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

Catalog No：E-BC-K001-M

Specification：48T(20 samples)/96T(44 samples)

Measuring instrument：Microplate reader（440-460 nm）

Elabscience®Inhibition And Production Of Superoxide Anionic Colorimetric Assay Kit(WST-1 Method)

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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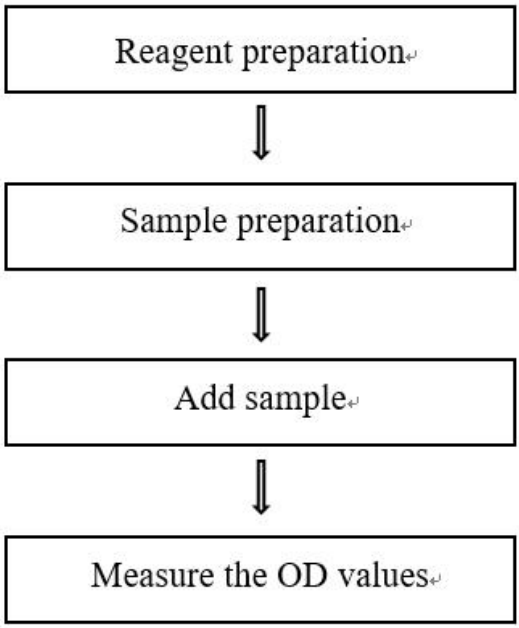
Assay summary



Intended use

This kit can be used to measure the activity of inhibition of superoxide anion radical in serum, plasma, urine, cells and cellular supernatant or the activity of production of superoxide anion radical in leucocyte samples.

Detection principle

Superoxide anion free radicals are produced through the reaction system of xanthine and xanthine oxidase. WST-1 (a water-soluble tetrazolium salt) can react with the generated superoxide anion to produce water-soluble formazan. When the tested sample contains the superoxide anion free radical inhibitor, it can inhibit the formation of formazan. When the tested sample contains the substance that produces superoxide anion free radical, it can promote the formation of formazan dye. By colorimetric analysis of WST-1 products, the units of activity of inhibition or production of superoxide anion radical in samples can be calculated.

Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Buffer Solution	12 mL × 1 vial	24 mL × 1 vial	2-8℃, 12 months
Reagent 2	Substrate Solution	0.07 mL × 1 vial	0.14 mL × 1 vial	2-8℃, 12 months shading light
Reagent 3	Enzyme Stock Solution	0.15 mL × 1 vial	0.3 mL × 1 vial	-20℃, 12 months
Reagent 4	Enzyme Diluent	1.5 mL × 1 vial	1.5 mL × 2 vials	2-8℃, 12 months
Reagent 5	VC Standard	Powder × 3 vials	Powder × 3 vials	-20℃, 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 (440-460 nm), Micropipettor, Multi-channel pipettor, Incubator, Vortex mixer, Centrifuge

Reagents:

Double distilled water, Normal saline (0.9% NaCl), PBS (0.01 M, pH 7.4)

Reagent preparation

① Keep enzyme stock solution on ice for use. Equilibrate all the reagents to room temperature before use

② The preparation of enzyme working solution：
Before testing, please prepare sufficient enzyme working solution according to the test wells. For example, prepare 55μL of enzyme working solution (mix well 5 μL of enzyme stock solution and 50 μL of enzyme diluent). Store at 2-8°C for 3 days. (enzyme stock solution should melt slowly on ice. It is recommended to aliquot the enzyme stock solution into smaller quantities for optimal storage. Avoid repeated freeze-thaw cycles.)

③ The preparation of substrate application solution：
Before testing, please prepare sufficient substrate application solution according to the test wells. For example, prepare 1005μL of substrate application solution (mix well 5 μL of substrate solution and 1000 μL of buffer solution). Store at 2-8°C for 7 days.

④ The preparation of 5 mg/mL standard solution：
Dissolve one vial of VC standard with 1 mL of double distilled water, mix well.

⑤ The preparation of 0.05 mg/mL standard solution：
Before testing, please prepare sufficient 0.05 mg/mL standard solution according to the test wells. For example, prepare 100 μL of 0.05 mg/mL

standard solution (mix well 1 μL of 5 mg/mL standard solution and 99 μL of double distilled water). VC standard is easy to oxidized, it is best to use within 30 min.

Sample preparation

① Sample preparation

Samples should not contain decontamination agents such as SDS, Tween-20, NP-40, Triton X-100, nor reductive reagents such as DTT, 2- mercaptoethanol.

Serum and plasma: detect directly. If not detected on the same day, the serum or plasma can be stored at -80°C for a month.

Saliva: Gargle with clear water, collect the saliva 30 min later, centrifuge at 10000×g for 10 min at 4℃. Take the supernatant and preserve it on ice for detection.

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 PBS (0.01 M, pH 7.4) with a dounce homogenizer at 4°C.

④ Centrifuge at 10000×g for 10 min 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 1×10^6 cells).

② Wash cells with PBS (0.01 M, pH 7.4).

③ Homogenize 1×10^6 cells in 300-500 μL PBS (0.01 M, pH 7.4) with a

ultrasonic cell disruptor at 4℃.

④ Centrifuge at 10000×g for 10 min 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 Inhibition ratio of sample

Before the formal experiment, it needs to choose 2-3 samples for diluting a series of diluent and determine the dilution factor when the SOD inhibition ratio is 30%~65% (the optimal inhibition ratio is the range of 40%~60%).

$$\text{Inhibition ratio} = \frac{(A_1 - A_2) - (A_5 - A_6)}{A_1 - A_2} \times 100\%$$

Adjust sampling volume: If inhibition ratio > 65%, need to dilute the sample or decrease the sampling volume than take the test. If inhibition ratio < 30%, need to increase the sampling volume.

③ Dilution of sample

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

Sample type	Dilution factor
Human serum	4-7
Mouse serum	15-25
Rat serum	25-35
Human saliva	1
HepG2 culture supernatant	1
10% Rat brain tissue homogenate	150-200
10% Rat liver tissue homogenate	500-600
10% Mouse liver tissue homogenate	500-600
10% Mouse heart tissue homogenate	150-200
10% Epipremnum aureum tissue homogenate	20-30

Note: The diluent is normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4). For the dilution of other sample types, please do pretest to confirm the dilution factor

The key points of the assay

① In order to reduce errors in different wells, the multi-channel pipettor is recommended.

② VC standard is easy to oxidized, it is best to use the standard solution within 30 min.

③ Prevent the formulation of bubbles when the supernatant is transferred into the microplate.

④ Samples should not contain decontamination agents such as SDS, Tween-20, NP-40, Triton X-100, nor reductive reagents such as DTT, 2- mercaptoethanol.

⑤ Before the formal experiment, it needs to choose one or two samples for diluting a series of diluent and determine the dilution factor when the SOD inhibition ratio is 30%~65% (the optimal inhibition ratio is the range of 40%~60%).

Operating steps

① Control well: add 20 μL of double distilled water and 20 μL of enzyme working solution to the corresponding wells.

$Blank_{control}$ well: add 20 μL of double distilled water and 20 μL of enzyme diluent to the corresponding wells.

Standard well: add 20 μL of 0.05 mg/mL standard solution and 20 μL of enzyme working solution to the corresponding wells.

$Blank_{standard}$ well: add 20 μL of 0.05 mg/mL standard solution and 20 μL of enzyme diluent to the corresponding wells.

Sample well: add 20 μL of sample and 20 μL of enzyme working solution to the corresponding wells.

$Blank_{Sample}$ well: add 20 μL of sample and 20 μL of enzyme diluent to the corresponding wells.

② Add 200 μL of substrate application solution with a multi-channel pipettor into each well and mix fully.

③ Incubate at 37℃ for 20 min. Measure the OD values of each well with microplate reader at 450 nm.

Calculation

1. Calculation formula for the activity of inhibition of superoxide anion radical in serum, plasma, cellular supernatant:

Definition: In the reaction system, the amount of superoxide anion radical inhibited by 1 L of sample in 20 min at 37℃ that equivalent to inhibited by 1 mg of VC is defined as 1 unit.

$$\text{The inhibition of superoxide anion radical (U/L)} = \frac{(A_1 - A_2) - (A_5 - A_6)}{(A_1 - A_2) - (A_3 - A_4)} \times C \times 1000 \times f$$

2. Calculation formula for the activity of inhibition of superoxide anion radical in tissue and cells

Definition: In the reaction system, the amount of superoxide anion radical inhibited by 1 g of sample in 20 min at 37℃ that equivalent to inhibited by 1 mg of VC is defined as 1 unit.

$$\text{The inhibition of superoxide anion radical (U/gprot)} = \frac{(A_1 - A_2) - (A_5 - A_6)}{(A_1 - A_2) - (A_3 - A_4)} \times C \times 1000 \div C_{pr} \times f$$

3. Calculation formula for the activity of production of superoxide anion radical:

(1) Liquid sample:

Definition: In the reaction system, the amount of superoxide anion radical producted by 1 L of substance in 20 min at 37℃ that equivalent to inhibited by 1 mg of VC is defined as 1 unit.

$$\text{The production of superoxide anion radical (U/L)} = \frac{(A_5 - A_6) - (A_1 - A_2)}{(A_1 - A_2) - (A_3 - A_4)} \times C \times 1000 \times f$$

(2) For solid samples

Definition: In the reaction system, the amount of superoxide anion radical producted by 1 g of substance in 20 min at 37℃ that equivalent to inhibited by 1 mg of VC is defined as 1 unit.

$$\text{The production of superoxide anion radical (U/gprot)} = \frac{(A_5 - A_6) - (A_1 - A_2)}{(A_1 - A_2) - (A_3 - A_4)} \times C \div C_1 \times f$$

[Note]

A_1: The OD value of control

A_2: The OD value of $blank_{Control}$

A_3: The OD value of standard

A_4: The OD value of $blank_{standard}$

A_5: The OD value of sample

A_6: The OD value of $blank_{Sample}$

C: The concentration of standard, 0.05 mg/mL.

1000: Unit conversion, 1 L=1000 mL.

Cpr: The concentration of protein in sample, gprot/L.

f: The dilution factor of sample before test.

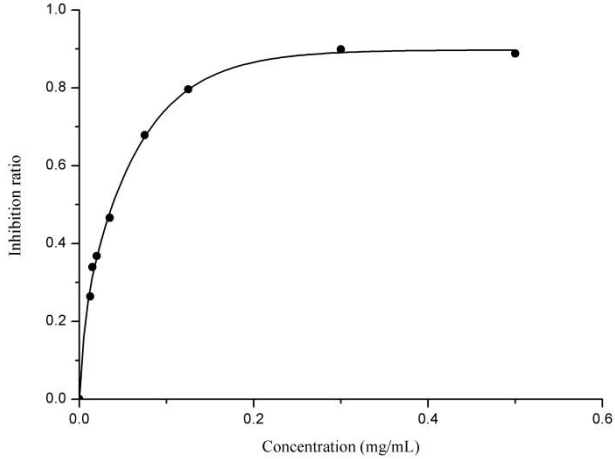
C_1: The concentration of sample, g/L.

Appendix I Performance Characteristics

1. 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 (mg/mL)	**0**	**0.0125**	**0.015**	**0.035**	**0.075**	**0.125**	**0.3**	**0.5**
OD of standard	0.567	0.411	0.386	0.311	0.218	0.167	0.202	0.31
	0.541	0.426	0.376	0.324	0.214	0.172	0.197	0.308
OD of blank$_{standard}$	0.039	0.039	0.041	0.042	0.052	0.064	0.15	0.252
	0.039	0.04	0.041	0.043	0.049	0.065	0.145	0.251
Average OD of standard	0.554	0.419	0.381	0.318	0.216	0.17	0.2	0.309
Average OD of blank$_{standard}$	0.039	0.04	0.041	0.043	0.051	0.065	0.148	0.252
Absolute OD	0.515	0.379	0.34	0.275	0.166	0.105	0.052	0.058
Inhibition ratio	0	26%	34%	47%	68%	80%	90%	89%



Appendix Ⅱ Example Analysis

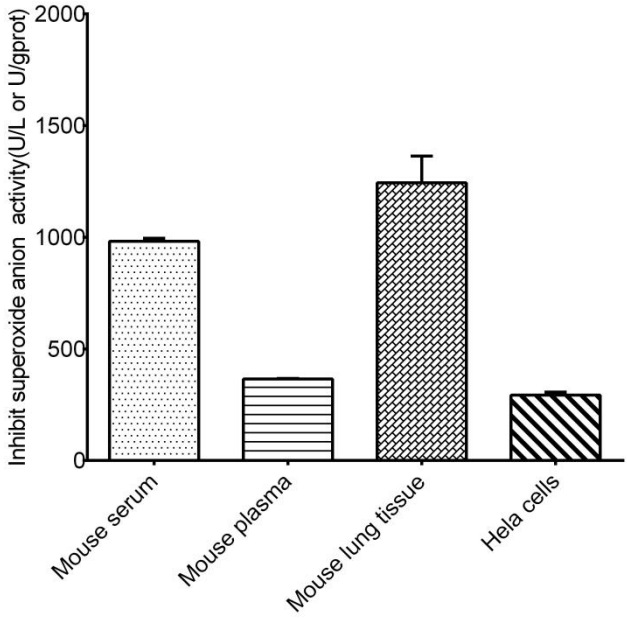
Example analysis：

Dilute 10% mouse lung tissue homogenate with normal saline (0.9% NaCl) for 200 times, take 20 μL of diluted sample and carry the assay according to the operation steps. The results are as follows:

The average OD value of control well is 0.588, the average OD value of $blank_{control}$ well is 0.045, the average OD value of standard well is 0.313, the average OD value of $blank_{standard}$ well is 0.043, the average OD value of $blank_{sample}$ well is 0.040, the concentration of protein in 10% mouse lung tissue homogenate is 7.04 gprot/L, and the calculation result is:

$$\text{The production of superoxide anion radical (U/L)} = \{(0.588-0.045) - (0.344-0.040)\} \div \{(0.588-0.045) - (0.313-0.043)\} \times 0.05 \times 1000 \times 200 \div 7.04 = 1243.5 \text{ U/gprot}$$

Detect mouse serum (dilute for 20 times), mouse plasma (dilute for 8 times), 10% mouse lung tissue homogenate (the concentration of protein is 7.04 gprot/L, dilute for 200 times) and Hela cells (the concentration of protein is 5.59 gprot/L, dilute for 30 times) according to the protocol, the result is as follows：


Inhibit superoxide anion activity(U/L or U/gprot)
2000
1500
1000
500
0
Mouse serum
Mouse plasma
Mouse lung tissue
Hela cells

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.