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

Catalog No：E-BC-K815-M

Specification：48T (24 samples)/96T (48 samples)

Measuring instrument：Microplate reader(440-460 nm)

Detection range：0.27-43.43 U/L

Elabscience® NADPH Oxidase (NAO) Activity Colorimetric Assay Kit

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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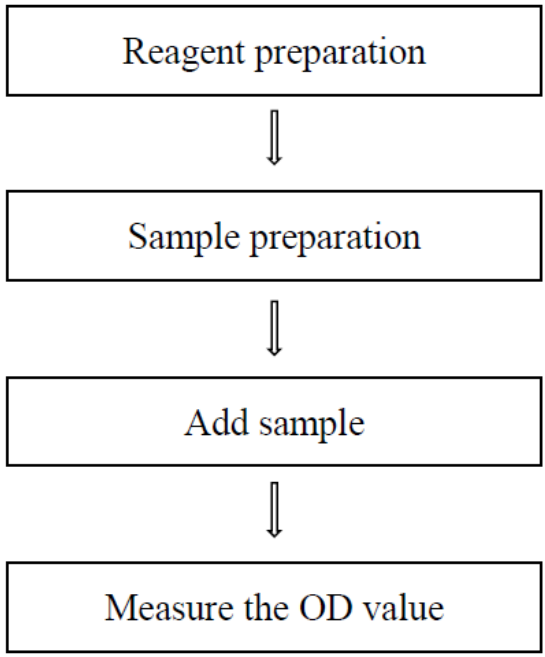
Assay summary



Intended use

This kit can be used to measure NADPH oxidase (NAO) activity in cells, animal and plant tissues samples.

Detection principle

NADPH Oxidase (NAO) is a membrane protein mainly found in mammalian neutrophils, plant cells and filamentous fungal cells, and was initially discovered in phagocytic cell membranes. NAO can oxidize NADPH to NAD^+ while generating superoxide anions, and it is one of the main sources of reactive oxygen species (ROS) in the body. NAO is involved in many important physiological processes, including host defense, post-translational modification of proteins, cell signal transduction, gene expression regulation and cell differentiation.

The detection principle of this kit is as follows: NAO catalyzes the substrate to produce product, which react with chromogenic agent to form chromogenic substance. It has the maximum absorption at 450 nm. The enzyme activity of NAO is calculated by measuring the OD value at 450 nm.

Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Extraction Solution	25 mL × 1 vial	50 mL × 1 vial	-20℃, 12 months
Reagent 2	Buffer Solution	11 mL × 1 vial	22 mL × 1 vial	-20℃, 12 months, shading light
Reagent 3	Inhibitor	0.3 mL × 1 vial	0.6 mL × 1 vial	-20℃, 12 months, shading light
Reagent 4	Substrate	Powder × 1 vial	Powder × 2 vials	-20℃, 12 months, shading light
Reagent 5	Chromogenic Agent	1.5 mL × 1 vial	3 mL × 1 vial	-20℃, 12 months, shading light

	Microplate	48 wells	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 prepared by users

Instruments:

Microplate reader (440-460 nm, optimum wavelength: 450 nm), Incubator

Reagent preparation

① Equilibrate all the reagents to 25°C before use.

② The preparation of substrate working solution:
Dissolve one vial of substrate with 550 μL of double distilled water, mix well to dissolve. Store at -20°C for a week protected from light.

③ The preparation of working solution:
For each well, prepare 80 μL of working solution (mix well 45 μL of buffer solution, 10 μL of substrate working solution and 25 μL of chromogenic agent). The working solution should be prepared on spot protected from light and used up within 30 min.

Sample preparation

① Sample preparation

Tissue sample:

① Harvest the amount of tissue needed for each assay (initial recommendation 20 mg).

② Homogenize 20 mg tissue in 180 μL extraction solution with a dounce homogenizer at 4°C.

③ Centrifuge at 10000×g for 10 min at 4°C to remove insoluble material. Collect supernatant and keep it on ice for detection. The supernatant should be used on the same day.

Cell samples:

① Harvest the number of cells needed for each assay (initial recommendation 1×10^6 cells).

② Homogenize 1×10^6 cells in 200 μL extraction solution with a ultrasonic cell disruptor at 4°C.

③ Centrifuge at 10000×g for 10 min at 4°C to remove insoluble material. Collect supernatant and keep it on ice for detection. The supernatant should be used on the same day.

② Dilution of sample

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

Sample type	Dilution factor
10% Mouse liver tissue homogenate	2-10
10% Mouse spleen tissue homogenate	1
10% Mouse heart tissue homogenate	1
10% Mouse brain tissue homogenate	1
10% Mouse kidney tissue homogenate	1
10% Mouse lung tissue homogenate	1
10% Potato tissue homogenate	1

10% Corn tissue homogenate	1
10% Garlic tissue homogenate	1
10% Broccoli tissue homogenate	1
1×10^6 Hela cells	1
1×10^6 Jurkat cells	1
1×10^6 RAW 264.7 cells	1

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

The key points of the assay

① Equilibrate Inhibitor to 25°C before use. On operating step ②, add 10 μL of inhibitor into control wells. It is not allowed to mix inhibitor and buffer solution together adding, otherwise, a large amount of precipitate will be produced.

② The control wells may have a small amount of precipitation that does not affect the experimental results.

Operating steps

① Sample well: Add 20 μL of samples into wells.
Control well: Add 20 μL of samples into wells.

② Add 10 μL of inhibitor into control wells.

③ Add 100 μL of buffer solution into sample wells. Add 90 μL of buffer solution into control wells.

④ Mix fully for 5 s with microplate reader and incubate at 37°C for 10 min.

⑤ Add 80 μL of working solution into each well.

⑥ Mix fully for 5 s with microplate reader and measure the OD value of each well at 450 nm, as A_1. Incubate at 37°C for 10 min and measure the OD value of each well at 450 nm, as A_2.

Calculation

The sample:

1. Tissue sample:

Definition: The amount of enzyme in 1 kg tissue samples per 1 min that catalyze the production of 1 μmol of product from the substrate at 37 °C is defined as 1 unit.

$$\text{NAO activity (U/kg wet weight)} = \frac{\Delta A_{sample} - \Delta A_{control}}{\varepsilon \times d} \times \frac{V_1}{V_2} \div \frac{m}{V} \div T \times f \times 1000$$

2. Cell sample:

Definition: The amount of enzyme in 1×10^9 cells samples per 1 min that catalyze the production of 1 nmol of product from the substrate at 37 °C is defined as 1 unit.

$$\text{NAO activity (U/10^9)} = \frac{\Delta A_{sample} - \Delta A_{control}}{\varepsilon \times d} \times \frac{V_1}{V_2} \div \frac{n}{V_3} \div T \times f \times 1000$$

[Note]

ΔA_{sample}: $\Delta A_{sample} = A_2 - A_1$.

$\Delta A_{control}$: $\Delta A_{control} = A_2 - A_1$.

ε: The molar extinction coefficient of chromogenic substance at 450 nm, 30.7 L/mmol/cm.

d: Optical path, 0.6 cm.

V_1: The volume of reaction system, μL.

V_2: The volume of sample added to the reaction system, μL.

m: The wet weight of sample, kg.

V: The volume of extraction solution in the preparation step of tissue, L.

T: Reaction time, 10 min.

n: The number of cell sample/10^6.

V_3: The volume of extraction solution in the preparation step of cell, L.

f: Dilution factor of sample before test.

1000：1 mmol/L = 1000 μmol/L

Appendix I Performance Characteristics

1. Parameter:

Intra-assay Precision

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

Parameters	Sample 1	Sample 2	Sample 3
Mean (U/L）	5.00	15.00	30.00
%CV	2.7	4.1	3.5

Inter-assay Precision

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

Parameters	Sample 1	Sample 2	Sample 3
Mean (U/L)	5.00	15.00	30.00
%CV	5.0	9.5	7.6

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 100%.

	Sample 1	Sample 2	Sample 3
Expected Conc. (UL)	2	10	20
Observed Conc. (U/L)	1.90	10.30	20.40
Recovery rate (%)	95	103	102

Sensitivity

The analytical sensitivity of the assay is 0.27 U/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.

Appendix Π Example Analysis

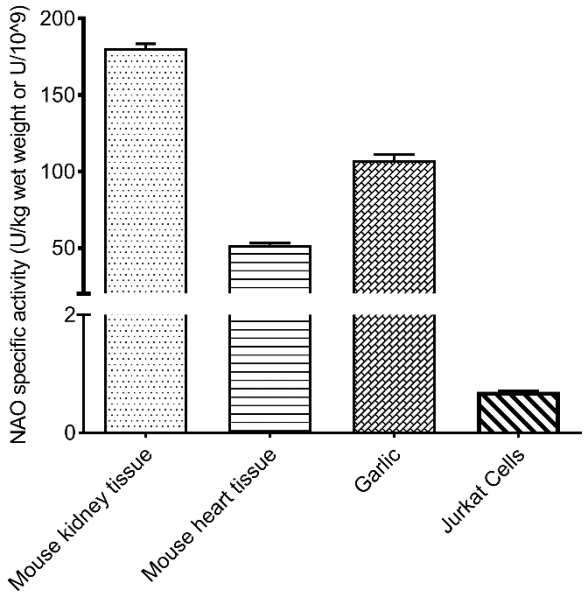
Example analysis：

Take 20 μL of 10% mouse kidney tissue homogenate which dilute 2 times and carry the assay according to the operation steps. The results are as follows:

The A_1 of the sample well is 0.171, the A_2 of the sample well is 0.379, $\Delta A_{sample} = A_2 - A_1 = 0.379 - 0.171 = 0.208$, The A_1 of the control well is 0.202, the A_2 of the control well is 0.225, $\Delta A_{control} = A_2 - A_1 = 0.225 - 0.202 = 0.023$, and the calculation result is:

NAO activity (U/kg wet weight) = (0.208 - 0.023) ÷ (30.7 × 0.6) × (200 ÷ 20) ÷ (0.0001 ÷ 0.0009) ÷ 10 × 2× 1000 = 180.78 U/kg wet weight

Detect 10% mouse kidney tissue homogenate (dilute 2 times), 10% mouse heart tissue homogenate, 10% garlic tissue homogenate and 2×10^6 Jurkat cells, according to the protocol, the result is as follows:



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.