TECHNICAL MANUAL

NAD+/NADH Colorimetric Assay Kit

- **SKU CODE:** MAES0233
- **SIZE:** 96 Tests
- **DETECTION PRINCIPLE:** Assay Kit
- **RUO:** Research-Use-Only

Revised: 01/2026

1. Assay summary

- Reagent preparation
- Sample preparation
- Standard curve preparation
- Add standards and samples
- Measure optical density at 450 nm
- ![img-0.jpeg](img-0.jpeg)

2. Intended use

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

3. Detection principle

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.

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 measuring the OD value at 450 nm.

Detect NADH

After treating sample, heat at 60°C 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 measuring 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 from the first two steps as well as the separate content of NADH.

Note: NADP+ and NADPH have no effect on the determination results.

4. Kit components & storage

Item	Component	Size (96 T)	Storage
Reagent 1	Extracting Solution	60 mL x 2 vials	-20°C, 12 months
Reagent 2	Buffer Solution	16 mL x 1 vial	-20°C, 12 months
Reagent 3	Chromogenic Agent	5 mL x 1 vial	-20°C, 12 months, shading light
Reagent 4	Enzyme Reagent	Powder x 2 vials	-20°C, 12 months, shading light
Reagent 5	Standard	Powder x 2 vials	-20°C, 12 months, shading light
	Microplate	96 wells	No requirement
	Plate Sealer	2 pieces	

5. Materials prepared by users

Instruments:

Micropipettor, 37°C water bath, Microplate reader (450 nm), 10 KD filter tubes

Reagents:

Double distilled water, PBS (0.01 M, pH 7.4)

6. 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°C 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 7 days 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.

7. Sample preparation

1. **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°C. 4. Centrifuge at 10000×g for 10 min at 4°C to remove insoluble material. Collect supernatant and keep it on ice for detection. 5. Meanwhile, determine the protein concentration of supernatant (MAES0177). 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 an ultrasonic cell disruptor at 4°C. 4. Centrifuge at 12000×g for 10 min at 4°C to remove insoluble material. Collect supernatant and keep it on ice for detection. 5. Meanwhile, determine the protein concentration of supernatant (MAES0177).
2. **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°C 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°C for 30 min, and cool with running water for detection.
3. **Dilution of sample:** The recommended dilution factor for different samples is as follows (for reference only): 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.

8. The key points of the assay

1. Keep enzyme working solution at 2-8°C with shading light for 5 h before use. Prepare in advance.
2. The sample must be fresh.
3. Heat the prepared sample at 60°C 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.

9. Operating 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°C for 30 min. Measure the OD value of each well at 450 nm with microplate reader.

10. Calculation

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 absolute OD value.

3. Plot the standard curve by using absolute 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] (μmol/gprot) = (ΔA - b) ÷ a × f ÷ C_{pr}

3. For NAD+:

[NAD+] (μmol/gprot) = [NAD]_total - [NADH]

4. For NAD+/NADH:

[NAD+]/[NADH] = ([NAD]_total - [NADH])/[NADH] × 100%

[Note]

f: Dilution factor of sample before test.

ΔA: OD_{Sample} – OD_{Blank} (OD_{Blank} is the OD value when the standard concentration is 0).

C_{pr}: Concentration of protein in sample supernatant before filter, gprot/L.

11. Appendix I Performance Characteristics

Parameter:

Intra-assay Precision

Three mouse kidney tissue samples were assayed in replicates of 20 to determine precision within an assay (CV = Coefficient of Variation).

Intra-assay Precision

Parameters	Sample 1	Sample 2	Sample 3
Mean (μmol/L)	0.55	2.10	4.30
%CV	2.3	1.7	1.4

Inter-assay Precision

Three mouse kidney tissue samples were assayed 20 times in duplicate by three operators to determine precision between assays.

Inter-assay Precision

Parameters	Sample 1	Sample 2	Sample 3
Mean (μmol/L)	0.55	2.10	4.30
%CV	8.5	9.2	9.6

Recovery

Three samples of high concentration, middle concentration and low concentration were tested, with each concentration tested 6 times in parallel to get an average recovery rate of 90%.

Recovery

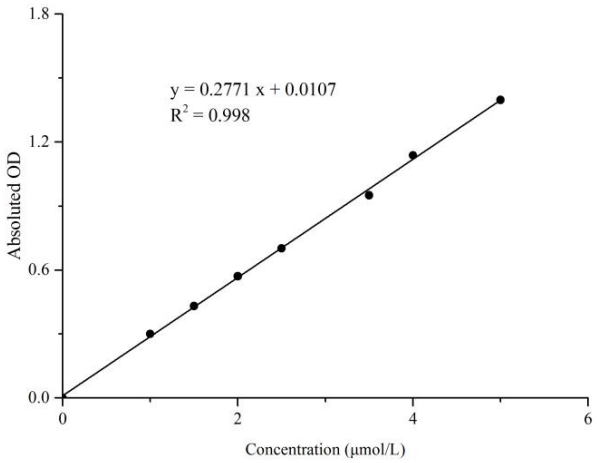
	Standard 1	Standard 2	Standard 3
Expected Conc. (µmol/L)	1.2	2.4	3.8
Observed Conc. (µmol/L)	1.1	2.2	3.4
Recovery rate (%)	89	92	89

Sensitivity

The analytical sensitivity of the assay is 0.02 µmol/L. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the corresponding concentration.

2. 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), the standard curve and data are provided below for reference only:



Standard curve data

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

12. Appendix II Example Analysis

Example analysis:

For mouse muscle tissue, take 20 µL of 10% mouse muscle tissue homogenate, and carry out 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 NAD_{total} 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:

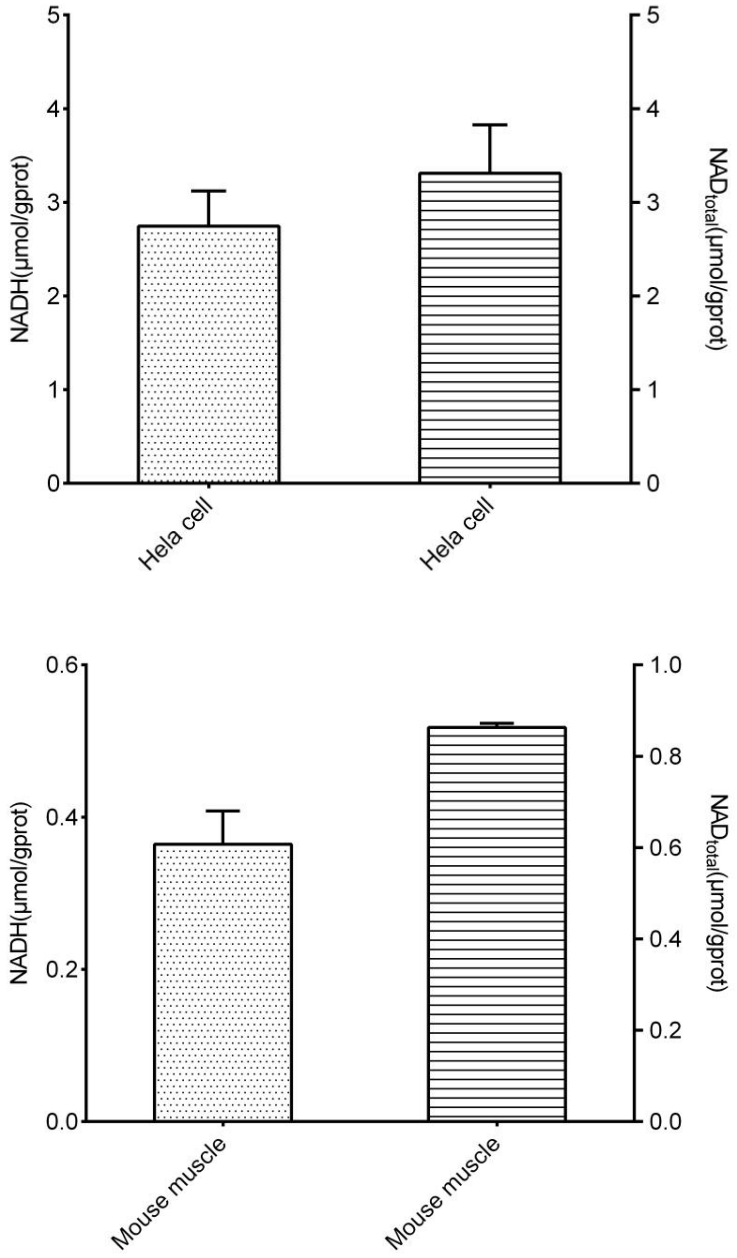
[NAD]_total (µmol/gprot) = (0.565 – 0.158 – 0.0107) ÷ 0.2771 ÷ 2.80 = 0.510 µmol/gprot

[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:



13. Statement

1. This assay kit is for Research Use Only. Assay Genie assumes no responsibility for any problems or legal liabilities arising from the use of this kit for clinical diagnosis or any other purpose.
2. Please read the instructions carefully and calibrate the instruments before performing the experiments. Follow the instructions strictly throughout the procedure.

3. Appropriate protective measures must be taken, including wearing a lab coat and latex gloves.
4. If the concentration of the substance falls outside the detection range, perform an additional dilution or concentration step on the sample.
5. It is recommended to perform a pre-test if your sample type is not listed in the instruction manual.
6. Experimental results are closely related to reagent quality, operator technique, environmental conditions, and other factors. Assay Genie guarantees the quality of the kits only and is NOT responsible for sample consumption resulting from use of the assay kits. It is advisable to estimate the expected sample usage and reserve sufficient samples before starting the experiment.

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Manufacturers Statement: This final kit system is assembled and quality-released by Assay Genie Limited.