Technical Manual

Inhibition And Production Of Superoxide Anionic Colorimetric Assay Kit

- Catalogue Code: MAES0014
- Size: 96T
- Research Use Only

1. Key features and Sample Types

Detection method:

Colorimetric method

Specification:

96T

Storage:

2-8°C and -20°C for 6 months

Expiry:

See Kit Label

Experiment Notes:

This kit is for **research use only.**

Instructions should be strictly followed. Changes of operation may result in unreliable results.

The validity of kit is 6 months.

Do not use components from different batches of kit.

2. 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.

3. 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.

4. 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.

5. Kit components & storage

Item	Specification	Storage
Buffer Solution	24 mL × 1 vial	2-8°C, 6 months
Substrate Solution	0.14 mL × 1 vial	2-8°C, 6 months, avoid direct sunlight
Enzyme Stock Solution	0.3 mL ×1 vial	-20°C, 6 months
Enzyme Diluent	1.5 mL × 2 vials	2-8°C, 6 months
VC Standard	Lyophilized × 3 vials	-20°C, 6 months, avoid direct sunlight
Microplate	96 wells	No requirement
Plate Sealer	2 pieces	

Materials required but not supplied

- Micropipettor
- Multi-channel pipettor
- Incubator
- Centrifuge
- Microplate Reader (440-460 nm)
- Tips (10 μL, 200 μL, 1000 μL)
- EP tubes (1.5 mL, 2 mL)
- Double distilled water
- Normal Saline (0.9% NaCl)
- PBS (0.01 M, pH 7.4)

6. Assay Notes:

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. 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%).

7. Reagent preparation:

1. Preparation of **substrate application solution:** Mix the buffer solution and substrate solution 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°C for 7 days.

2. Preparation of **enzyme working solution:** (Operate on ice) Mix the enzyme stock solution and enzyme diluent 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°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. Preparation of **standard solution (5 mg/mL):** Dissolve a vial of standard with 1 mL of double distilled water fully.

4. Preparation of **standard solution (0.05 mg/mL):** Dilute standard solution (5 mg/mL) with double distilled water for 100 times.

8. Sample Preparation

Sample requirements: Samples should not contain decontamination agents such as SD S, Tween-20, NP-40, Triton X-100, nor reductive reagents such as DTT, 2- mercaptoeth anol.

1. Serum sample:
Collect fresh blood and stand at 25°C for 30 min to clot the blood. Then centrifuge at 2000 g for 15 min at 4°C. 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°C for a month.

2. Plasma sample:
Take fresh blood into the tube which has anticoagulant (Heparin is used as anticoagulant), centrifuge at 700-1000 g for 10 min at 4°C. 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°C for a month.

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

4. Cell sample:
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 (2×10^6): normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4) (μL) =1: 300. Sonicate the sample on an ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant and preserve it on ice for detection. If not detected on the same day, the cells sample (without homogenization) can be stored at -80°C for a month.

5. Tissue sample:
Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2-8°C. Absorb the water with filter paper and weigh. Homogenize at the ratio of the volume of normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4) (2-8°C) (mL): the weight of the tissue (g) =9:1, then centrifuge the tissue homogenate for 10 min at 10000 g at 4°C. Take the supernatant to preserve it on ice for detection. If not detected on the same day, the tissue sample (without homogenization) can be stored at -80°C for a month.

Sample Notes:

The concentration should be determined before preforming the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.

Dilution of Samples:

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.

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 of is normal saline (0.9% NaCl) or PBS (0.01 M, pH 7.4).

9. Assay Protocol

Ambient Temperature: 25-30°C

Optimum detection wavelength: 450 nm

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.

10. Operation 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 enzyme diluent.
   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 enzyme diluen.
   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 enzyme diluent.

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

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

Operation Table

	Control	Blank Control	Standard	Blank Standard	Sample	Blank Sample
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	
Enzyme diluent (μL)		20		20		20
Substrate application solution (μL)	200	200	200	200	200	200
Mix fully and incubate at 37°C for 20 min. Measure the OD values of each well with microplate reader at 450 nm.						

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

11. Calculations

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

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

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

1) 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°C 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 produced by 1 g of substance in 20 min at 37°C that equivalent to inhibited by 1 mg of VC is defined as 1 unit.

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

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

12. Performance Characteristics

Average recovery rate (%)	100
Average inter-assay CV (%)	5.8
Average intra-assay CV (%)	2.0

Analysis

Dilute 10% mouse lung tissue homogenate with normal saline (0.9% NaCl) for 200 times, take 20 μL of diluted sample, 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)

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

$$= 1243.5 \text{ U/gprot}$$

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Safety Notes

Some of the reagents in the kit contain dangerous substances. Prevent touching 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.

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

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