standard with extracting solution to a serial concentration. The recommended dilution gradient is as follows: 0, 1, 1.5, 2, 2.5, 3.5, 4, 5 μmol/L. Reference is as follows:

| Item | ① | ② | ③ | ④ | ⑤ | ⑥ | ⑦ | ⑧ |
|---|---|---|---|---|---|---|---|---|
| **Concentration (μmol/L)** | **0** | **1.0** | **1.5** | **2.0** | **2.5** | **3.5** | **4.0** | **5.0** |
| **5 μmol/L standard (μL)** | 0 | 40 | 60 | 80 | 100 | 140 | 160 | 200 |
| **Extracting solution (μL)** | 200 | 160 | 140 | 120 | 100 | 60 | 40 | 0 |

# 8. Sample Preparation

**Tissue sample:**

1. Harvest the amount of tissue needed for each assay (initial recommendation 20 mg).
2. Wash tissue in cold PBS (0.01 M, pH 7.4).
3. Homogenize 20 mg tissue in 180 µL extracting solution with a dounce homogenizer at 4℃.
4. Centrifuge at 10000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it on ice for detection.
5. Meanwhile, determine the protein concentration of supernatant.

**Cell (adherent or suspension) samples:**

1. Harvest the number of cells needed for each assay (initial recommendation 1.5×10^6 cells).
2. Wash cells with PBS (0.01 M, pH 7.4).
3. Homogenize 1.5×10^6 cells in 400 µL extracting solution with a ultrasonic cell disruptor at 4℃.
4. Centrifuge at 12000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it on ice for detection.
5. Meanwhile, determine the protein concentration of supernatant.

**Sample ultrafiltration:**

Tissue and cell homogenate contains enzymes that can decompose NAD+. It is recommended that after sample extraction and centrifugation, the supernatant be centrifuged with 10 KD ultrafiltration tube at 10000×g for 10 min at 4℃ to remove the catabolase.
Measure total of NAD+ and NADH: Detect the filtered sample supernatant directly.
Measure NADH: Take amount of filtered sample supernatant into EP tube, heat at 60 ℃ for 30 min, and cool with running water for detection.

**Dilution of sample:**

The recommended dilution factor for different samples is as follows (for reference only):

| Sample type | Dilution factor |
|---|---|
| 10% Mouse muscle tissue homogenate | 1 |
| 10% Mouse kidney tissue homogenate | 1 |
| 293T cell | 1 |
| Hela cell | 1 |

**Note:** The diluent is extracting solution. For the dilution of other sample types, please do pretest to confirm the dilution factor.

## 9. Operation Steps

1. Standard well: Take 20 µL of standard solution with different concentrations into corresponding standard wells.
   Sample well: Take 20 µL of sample supernatant into corresponding sample wells.
2. Take 120 µL of reaction working solution into each well.
3. Add 40 µL of chromogenic agent to each well.
4. Mix fully with microplate reader for 5 s and incubate at 37℃ for 30 min. Measure the OD value of each well at 450 nm with microplate reader.

## 10. Calculations

**The standard curve:**

1. Average the duplicate reading for each standard.
2. Subtract the mean OD value of the blank (Standard # 1) from all standard readings. This is the absoluted OD value.
3. Plot the standard curve by using absoluted OD value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve (y = ax + b) with graph software (or EXCEL).

**The sample:**

**1.** For total content of NAD+ and NADH:

$$[NAD]total\ (\mu mol/gprot) = (\Delta A - b) \div a \times f \div C_{pr}$$

**2.** For NADH:

$$[NADH]\ (\mu mol/gprot) = (\Delta A - b) \div a \times f \div C_{pr}$$

**3.** For NAD+:

$$[NAD+]\ (\mu mol/gprot) = [NAD]total - [NADH]$$

**4.** For NAD+/ NADH:

$$[NAD+]/[NADH] = ([NAD] - [NADH])/[NADH] \times 100\%$$

[Note]
f: Dilution factor of sample before test.
ΔA: ODSample – ODBlank (ODBlank is the OD value when the standard concentration is 0).
$C_{pr}$: Concentration of protein in sample supernatant before filter, gprot/L.

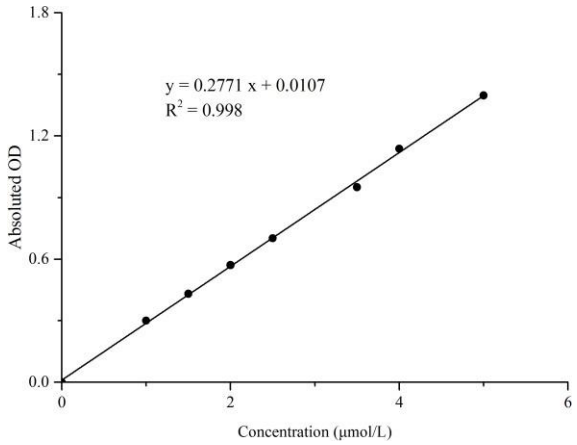
# 11. Performance Characteristics

| | |
|---|---|
| **Detection Range** | 0.02-5.0 umol/L |
| **Sensitivity** | 0.02 umol/L |
| **Average recovery rate (%)** | 90 |
| **Average inter-assay CV (%)** | 9.1 |
| **Average intra-assay CV (%)** | 1.8 |

## Standard curve:

As the OD value of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique or temperature effects), so the standard curve and data are provided as below for reference only:

| **Concentration (µmol/L)** | **0** | **1.0** | **1.5** | **2.0** | **2.5** | **3.0** | **4.0** | **5.0** |
|---|---|---|---|---|---|---|---|---|
| **Average OD** | 0.153 | 0.454 | 0.584 | 0.724 | 0.854 | 1.103 | 1.290 | 1.550 |
| **Absoluted OD** | 0.000 | 0.301 | 0.431 | 0.571 | 0.701 | 0.950 | 1.137 | 1.397 |



## Analysis:

For mouse muscle tissue, take 20 µL of 10% mouse muscle tissue homogenate, and carry the assay according to the operation steps. The results are as follows:
Standard curve: y = 0.2771 x + 0.0107, the average OD value of the blank is 0.158, the average OD value of the sample for NADtotal is 0.565, the average OD value of the sample for NADH is 0.466, the concentration of protein in sample is 2.80 gprot/L, and the calculation result is:

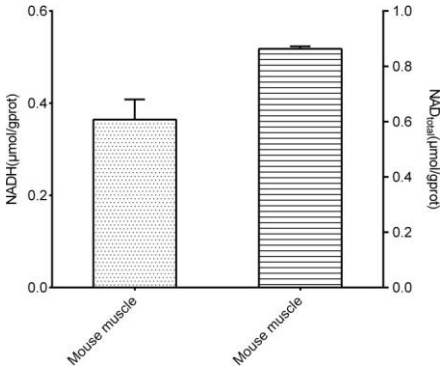
[NAD]total (µmol/gprot) = (0.565 – 0.158 – 0.0107) ÷ 0.2771 ÷ 2.80 = 0.510 µmol/gprot

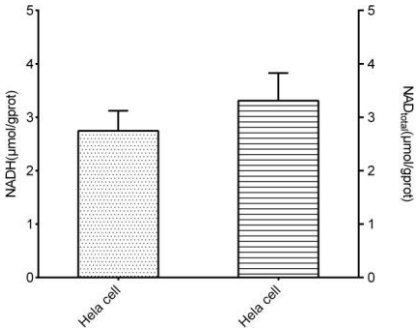
[NADH] (µmol/gprot) = (0.466 – 0.158 – 0.0107) ÷ 0.2771 ÷ 2.80 = 0.383 µmol/gprot

[NAD+] (µmol/gprot) = 0.510- 0.383 = 0.127 µmol/gprot

[NAD+] / [NADH] = (0.510 – 0.383) ÷ 0.383 ×100% = 33.2%

Detect 10% mouse muscle tissue homogenate (the concentration of protein is 2.80 gprot/L) and Hela cell (the concentration of protein is 0.05 gprot/L) according to the protocol, the result is as follows:



## Safety Notes

Some of the reagents in the kit contain dangerous substances. Prevent touching skin and clothing.

Wash immediately with plenty of water if touching it carelessly.

All the samples and waste material should be treated according to the relevant rules of laboratory's biosafety.

Before the experiment, read the instructions carefully, and wear gloves and work clothes.

## Notes:



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