CheKine™ Micro Coenzyme Ⅰ NAD(H) Assay Kit

Cat #: KTB1020 Size: 48 T/96 T

	Micro Coenzyme Ⅰ NAD(H) Assay Kit		
REF	**Cat #:** KTB1020	LOT	**Lot #:** Refer to product label
	Detection range: 0.78 μM-50 μM		**Sensitivity:** 0.78 μM
	Applicable samples: Animal Tissues, Plant Tissues, Cells, Bacteria, Serum (Plasma)		
	Storage: Stored at -20℃ for 6 months, protected from light		

Assay Principle

Nicotinamide adenine dinucleotide (NAD) is a coenzyme found in all cells, including two forms, NAD^+ (oxidized) and NADH (reduced). NAD^+ is not only a coenzyme that transfers electrons during REDOX reaction, but also a substrate for many enzymes to participate in intracellular reactions. NAD^+ plays an important role in cells and in vivo. Its synthesis, degradation and its products are participated in apoptosis, metabolism regulation and gene expression regulation, and the reduction of NAD^+ is one of the main factors of cell death. The importance of NAD^+ in regulating the REDOX state of cells and its function in regulating signaling pathways and transcription make NAD^+, its synthesis and consumption enzymes to be potential drug targets for a variety of diseases. CheKine™ Micro Coenzyme Ⅰ NAD(H) Assay Kit is a WST-8 based chromogenic reaction that uses colorimetry to measure the amount, ratio, and total amount of NAD^+(oxidized coenzyme Ⅰ) and NADH(reduced Coenzyme Ⅰ) in cells, tissues, or other samples. The NAD^+/NADH assay kit is based on an enzyme cycle reaction (which does not recognize $NADP^+$/NADPH) in which NAD^+ is reduced to NADH and NADH reduces WST-8 to orange formazan with a maximum absorption peak detected at around 450 nm. The formation of Formazan in the reaction system is proportional to the total amount of NAD^+ or NADH in the sample.

Materials Supplied and Storage Conditions

Kit components	Size		Storage conditions
	48 T	96 T	
Assay Buffer	7.5 mL	15 mL	4℃
EtOH Solution	1 mL	2 mL	4℃
WST-8	300 μL	600 μL	-20℃, protected from light
Enhancer	60 μL	120 μL	-20℃, protected from light
NAD Cycling Enzyme Mix	60 μL	120 μL	-20℃, protected from light

NAD Standard (10 mM)	100 µL	200 µL	-20℃, protected from light
NAD Extraction Buffer	6 mL	12 mL	4℃
NADH Extraction Buffer	6 mL	12 mL	4℃

Materials Required but Not Supplied

- Microplate reader or visible spectrophotometer capable of measuring absorbance at OD450 nm
- Water bath, ice maker, refrigerated centrifuge
- 96-well plate or microglass cuvette, precision pipettes, disposable pipette tips
- Deionized water
- Dounce homogenizer (for tissue samples)

Reagent Preparation

Note: Briefly centrifuge small vials at low speed prior to opening.

Assay Buffer: Ready to use as supplied. Equilibrate to room temperature before use. Store at 4℃.

EtOH Solution: Ready to use as supplied. Equilibrate to room temperature before use. Store at 4℃.

WST-8: Ready to use as supplied. Keep on ice protected from light during the assay. Store aliquots at -20℃, protected from light.

Enhancer: Ready to use as supplied. Keep on ice protected from light during the assay. Store aliquots at -20℃, protected from light.

NAD Cycling Enzyme Mix: Ready to use as supplied. Keep on ice protected from light during the assay. Store aliquots at -20℃, protected from light.

NAD Standard: Ready to use as supplied. Keep on ice protected from light during the assay. Store aliquots at -20℃, protected from light.

NAD Extraction Buffer: Ready to use as supplied. Equilibrate to room temperature before use. Store at 4℃.

NADH Extraction Buffer: Ready to use as supplied. Equilibrate to room temperature before use. Store at 4℃.

Working Reagent: For each well of reaction, prepare 85 µL Working Reagent by mixing 68 µL Assay Buffer, 1 µL NAD Cycling Enzyme Mix, 5 µL WST-8, 1 µL Enhancer. The mixtures were preincubated 5 min at room temperature then add 10 µL EtOH Solution. Working Reagent is freshly prepared.

Calibration NAD Curve: Prepare 1,000 µL 50 µM NAD Premix by mixing 5 µL 10 mM Standard and 995 µL Assay Buffer. Dilute standard as follows.

Num.	Volume of Standard (µL)	Assay Buffer (µL)	Concentration (µM)
Std.1	5 µL 10 mM NAD	995	50
Std.2	100 µL of Std.1 (50 µM)	100	25
Std.3	100 µL of Std.2 (25 µM)	100	12.5
Std.4	100 µL of Std.3 (12.5 µM)	100	6.25
Std.5	100 µL of Std.4 (6.25 µM)	100	3.13
Std.6	100 µL of Std.5 (3.13 µM)	100	1.56
Std.7	100 µL of Std.6 (1.56 µM)	100	0.78

Sample Preparation

1. **Extraction method of tissue samples:** after washing the tissue in pre-chilled PBS on ice, weigh about 20 mg of tissue

sample, cut with scissors and place in homogenizer.

Extraction of NAD: Samples were homogenized by adding 100 μL NAD Extraction Buffer. Heat extracts at 100℃ for 5 min and then add 20 μL Assay Buffer and 100 μL NADH Extraction Buffer to neutralize the extracts. Centrifuge at 14,000 rpm for 5 min at 4℃, and the supernatant was transferred to a new centrifuge tube and place it on ice to be tested.

Extraction of NADH: Samples were homogenized by adding 100 μL NADH Extraction Buffer. Heat extracts at 100℃ for 5 min and then add 20 μL Assay Buffer and 100 μL NAD Extraction Buffer to neutralize the extracts. Centrifuge at 14,000 rpm for 5 min at 4℃, and the supernatant was transferred to a new centrifuge tube and place it on ice to be tested.

2. **Extraction method for cells or bacteria samples:** collect cells or bacteria samples (sample size is recommended to be 1 x 10^6 cells /time), wash the cells or bacteria with cold PBS, centrifuge at low speed for 5 min, and discard the supernatant.

Extraction of NAD: Add 100 μL NAD Extraction Buffer and ultrasonic crushing was performed for 5 min (20% or 200 W, ultrasonic 3 s, interval 7 s, repeat 30 times). Heat extracts at 100℃ for 5 min and then add 20 μL Assay Buffer and 100 μL NADH Extraction Buffer to neutralize the extracts. Centrifuge at 14,000 rpm for 5 min at 4℃, and the supernatant was transferred to a new centrifuge tube and place it on ice to be tested.

Extraction of NADH: Add 100 μL NADH Extraction Buffer and ultrasonic crushing was performed for 5 min (20% or 200 W, ultrasonic 3 s, interval 7 s, repeat 30 times). Heat extracts at 100℃ for 5 min and then add 20 μL Assay Buffer and 100 μL NAD Extraction Buffer to neutralize the extracts. Centrifuge at 14,000 rpm for 5 min at 4℃, and the supernatant was transferred to a new centrifuge tube and place it on ice to be tested.

3. **Extraction method of serum (plasma) samples:**

Extraction of NAD: Take about 20 μL serum (plasma), and add 100 μL NAD Extraction Buffer. Heat extracts at 100℃ for 5 min and then add 20 μL Assay Buffer and 100 μL NADH Extraction Buffer to neutralize the extracts. Centrifuge at 14,000 rpm for 5 min at 4℃, and the supernatant was transferred to a new centrifuge tube and place it on ice to be tested.

Extraction of NADH: Take about 20 μL serum (plasma), and add 100 μL NADH Extraction Buffer. Heat extracts at 100℃ for 5 min and then add 20 μL Assay Buffer and 100 μL NAD Extraction Buffer to neutralize the extracts. Centrifuge at 14,000 rpm for 5 min at 4℃, and the supernatant was transferred to a new centrifuge tube and place it on ice to be tested.

Note: We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80℃. When you are ready to test your samples, thaw them on ice. However, this might affect the stability of your samples and the readings can be lower than expected. For additional measurement, it is recommended to use Abbkine Protein Quantification Kit (BCA Assay) (Cat #: KTD3001).

Assay Procedure

1. Preheat the microplate reader or visible spectrophotometer for more than 30 min, and adjust the wavelength to 450 nm. Visible spectrophotometer was returned to zero with deionized water.

2. Add the following reagents to the 96-well plate or microglass cuvette:

Reagent	Blank Well (μL)	Standard Well (μL)	Test Well (μL)
Assay Buffer	40	0	0
Different Concentration of Standard	0	40	0
Sample	0	0	40
Working Reagent	80	80	80

3. Mix Well. Incubate at room temperature for 30 min, and the record the absorbance values as A. Calculate $\Delta A_{Test}=A_{Test}-A_{Blank}$, $\Delta A_{Standard}=A_{Standard}-A_{Blank}$.

Note: Addition of Working Reagent should be quick and mixing should be brief but thorough. Use of multi-channel pipettor is recommended; The following substances interfere and should be avoided in sample preparation: EDTA (>0.5 mM), ascorbic acid, SDS (>0.2%), sodium azide, NP-40 (>1%) and Tween-20 (>1%).

Data Analysis

Note: We provide you with calculation formulae, including the derivation process and final formula. The two are exactly equal. It is suggested that the concise calculation formula in bold is final formula.

1．Drawing of standard curve

With the concentration of the standard solution as the y-axis and the $\Delta A_{Standard}$ as the x-axis, draw the standard curve. Use the ΔA_{Test} values to determine sample NAD/NADH concentration y from the standard curve.

Note: If the $\Delta\Delta A_{Test}$ of the sample is higher than the $\Delta A_{Standard}$ of the 50 μM standard, dilute the sample with deionized water before performing the experiment.

2．Calculation of NAD/NADH content

(1) By sample fresh weight

NAD/NADH (nmol/g) $=(y\times V_{Sample})\div(W\times V_{Sample}\div V_{Sample\ Total})\times n$**$=0.22\times y\div W\times n$**

(2) By protein concentration

NAD/NADH (nmol/mg prot)$=(y\times V_{Sample})\div(V_{Sample}\times Cpr)\times n$**$=y\div Cpr\times n$**

(3) By number of cells or bacteria

NAD/NADH (nmol/10^4)$=(y\times V_{Sample})\div(number\times V_{Sample}\div V_{Sample\ Total})\times n$**$=0.22\times y\div number\times n$**

(4) By number of serum (plasma)

NAD/NADH (nmol/mL)$=y\times(V_{Extraction}+V_{Sample\ Total})\div V_{Extraction}\times n$**$=12\times y\times n$**

Where: nmol,1μM=1 nmol/mL; V_{Sample}: add sample volume, 0.04 mL; $V_{Sample\ Total}$: Total volume of sample, 0.22 mL;$V_{Extraction}$: volume of sample Extraction, 0.02 mL; Cpr: The protein concentration of the sample, mg/mL; W: Weight of sample, g; Number: the number of cells or bacteria; n: The sample dilution factor.

Typical Data

Typical standard curve-data provided for demonstration purposes only. A new standard curve must be generated for each assay.

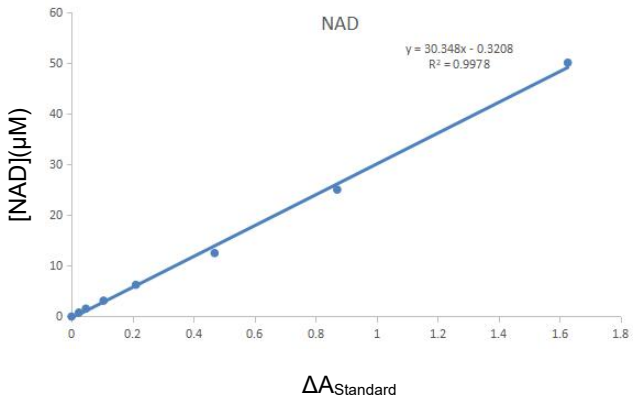

Figure 1. Standard Curve of NAD

Note: At these concentrations, the standard curves for NAD and NADH are identical. we provide only NAD as the standard.

Recommended Products

Catalog No.	Product Name
KTB1010	CheKine™ Micro Coenzyme II NADP(H) Assay Kit
KTB1021	CheKine™ Micro NADH Oxidase (NOX) Assay Kit

Disclaimer

The reagent is only used in the field of scientific research, not suitable for clinical diagnosis or other purposes.