CO-K022-S CuZn/Mn Superoxide Dismutase (CuZn-SOD/Mn-SOD) Activity Assay Kit (Hydroxylamine Method)

Size: 100Assays **(**Can detect 50 samples for CuZn-SOD only without duplication, or detect 25 samples for both CuZn-SOD and Mn-SOD without duplication)

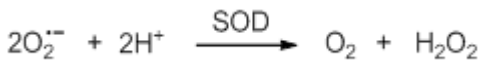
Method: Colorimetric method
Measuring instrument: Spectrophotometer
Sensitivity: 2.03 U/mL
Detection range: 2.03-155 U/mL

Intended use

This kit can be used to measure T-SOD, CuZn-SOD, Mn-SOD activity in serum, plasma, urine, cells, cell culture supernatant and tissue homogenate samples.

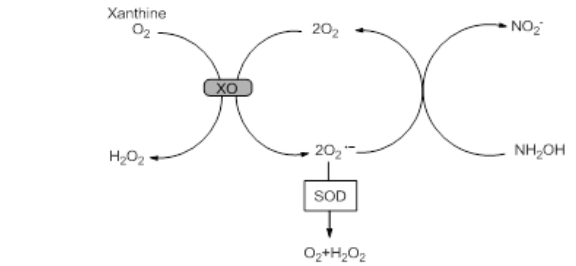
Background

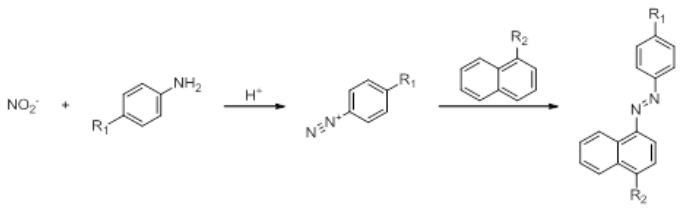
According to the literature, superoxide dismutase exists in all oxygen-metabolizing cells to protect cells from excessive superoxide. Under the action of SOD, two superoxide anions were converted to oxygen and hydrogen peroxide.
In mammals, there are three different forms of SOD: CuZn-SOD, Mn-SOD and EC-SOD (an extracellular form of SOD). Cu-Zn SOD exists in the cytoplasmic and mitochondrial membrane spaces of the cells, while Mn-SOD is located in the mitochondrial matrix.



Detection principle

Superoxide anion (O2•-) produced by xanthine and xanthine oxidase system can oxidize hydroxylamine to form nitrite which appear purplish red after chromogenic reaction. The SOD in the sample has a specific inhibitory effect on superoxide anion (O2•-), can reduce the content of nitrite. The OD value is lower than control and the activity of SOD is calculated through the formula. There are two kinds of SOD (CuZn-SOD, Mn-SOD) in the cells of higher animals, and the sum of them is equal to the total SOD. The activity of Mn-SOD in the sample will lost after sample pretreatment, but the activity of CuZn-SOD will not.





Kit components & storage

Item	Component	Specification	Storage
Reagent 1	Buffer Solution	12 mL × 1 vial	2-8℃, 12 months
Reagent 2	Nitrosogenic Agent	12 mL × 1 vial	2-8℃, 12 months
Reagent 3	Substrate Solution	12 mL × 1 vial	2-8℃, 12 months
Reagent 4	Enzyme Stock Solution	0.6 mL × 1 vial	-20℃, 12 months
Reagent 5	Enzyme Diluent	12 mL × 1 vial	2-8℃, 12 months
Reagent 6	Chromogenic Agent A	Powder × 1 vial	2-8℃, 12 months, shading light
Reagent 7	Chromogenic Agent B	Powder × 1 vial	2-8℃, 12 months, shading light
Reagent 8	Chromogenic Agent C	60 mL × 1 vial	2-8℃, 12 months
Reagent 9	Extracting Solution	25 mL × 1 vial	2-8℃, 12 months, shading light
	Microplate	96 wells	
	Plate Sealer	2 pieces	

Materials prepared by users

Instruments:
Spectrophotometer (550 nm), Micropipettor, Centrifuge, Incubator, Vortex mixer
Consumptive material:
Tips (10 μL, 200 μL, 1000 μL), EP tubes (1.5 mL, 5 mL)
Reagents:
Double distilled water, Normal saline (0.9% NaCl)

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. Determine optimal sampling volume of each sample before formal experiment. Calculate the inhibition ratio of serial sampling volume, and choose the optimal sampling volume when inhibition ratio in the range of 25%~45%.
2. The optimal sampling volume are different for different species, the SOD also are different for different samples. So it is best to do a pre-test to determining optimal sampling volume for a new sample.
3. It is best to reserve 3 paralleled tubes with different sampling volumes in pre-test for determining the optimal sampling volume.
4. Adjust sampling volume: If inhibition ratio >55%, need to dilute the sample or decrease the sampling volume than take the test. If inhibition ratio < 15%, need to increase the sampling

volume.

5. There should be no bubbles in the wells of the microplate when measuring the OD value.
6. The multichannel pipeter is recommended to shorten the time and reduce the error between wells.

Pre-assay preparation

Reagent preparation

1. **Preparation of reagent 1 working solution:**
   Dilute the reagent 1 with double distilled water at a ratio of 1:9 before use. Prepared solution can be stored at 2-8℃ for 3 months.
2. **Preparation of reagent 4 working solution:**
   (Operate on ice) Dilute reagent 4 with reagent 5 at a ratio of 1:19. Prepare the fresh solution before use. Unused reagent can be stored at 2-8℃ for 3 days.
3. **Preparation of reagent 6 application solution:**
   Dissolve a vial of powder with 70-80℃ double distilled water to a final volume of 90 mL. It can be store at 2-8℃ with shading light for 3 months.
4. **Preparation of reagent 7 application solution:**
   Dissolve a vial of powder with double distilled water to a final volume of 90 mL. It can be store at 2-8℃ with shading light for 1 months.
5. **Preparation of chromogenic agent:**
   Prepare chromogenic agent at ratio of reagent 6 application solution: reagent 7 application solution: reagent 8 =3:3:2. Prepare the fresh solution before use and the prepared chromogenic agent can be stored at 2-8℃ in the dark.

Sample preparation

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

Sample requirements

1. Samples should not contain decontamination agents such as SDS, Tween-20, NP-40, Triton X-100, nor reductive reagents such as DTT, 2- mercaptoethanol.
2. EDTA should not be as anticoagulation, suggest to use heparin.

Dilution of sample

The optimal sampling volume are different for different species, the SOD also are different for different samples. It is recommended to take 2~3 samples to do a pre-experiment, diluting a series of diluent and determine the dilution factor when the SOD inhibition ratio is 15%~55% (the optimal inhibition ratio is the range of 25%~45%.) before formal experiment.
The recommended dilution factor for different samples is as follows (for reference only):

Sample type	Dilution factor	The volume added to the reaction (μL)
Mouse serum	3-5	20-30
Human serum	1	30-40
Rat serum	3-5	20-30
Rat plasma	1-2	20-30
Human hydrothorax	1	30-50
Human urine	1	30-50
10% Mouse liver tissue homogenate	50-80	20-30
10% Mouse brain tissue homogenate	8-12	20-30
10% Mouse kidney tissue homogenate	10-20	20-30
HepG2 cells (3.27 mgprot/mL)	5-10	20-30

Note: The diluent is normal saline (0.9% NaCl).

Assay protocol	
Ambient temperature	25-30℃
Optimum detection wavelength	550 nm

Instructions for the use of transferpettor:

(1) Equilibrate the pipette tip in that reagent before pipetting each reagent.

(2) Don't add the liquid outside the tips into the reaction system when pipetting each reagent.

Assay protocol

Operating steps

1. Sample pretreatment

1) Take 0.1 mL sample and add 0.1 mL reagent 9. Mix thoroughly with a vortex mixer for 1 min by

vortex mixer and centrifuge at 3500 g for 15 min. Take **supernatant for CuZn-SOD** measurement.

2) Take 0.1 mL normal saline and add 0.1 mL reagent 9. Mix thoroughly for 1 min with a vortex mixer and centrifuge at 3500 g for 15 min. Take **supernatant as control of CuZn-SOD**.

2. The measurement of samples

1) Sample tube of T-SOD: take 1 mL of reagent 1 working solution and a* μL of sample to the corresponding tubes.
Control tube of T-SOD: take 1 mL of reagent 1 working solution and a* μL of double distilled water to the corresponding tubes.
Sample tube of CuZn-SOD: take 1 mL of reagent 1 working solution and a* μL of **supernatant for CuZn-SOD** to the corresponding tubes.
Control tube of CuZn-SOD: take 1 mL of reagent 1 working solution and a* μL of **supernatant as control of CuZn-SOD** to the corresponding tubes.
2) Add 0.1 mL of reagent 2, 0.1 mL of reagent 3, 0.1 mL of reagent 4 working solution to each tube.
3) Mix fully with a vortex mixer and incubate for 40 min at 37℃. (*When the room temperature is below 20℃, the time of incubation can be extended to 45 min*.)
4) Add 2 mL of chromogenic agent to each tube.
5) Mix fully and stand for 10 min at room temperature. Set the spectrophotometer to zero with double distilled water and measure the OD values of each tube at 550 nm with 1 cm optical path cuvette.

Operation table

	T-SOD sample tube	T-SOD control tube	CuZn-SOD sample tube	CuZn-SOD control tube
Reagent 1 working solution (mL)	1.0	1.0	1.0	1.0
Sample (mL)	a*			
Double distilled water (mL)		a*		
Sample supernatants (mL)			a*	
Control supernatants (mL)				a*
Reagent 2 (mL)	0.1	0.1	0.1	0.1
Reagent 3 (mL)	0.1	0.1	0.1	0.1
Reagent 4 working solution (mL)	0.1	0.1	0.1	0.1

Mix fully with a vortex mixer and incubate for 40 min at 37℃. (*When the room temperature is below 20 ℃, the time of incubation can be extended to 45 min.*)				
Chromogenic agent (mL)	2	2	2	2
Mix fully and stand for 10 min at room temperature. Set the spectrophotometer to zero with double distilled water and measure the OD values of each tube at 550 nm with 1 cm optical path cuvette.				

Calculation

1. **Calculation formula of SOD activity in serum (plasma), cell culture medium and other liquid samples**

   Definition: When SOD inhibition ratio in 1 mL reaction system reach 50%, the corresponding enzyme level is 1 SOD activity unit (U).

$$\text{T-SOD activity (U/mL)} = i_1 \div 50\% \times \frac{V_1}{V_2} \times f$$

$$\text{CuZn-SOD activity (U/mL)} = i_2 \div 50\% \times \frac{V_1}{V_2} \times f$$

2. **Calculation formula of SOD activity in tissue and cell samples**

   Definition: When SOD inhibition ratio of 1 mg of tissue protein in 1 mL reaction system reach 50%, the corresponding enzyme level is 1 SOD activity unit (U).

$$\text{T-SOD activity (U/mgprot)} = i_1 \div 50\% \times \frac{V_1}{V_2} \times f \div C_{pr}$$

$$\text{CuZn-SOD activity (U/mgprot)} = i_2 \div 50\% \times \frac{V_1}{V_2} \times f \div C_{pr}$$

Mn-SOD activity =T-SOD activity -CuZn-SOD activity

[Note]:

i_1: Inhibition ratio of T-SOD

$$i_1 (\%) = \frac{(A_1 - A_2)}{A_1} \times 100\%$$

i_2: Inhibition ratio of CuZn-SOD

$$i_2 (\%) = \frac{(A_3 - A_4)}{A_3} \times 100\%$$

A_1: The OD value of $\text{T-SOD}_{\text{Control}}$

A_2: The OD value of $\text{T-SOD}_{\text{Sample}}$

A_3: The OD value of $\text{CuZn-SOD}_{\text{Control}}$

A_4: The OD value of CuZn-SOD_{Sample}

V_1: The total volume of the reaction system (mL)

V_2: The volume of sample added to the reaction system (mL)

C_{pr}: Protein concentration of sample (mgprot/mL)

f: Dilution factor of sample before tested

Notes

1. This kit is for research use only.
2. Instructions should be followed strictly, changes of operation may result in unreliable results.
3. The validity of kit is 12 months.
4. Do not use components from different batches of kit.

Appendix I Performance characteristics

Appendix I Performance characteristics			
Detection range	2.03-155 U/mL	**Average intra-assay CV (%)**	4.0
Sensitivity	2.03 U/mL	**Average inter-assay CV (%)**	7.2
Average recovery rate (%)	96		

Inter-assay CV

Take three kits of different batches to detect the samples of high concentration, middle concentration and low concentration respectively. The concentrations should be within the detection range. The samples of each concentration were tested 3 times (n=3) parallelly with each kit. Then calculate the corresponding values according to the following formula. The average Inter-assay CV ($\bar{R}$) is 7.2%.

$$\bar{x}_T = \frac{\bar{x}_1 + \bar{x}_2 + \bar{x}_3}{3}$$

$$R = \frac{\bar{x}_{max} - \bar{x}_{min}}{\bar{x}_T} \times 100\%$$

$$\bar{R} = \frac{R_1+R_2+R_3}{3} \quad (n=3)$$

$\bar{x}_{max}$--- The max values of $\bar{x}_i$

$\bar{x}_{min}$--- The min values of $\bar{x}_i$

$\bar{x}_T$---The average values of $\bar{x}_i$

R_i---The value of each batch number kit

Intra-assay CV

Take one kit to detect the samples of high concentration, middle concentration and low concentration respectively. The concentrations should be within the detection range. The samples of each concentration were tested 6 times (n=6) parallelly, and the average Intra-assay CV is 4.0%, which was calculated according to the following formula.

$$\mathrm{CV} = \frac{s}{\bar{x}} \times 100\%$$

s--- Standard deviation

Sensitivity

The volume of sample added to reaction is 5 μL, carry the assay according to the operation steps for multiple experiments (n=20), and the absolute OD value is 0.409-0.45, the minimum detected absorbance is 0.005 (ΔA_{550}=0.005), calculate the sensitivity according to the formula of inhibition ratio is 2.03 U/mL.

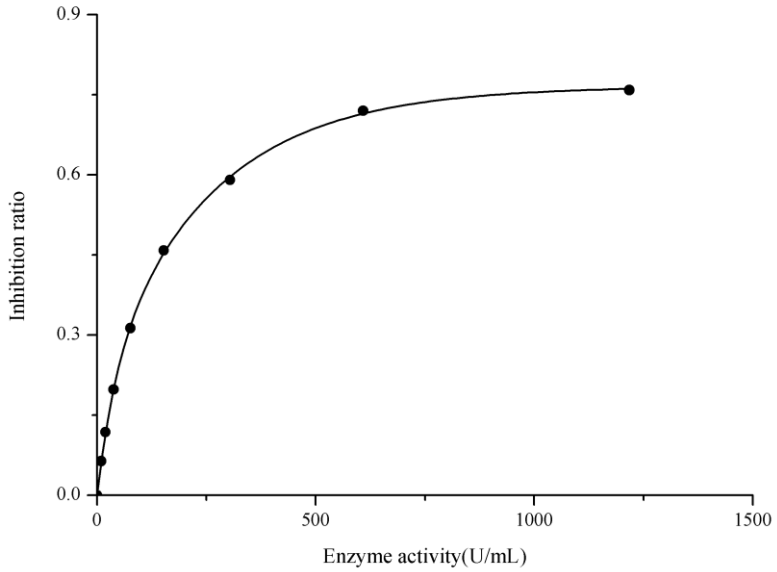
Recovery rate

Take three samples of high concentration, middle concentration and low concentration (which inhibition rate of SOD is kept between 20-47%) to test the samples of each concentration for 3 times parallelly to get the average recovery rate of 96%.

Standard curve

(It doesn't need to prepare the standard curve for this kit and the provided standard curve is fo r reference only)

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.



Example analysis

The detection of T-SOD: Take 10% mouse heart tissue homogenate, dilute for 10 times with normal saline (0.9% NaCl), then 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 T-SOD$_{Sample}$ is 0.252, the average OD value of T-SOD$_{Control}$ is 0.522, the concentration of protein in 10% mouse heart tissue homogenate is 6.84 mgprot/mL, and the calculation result is:

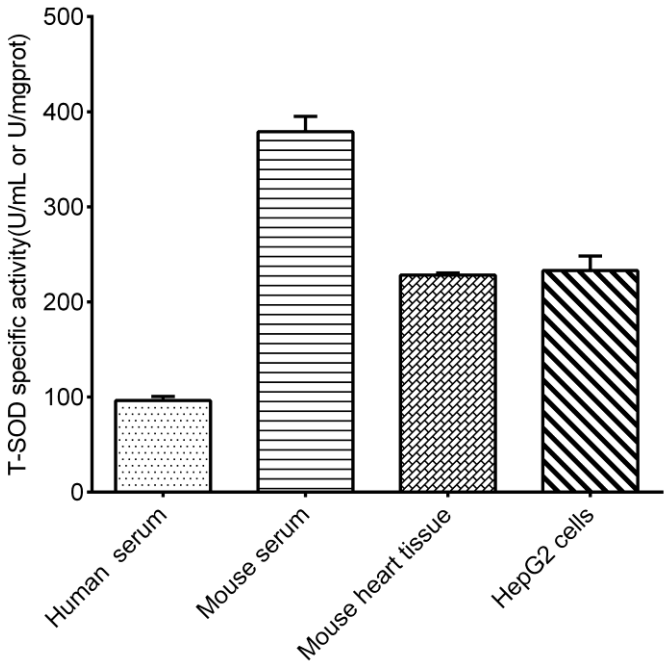
$$\text{T-SOD activity (U/mgprot)} = \left(\frac{0.522-0.252}{0.522}\right) \div 50\% \times \frac{3.02}{0.02} \times 10 \div 6.84 = 228.37 \text{ U/mgprot}$$

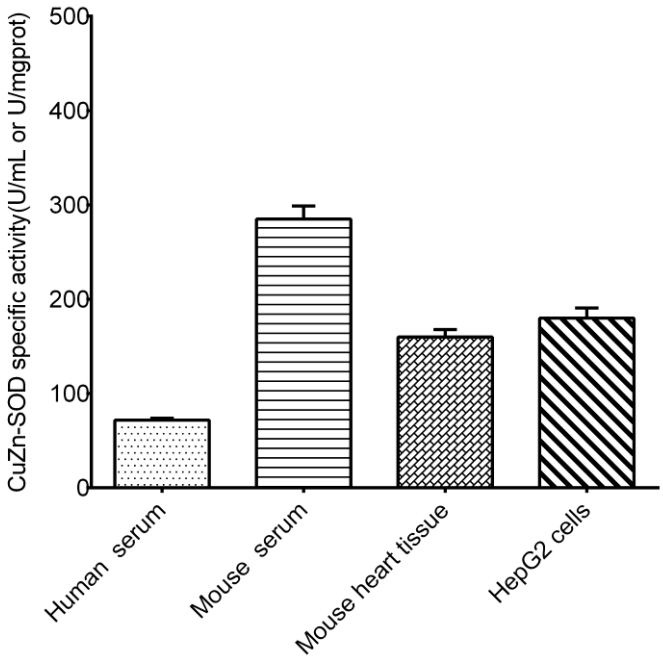
The detection of CuZn-SOD: pretreat the diluted sample with reagent 9 and carry the assay according to the operation table. The results are as follows:

The average OD value of CuZn-SOD$_{Sample}$ is 0.365, the average OD value of CuZn-SOD$_{Control}$ is 0.571, the concentration of protein in 10% mouse heart tissue homogenate is 6.84 mgprot/mL, and the calculation result is:

$$\text{CuZn-SOD activity (U/mgprot)} = \left(\frac{0.571-0.365}{0.571}\right) \div 50\% \times \frac{3.02}{0.02} \times 10 \div 6.84 = 159.29 \text{ U/mgprot}$$

Detect human serum (V_2=30 μL), mouse serum (dilute for 4 times, V_2=20 μL), 10% mouse heart tissue homogenate (the concentration of protein is 6.84 mgprot/mL, dilute for 10 times, V_2=20 μL) and HepG2 cells (the concentration of protein is 3.27 mgprot/mL, dilute for 10 times, V_2=25 μL) according to the protocol, the result is as follows:





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 (do not use EDTA as anticoagulation, heparin is recommended), centrifuge at 1000-2000 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 10000 g at 4℃. Take the supernatant to preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant. 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 1500 g for 10 min, then take the supernatant and preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant. 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).
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 10 min).

Note for sample

1. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
2. If a lysis buffer is used to prepare tissue or cell homogenates, there is a possibility of causing a deviation due to the introduced chemical substance.

Appendix III References

1. Perry J J, Shin D S, Getzoff E D, et al. The structural biochemistry of the superoxide dismutases[J]. Biochim Biophys Acta, 2010, 1804: 245-262.
2. Miller A F. Superoxide dismutases: ancient enzymes and new insights[J]. FEBS Lett, 2012, 586: 585-595.
3. Cristiana F, Elena A, Nina Z. Superoxide Dismutase: Therapeutic Targets in SOD Related Pathology[J]. Scientific Research, 2014, 06: 975-988.