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

Catalog No：E-BC-K863-M

Specification：96T(38 samples)

Measuring instrument：Microplate reader(530-550 nm)

Detection range：0.045-9 μmol/h/g

Elabscience® Nitrite Reductase(NiR) 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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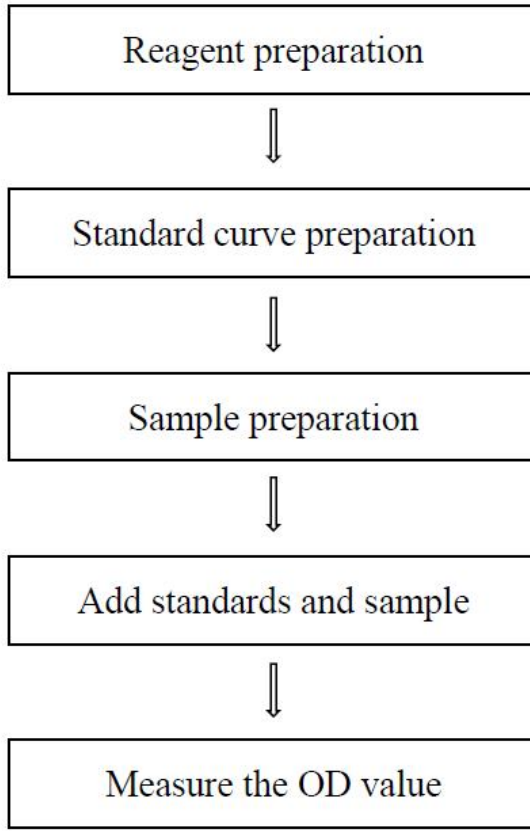
Assay summary



Intended use

This kit can be used to measure nitrite reductase (NiR) activity in plant tissue samples.

Detection principle

Nitrite reductase (NiR) in the sample can reduce NO^{2-} to NO, so that the NO^{2-} involved in the diazotization reaction to produce purplish red compounds is reduced, that is, the change of absorbance value at 540 nm can reflect the activity of the sample nitrite reductase.

Kit components & storage

Item	Component	Size (96 T)	Storage
Reagent 1	Extraction Solution	55 mL × 2 vials	2-8°C, 12 months, shading light
Reagent 2	Buffer A	24 mL × 1 vial	2-8°C, 12 months, shading light
Reagent 3	Buffer B	24 mL × 1 vial	2-8°C, 12 months
Reagent 4	Substrate A	Power × 2 vials	2-8°C, 12 months, shading light
Reagent 5	Substrate B	Powder × 6 vials	2-8°C, 12 months, shading light
Reagent 6	Chromogenic Agent A	10 mL × 1 vial	2-8°C, 12 months, shading light
Reagent 7	Chromogenic Agent B	10 mL × 1 vial	2-8°C, 12 months, shading light
Reagent 8	Standard	Power × 1 vial	2-8°C, 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 prepared by users

Instruments:

Microplate reader (530-550 nm, optimum wavelength: 540 nm), Incubator

Reagents:

PBS (0.01 M, pH 7.4)

Reagent preparation

① Equilibrate all the reagents to 25℃ before use.

② The preparation of standard working solution:
Dissolve one vial of standard with 2 mL of ultrapure water, mix well to dissolve. Store at 2-8°C for 2 weeks protected from light.

③ The preparation of substrate A working solution:
Dissolve one vial of substrate A with 12 mL of buffer A, mix well to dissolve. Store at 2-8°C for 2 weeks protected from light.

④ The preparation of substrate B working solution:
Dissolve one vial of substrate B with 4 mL of buffer B, mix well to dissolve. The substrate B working solution should be prepared on spot and use it up the same day.

⑤ The preparation of chromogenic working solution:
For each well, prepare 160 μL of chromogenic working solution (mix well 80 μL of chromogenic agent A and 80 μL of chromogenic agent B). Store at 2-8°C for 1 day protected from light.

⑥ The preparation of 0.2 μmol/mL standard solution:
Before testing, please prepare sufficient 0.2 μmol/mL standard solution. For example, prepare 1250 μL of 0.2 μmol/mL standard solution (mix well 5 μL of standard working solution and 1245 μL of ultrapure water). The

0.2 μmol/mL standard solution should be prepared on spot.

⑦ The preparation of standard curve：

Always prepare a fresh set of standards. Discard working standard dilutions after use.

Dilute 0.2 μmol/mL standard with extraction solution to a serial concentration. The recommended dilution gradient is as follows: 0, 0.025, 0.05, 0.10, 0.12, 0.14, 0.16, 0.2 μmol/mL. Reference is as follows:

Item	①	②	③	④	⑤	⑥	⑦	⑧
Concentration (μmol/mL)	**0**	**0.025**	**0.05**	**0.10**	**0.12**	**0.14**	**0.16**	**0.2**
0.2 μmol/mL standard (μL)	0	62.5	125	250	300	350	400	500
Extraction Solution (μL)	500	437.5	375	250	200	150	100	0

Sample preparation

① Sample preparation:

Tissue samples:

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℃.

③ Centrifuge at 10000×g for 10 min at 4℃ to remove insoluble material. Collect supernatant and keep it on ice for detection.

④ Meanwhile, determine the protein concentration of supernatant (E-BC-K168-M).

② Dilution of sample

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

Sample type	Dilution factor
10% Corn tissue homogenate	1
10% Cabbages tissue homogenate	1
10% Green pepper tissue homogenate	1
10% Radish tissue homogenate	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 The substrate B working solution should be prepared on spot.

② Sample extraction and centrifugation process at 4℃.

Operating steps

Enzymatic reaction

① Matrix tube: Add 100 μL of ultrapure water to the 1.5 mL EP tubes.
Matrix blank tube: Add 300 μL of ultrapure water to the 1.5 mL EP tubes.
Sample tube: Add 100 μL of sample to the 1.5 mL EP tubes.
Control tube: Add 200 μL of ultrapure water and 100 μL sample to the 1.5 mL EP tubes.

② Add 200 μL of substrate A working solution to matrix tube and sample tube.

③ Add 200 μL of substrate B working solution to each tube.

④ Mix gently, incubate at 37°C for 1 h, then remove the supernatant after vigorous shaking for 30 s, and the chromogenic reaction was carried out.

Chromogenic reaction

① Standard well: Take 80 μL of different concentrations of standard solutions to the corresponding wells.
Matrix well: Take 80 μL of solutions from the matrix tubes to the corresponding wells.
Matrix blank wells: Take 80 μL of solutions from the matrix blank tubes to the corresponding wells.
Sample wells: Take 80 μL of solutions from the sample tubes to the corresponding wells.
Control well: Take 80 μL of solutions from the control tubes to the corresponding wells.

② Add 160 μL of chromogenic working solution to each well.

③ Mix fully with microplate reader for 10 s, standing for 5 min. Measure the OD value of each well at 540 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. Tissue samples(Calculate by tissue wet weight):

Definition: The amount of enzyme in 1 g of tissue that catalyze the substrate to produce 1 μmol NO^{2-} at 37 ℃ for 1 h is defined as 1 unit.

$$\text{nitrite reductase (NiR) activity (μmol/h/g)} = (\Delta A - b) \div a \times V_1 \div V_2 \times V_3 \div m \times f \div t$$

2. Tissue samples(Calculate by protein concentration):

Definition: The amount of enzyme in 1 mg of protein that catalyze the substrate to produce 1 μmol NO^{2-} at 37 ℃ for 1 h is defined as 1 unit.

$$\text{nitrite reductase (NiR) activity (μmol/h/mgprot)} = (\Delta A - b) \div a \times V_1 \div V_2 \div C_{pr} \times f \div t$$

[Note]

ΔA: (the OD value of matrix well - the OD value of matrix blank well) – (the OD value of sample well - the OD value of control well).

m: The tissue wet weight, g.

V_1: The reaction system volume of before taking supernatant, 0.5 mL.

V_2: The volume of sample, 0.1 mL.

V_3: The volume of extraction solution during sample preparation, mL.

f: Dilution factor of sample before test.

C_{pr}: The concentration of protein in sample, mgprot/mL.

t: Reaction time, 1 h.

Appendix I Performance Characteristics

1. Parameter:

Intra-assay Precision

Three corn 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/h/g)	0.56	1.53	4.85
%CV	0.6	0.9	1.8

Inter-assay Precision

Three corn 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/h/g)	0.56	1.53	4.85
%CV	3.5	5.8	7.7

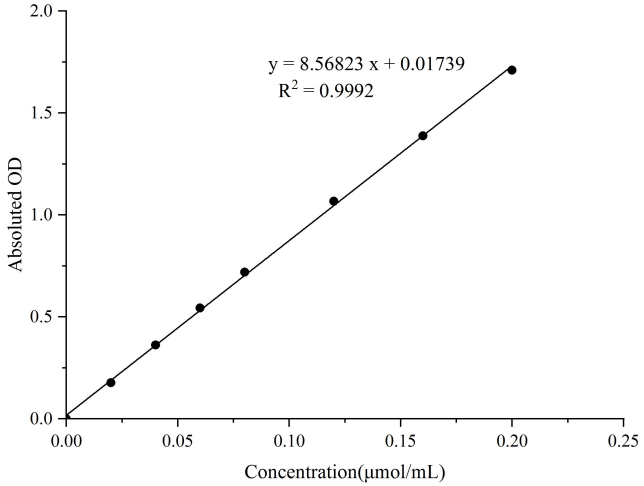
Sensitivity

The analytical sensitivity of the assay is 0.045 μmol/h/g. 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 (mmol/L)	0	0.025	0.05	0.10	0.12	0.14	0.16	0.2
OD	0.039	0.213	0.399	0.586	0.755	1.107	1.426	1.766
	0.037	0.218	0.400	0.577	0.759	1.102	1.426	1.729
Average OD	0.038	0.215	0.400	0.582	0.757	1.105	1.426	1.748
Absoluted OD	0.000	0.177	0.362	0.544	0.719	1.067	1.388	1.710



Appendix II Example Analysis

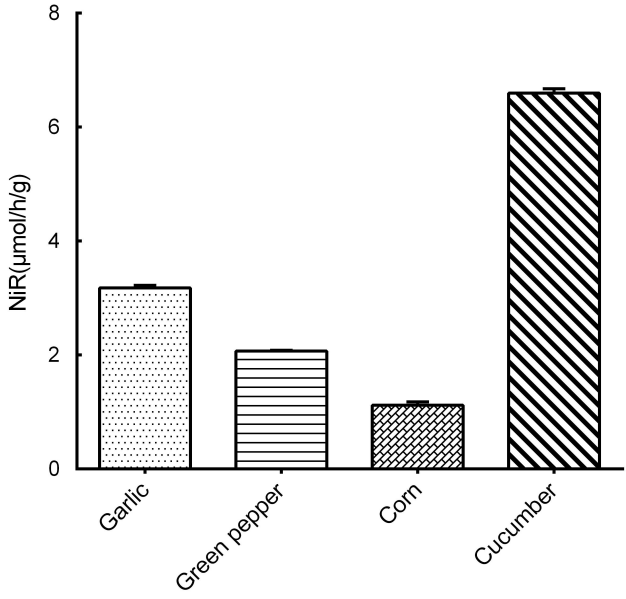
Example analysis:

Take 100 μL of epipremnum aureum tissue homogenate, carry the assay according to the operation steps. The results are as follows:

Standard curve: y = 8.56823x + 0.01739, the average OD value of the matrix wells is 1.134, the average OD value of the matrix blank wells is 0.043, the average OD value of the sample wells is 0.239, the average OD value of the control wells is 0.040, ΔA= (1.134-0.043) - (0.239-0.040) = 0.892and the calculation result is:

$$\text{NiR activity (μmol/h/g)} = (0.892-0.01739) \div 8.56823 \times 0.5 \div 0.1 \times 0.9 \div 0.1 \div 1 = 4.59\ \mu\text{mol/h/g}$$

Detect 10% garlic tissue homogenate, 10% green pepper tissue homogenate, 10% corn tissue homogenate, 10% cucumber tissue homogenate, 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.