（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Catalog No：E-BC-K804-M

Specification：96T(80 samples)

Measuring instrument：Microplate reader（450 nm）

Detection range：0.02-5.0 μmol/L

Elabscience®NAD^+/NADH Colorimetric Assay Kit (WST-8)

This manual must be read attentively and completely before using this product.

If you have any problem, please contact our Technical Service Center for help：

Toll-free: 1-888-852-8623

Tell: 1-832-243-6086

Fax: 1-832-243-6017

Email: techsupport@elabscience.com

Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

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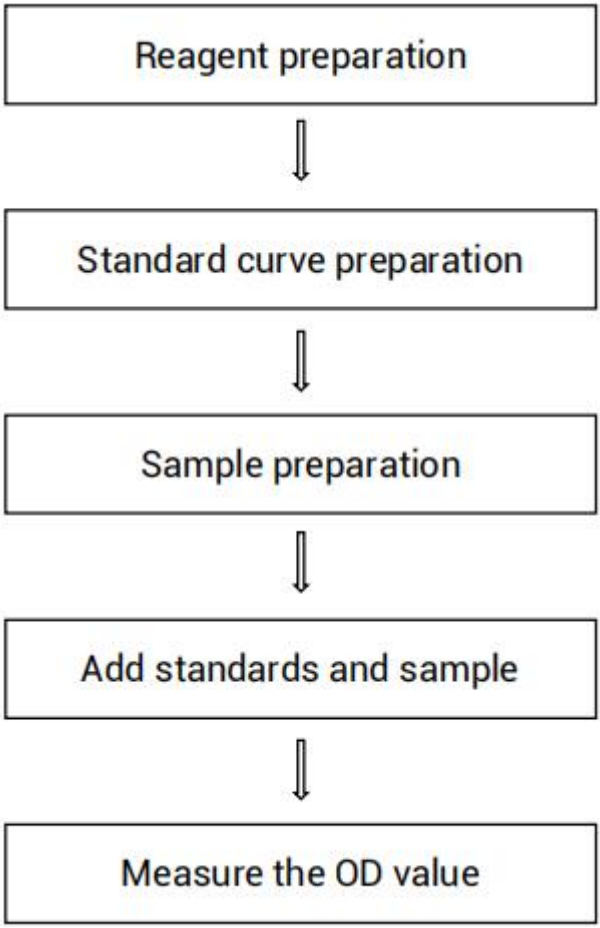
Assay summary



Intended use

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

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 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.

Kit components & storage

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

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 prepared by users

Instruments:

Micropipettor, 37℃ water bath, Microplate reader (450 nm), 10 KD filters tube

Reagents:

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

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

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℃.

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 (E-BC-K318-M).

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 (E-BC-K318-M).

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℃ 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.

③ **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.

The key points of the assay

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..

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℃ for 30 min. Measure the OD value of each well at 450 nm with microplate reader.

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 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:

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

2. For NADH:

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

3. For NAD^+:

$$\frac{[NAD^+]}{(\mu mol/gprot)} = [NAD]_{total} - [NADH]$$

4. For NAD^+/ NADH:

$$[NAD^+]/[NADH] = ([NAD]_{total} - [NADH])/[NADH] \times 100\%$$

[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.

Appendix I Performance Characteristics

1. 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).

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.

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

Recovery

Take three samples of high concentration, middle concentration and low concentration to test the samples of each concentration for 6 times parallelly to get the average recovery rate of 90%.

	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

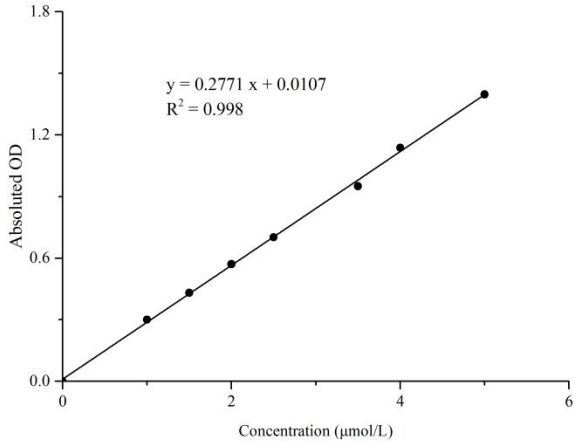
The analytical sensitivity of the assay is 0.02 μmol/L. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the

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), 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



Appendix Π Example Analysis

Example 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 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:

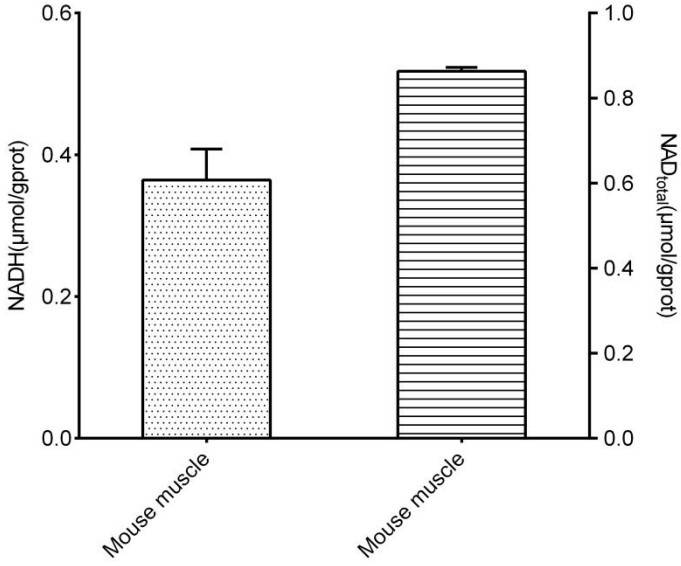
$[NAD]_{total}$ (μmol/gprot) = (0.565 – 0.158 – 0.0107) ÷ 0.2771 ÷ 2.80 = 0.510 μmol/gprot

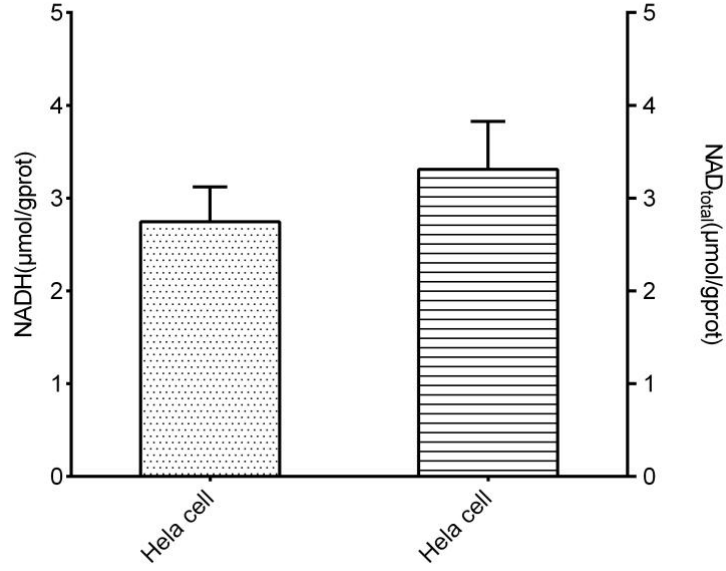
[NADH] (μmol/gprot) = (0.466 – 0.158 – 0.0107) ÷ 0.2771 ÷ 2.80 = 0.383 μmol/gprot

$[NAD^+]$ (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：


0.6
0.4
0.2
0.0
NADH(μmol/gprot)
1.0
0.8
0.6
0.4
0.2
0.0
NADtotal(μmol/gprot)
Mouse muscle
Mouse muscle


5
4
3
2
1
0
NADH(μmol/gprot)
5
4
3
2
1
0
NADtotal(μmol/gprot)
Hela cell
Hela cell

Appendix III Publications

1. Wang Z, Yan C, Wang X, et al. Double-edged sword effects of sulfate reduction process in sulfur autotrophic denitrification system: Accelerating nitrogen removal and promoting antibiotic resistance genes spread[J]. Bioresource Technology, 2024, 409: 131239.
2. Chang H Y, Hsu H C, Fang Y H, et al. Empagliflozin attenuates doxorubicin-induced cardiotoxicity by inhibiting the JNK signaling pathway[J]. Biomedicine & Pharmacotherapy, 2024, 176: 116759.
3. Fang Y, Tang W Q, Qu S, et al. RBBP7, regulated by SP1, enhances the Warburg effect to facilitate the proliferation of hepatocellular carcinoma cells via PI3K/AKT signaling[J]. Journal of Translational Medicine, 2024, 22(1): 170.
4. Wenhui Y A O, Rongpin T A O, Kai W, et al. Icariin attenuates vascular endothelial dysfunction by inhibiting inflammation through GPER/Sirt1/HMGB1 signaling pathway in type 1 diabetic rats[J]. Chinese Journal of Natural Medicines, 2024, 22(4): 293-306.
5. Chen J, Deng X, Lin T, et al. Ferrostatin-1 reversed chronic intermittent hypoxia-induced ferroptosis in aortic endothelial cells via reprogramming mitochondrial function[J]. Nature and Science of Sleep, 2024: 401-411.
6. Gu W, Cong X, Pei Y, et al. Impaired Mitochondrial Energy Metabolism Regulated by p70S6K: A Putative Pathological Feature in Alzheimer's Disease[J]. Metabolites, 2024, 14(7): 369.

Statement

1. This assay kit is for Research Use Only. We will not response for any arising problems or legal responsibilities causing by using the kit for clinical diagnosis or other purpose.
2. Please read the instructions carefully and adjust the instruments before the experiments. Please follow the instructions strictly during the experiments.
3. Protection methods must be taken by wearing lab coat and latex gloves.
4. If the concentration of substance is not within the detection range exactly, an extra dilution or concentration should be taken for the sample.
5. It is recommended to take a pre-test if your sample is not listed in the instruction book.
6. The experimental results are closely related to the situation of reagents, operations, environment and so on. Elabscience will guarantee the quality of the kits only, and NOT be responsible for the sample consumption caused by using the assay kits. It is better to calculate the possible usage of sample and reserve sufficient samples before use.