PRODUCT INFORMATION & MANUAL

Inhibition and Production of Superoxide Ion Assay Kit (Colorimetric)

NBP3-25833

For research use only.
Not for diagnostic or therapeutic procedures.

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Inhibition and Production of Superoxide Ion Assay Kit (Colorimetric)

Catalog No: NBP3-25833

Method: Colorimetric method

Specification: 96T (Can detect 44 samples without duplication)

Measuring instrument: Microplate reader

Average recovery rate (%): 100

- This kit is for research use only.
- Instructions should be followed strictly, changes of operation may result in unreliable results.
- Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

General information

▲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.

▲Background

Superoxide anion radical is a kind of reactive oxygen, which is formed by the reduction of molecular oxygen. Excessive accumulation of reactive oxygen species will lead to oxidative stress.

▲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	Specification	Storage
Reagent 1	Buffer Solution	24 mL × 1 vial	2-8℃ , 12 months
Reagent 2	Substrate Solution	0.14 mL × 1 vial	2-8℃ , 12 months, shading light
Reagent 3	Enzyme Stock Solution	0.3 mL ×1 vial	-20℃ , 12 months
Reagent 4	Enzyme Diluent	1.5 mL × 2 vials	2-8℃ , 12 months
Reagent 5	VC Standard	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.			

▲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)

▲Safety data

Some of the reagents in the kit contain dangerous substances. It should be avoided to touch the skin and clothing. Wash immediately with plenty of water if touching it carelessly. All the samples and waste material should be treated according to the relevant rules of laboratory's biosafety.

▲Precautions

Before the experiment, please read the instructions carefully, and wear gloves and work clothes.

▲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%).

Pre-assay preparation

▲Reagent preparation

1. Preparation of substrate application solution:
   Mix the reagent 1 and reagent 2 at the ratio of 200:1 thoroughly. Prepare the fresh solution before use and the unused substrate application solution can be stored at 2~8℃ for 7 days.
2. Preparation of enzyme working solution:
   (Operate om ice) Mix the reagent 3 and reagent 4 at the ratio of 1:10 thoroughly. Prepare the fresh solution before use and the unused enzyme working solution can be stored at 2-8℃ for 3 days. (Reagent 3 should melt slowly on ice. It is recommended to aliquot the reagent 3 into smaller quantities for optimal storage. Avoid repeated freeze-thaw cycles.)
3. Preparation of 5 mg/mL standard solution:
   Dissolve a vial of reagent 5 with 1 mL of double distilled water fully.
4. Preparation of 0.05 mg/mL standard solution:
   Dilute 5 mg/mL standard solution with double distilled water for 100 times.
   [Note]: VC standard is easy to oxidized, it is best to use the standard solution within 30 min.

▲Sample preparation

The samples should be prepared as conventional methods. Also please refer to appendix II.

Sample requirements

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

▲Dilution 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%).

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

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.

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).

Assay protocol

▲Plate set up

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A'	S5	S5'	S13	S13'	S21	S21'	S29	S29'	S37	S37'
B	A	A'	S6	S6'	S14	S14'	S22	S22'	S30	S30'	S38	S38'
C	B	B'	S7	S7'	S15	S15'	S23	S23'	S31	S31'	S39	S39'
D	B	B'	S8	S8'	S16	S16'	S24	S24'	S32	S32'	S40	S40'
E	S1	S1'	S9	S9'	S17	S17'	S25	S25'	S33	S33'	S41	S41'
F	S2	S2'	S10	S10'	S18	S18'	S26	S26'	S34	S34'	S42	S42'
G	S3	S3'	S11	S11'	S19	S19'	S27	S27'	S35	S35'	S43	S43'
H	S4	S4'	S12	S12'	S20	S20'	S28	S28'	S36	S36'	S44	S44'

Note: A, control well; A', $blank_{control}$ well; B, standard well; B', $blank_{standard}$ well; S1-S44, sample well; S1'-S44', $blank_{sample}$ well.

▲Detailed operating steps

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

   $Blank_{control}$ well: add 20 μL of double distilled water and 20 μL of reagent 4.

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

   $Blank_{standard}$ well: add 20 μL of 0.05 mg/mL standard solution and 20 μL of reagent 4.

   Sample well: add 20 μL of sample and 20 μL of enzyme working solution.

   $Blank_{Sample}$ well: add 20 μL of sample and 20 μL of reagent 4.

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.

▲Summary operation table

	Control well	$Blank_{Control}$ well	Standard well	$Blank_{standard}$ well	Sample well	$Blank_{Sample}$ well
Sample (μL)					20	20
0.05 mg/mL Standard solution (μL)			20	20		
Double distilled water (μL)	20	20				
Enzyme working solution (μL)	20		20		20	
Reagent 4 (μL)		20		20		20
Substrate application solution (μL)	200	200	200	200	200	200
Mix fully and incubate at 37℃ for 20 min. Measure the OD values of each well with microplate reader at 450 nm.						

Notices: Control, $Blank_{Control}$, Standard well, $Blank_{standard}$ only need 1-2 wells for each experiment. Every sample well need a $blank_{Sample}$ well.

▲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.

Calculation formula:

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

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 tissue protein in 20 min at 37℃ that equivalent to inhibited by 1 mg of VC is defined as 1 unit.

Calculation formula:

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

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

For liquid sample

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

Calculation formula:

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

For solid sample

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

Calculation formula:

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

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.

C_{pr}: 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 Data

▲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 table. 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 sample well is 0.344, 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:

The inhibition of superoxide anion radical(U/gprot)

$$= \frac{(0.588 - 0.045) - (0.344 - 0.040)}{(0.588 - 0.045) - (0.313 - 0.043)} \times 0.05 \times 1000 \times 200 \div 7.04 = 1243.5 \text{ U/gprot}$$

Appendix II Sample preparation

The following sample pretreatment methods are for reference only.

▲ Serum

Collect fresh blood and stand at 25℃ for 30 min to clot the blood. Then centrifuge at 2000 g for 15 min at 4℃ . Take the serum (which is the upper light yellow clarified liquid layer) to preserve it on ice for detection. If not detected on the same day, the serum can be stored at -80℃ for a month.

▲ Plasma

Take fresh blood into the tube which has anticoagulant (Heparin is used as anticoagulant), centrifuge at 700-1000 g for 10 min at 4℃ . Take the plasma (which is the upper light yellow clarified liquid layer, don't take white blood cells and platelets in the middle layer) to preserve it on ice for detection. If not detected on the same day, the plasma can be stored at -80℃ for a month.

▲Urine

Collect fresh urine and centrifuge at 10000 g for 15 min at 4℃ . Take the supernatant to preserve it on ice for detection. If not detected on the same day, the urine can be stored at -80℃ for a month.

▲ Tissue sample

Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2-8℃ . Absorb the water with filter paper and weigh. Homogenize at the ratio of the volume of homogenized medium (2-8℃) (mL): the weight of the tissue (g) =9:1, then centrifuge the tissue homogenate for 10 min at 1500 g at 4℃ . Take the supernatant to preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M). If not detected on the same day, the tissue sample (without homogenization) can be stored at -80℃ for a month.

▲ Cells

Collect the cells and wash the cells with PBS (0.01 M, pH 7.4) for 1~2 times. Centrifuge at 1000 g for 10 min and then discard the supernatant and keep the cell sediment. Add homogenization medium at a ratio of cell number (10^6): homogenization medium (μL) =1: 300-500. Sonicate or grind with hand-operated in ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant and preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M). If not detected on the same day, the cells sample (without homogenization) can be stored at -80℃ for a month.

Note:

1. Homogenized medium: Normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4).

2. Homogenized method:

(1) Hand-operated: Weigh the tissue and mince to small pieces (1 mm^3), then put the tissues pieces to glass homogenized tube. Add homogenized medium into homogenized tube, place the tube into the ice bath with left hand, and insert the glass tamping rod vertically into the homogenized tube with the right hand to grind up and down for 6-8 min.
Or put the tissue into the mortar, and add liquid nitrogen to grind fully. Then add the homogenized medium to homogenize.

(2) Mechanical homogenate: Weigh the tissue to EP tube, add the homogenized medium to homogenize the tissue with homogenizer instrument (60 Hz, 90s) in the ice bath. (For samples of skin, muscle and plant tissue, the time of homogenization can be properly prolonged.)

(3) Ultrasonication: Treat the cells with ultrasonic cell disruptor (200 W, 2 s/ time, interval for 3 s, the total time is 5 min).

Appendix III References

1. Han D, WILLIAMS E, CADENAS E. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space[J]. Biochemical Journal, 353(2): 411-416.
2. Betteridge D J. What Is Oxidative Stress ?[J]. Metabolism, 2000: 3-8.