Superoxide Dismutase (T-SOD) assay kit

(Cat/No.:BC094 Size:100T/96S，50T/48S)

1. Introduction of SOD assay kit

Xanthine Oxidase method is used in this kit to measure superoxide dismutase (SOD) activity. This kit applies to measure SOD activities in blood serum (or plasma), cerebrospinal fluid, hydrothorax, hydroperitoneum, kidney dialysate, urine, erythrocytes, leucocytes, blood platelets, cardiac muscle cultured cells, tumor cultured cells, various kinds of animal or plant tissue cells and subcellsular level (mitochondria, microsome), it also can measure SOD activities in microbes, medicines, foods, drinks, cosmetics.

2. Assay significance

Superoxide dismutase (SOD) plays an important role in oxidation-antioxidation balance in organisms, this enzyme can remove superoxide anion radicals (O2 − ·) to protect cells away from damage.

3. Assay principle (Hydroxylamine method)

Use xanthine and xanthine oxidase reaction system to produce superoxide anion radicals (O2 − ··) , the latter will oxidate hydroxylamine to form nitrite, appears prunosus color under effect of chromogenic agent, its absorbance can be measured by visible range spectrophotometer. If sample to assay contains SOD, then it has a narrow spectrum depressant effect for superoxide anion radicals, as the result, absorbance in sample tube will be lower than absorbance in contrast tube, SOD activity can be calculated by formula.

In higher animal cells, there are only two types of SOD: copper-zinc superoxide dismutase (CuZn-SOD) and manganese superoxide dismutase (Mn-SOD). In lower animals, unicellular organisms, and plants, iron superoxide dismutase (Fe-SOD) is present in addition to CuZn-SOD and Mn-SOD. By determining total SOD (T-SOD), Mn-SOD, CuZn-SOD, and Fe-SOD activities, the activity of each SOD isoenzyme can be calculated.

4. Expiry date and storage condition

This kit's expiry date: **6 months;** storage temperature : **4℃.**

Note that Reagent 4 stock solution must be stored frozen below −20 °C (the lower the temperature, the longer the storage time; repeated freeze–thaw cycles must be avoided), otherwise it may easily lose activity. The pipette tips used must be clean, preferably sterilized, or new tips should be thoroughly rinsed repeatedly with distilled water and dried before use. It is essential to avoid contamination by microorganisms and heavy metal ions.

5. Required equipments and reagents in experiment

1. Adjustable-wavelength spectrophotometer or microplate reader capable of 550 nm
2. constant-temperature water bath or air incubator
3. Centrifuge

4. Distilled water, glacial acetic acid (analytical grade, acetic acid concentration ≥99.5%)

5. Anhydrous ethanol and chloroform (both analytical grade) are required for hyperlipidemic serum or red blood cell samples

6. Composition and preparation

	Component	**50 tubes/48 samples**	**100 tubes/96 samples**	**Storage conditions**
Reagent 1	Substrate stock solution	5 mL × 1 bottle	10 mL × 1 bottle	4℃
	Preparation of Reagent 1 working solution: dilute with distilled water at a volume ratio of 1:9 before use, store at 4 ℃. **(Note: crystals may precipitate from the Reagent 1 stock solution in cold weather or after storage at 4 ℃ for a period of time; dissolve completely by warming in a 37 ℃ water bath before use.)**			
Reagent 2	Liquid	5 mL × 1 bottle	10 mL × 1 bottle	4℃
Reagent 3	Liquid	5 mL × 1 bottle	10 mL × 1 bottle	4℃
Reagent 4	Stock solution	350 μL × 1 vial	350 μL × 2 vial	Below −20 ℃
	Diluent	5 mL × 1 bottle	10 mL × 1 bottle	4℃
	Preparation of **Reagent 4 working solution**: prepare freshly before use by mixing stock solution and diluent at a ratio of 1:14. **Prepare only the required amount and use immediately. (Repeated freeze–thaw cycles of Reagent 4 stock solution must be avoided.)**			
Reagent 5	White powder	1 vial	1 vial	4 ℃, protected from light
		Add distilled water 37.5 mL per vial, heat above 70 ℃ to dissolve before use	Add distilled water 75 mL per vial, heat above 70 ℃ to dissolve before use	4 ℃, protected from light
Reagent 6	Light gray powder	1 vial	1 vial	4 ℃, protected from light
		Add distilled water 37.5 mL per vial and dissolve at room temperature before use	Add distilled water 75 mL per vial and dissolve at room temperature before use	4 ℃, protected from light
Preparation of **chromogenic reagent: prepare according to the volume ratio of Reagent 5 solution : Reagent 6 solution : glacial acetic acid = 3:3:2. Prepare freshly before use. The prepared chromogenic reagent can also be stored at 4 ℃ protected from light for up to 3 months. (Note:** Reagent 5 and Reagent 6 must be prepared separately and must not be mixed before preparation; otherwise, no color will develop.**)**				

7. Assay procedure

I. Sample pretreatment:

Liquid samples such as serum (plasma), myocardial perfusate, renal dialysate, and cell culture medium: test directly (determine the optimal sampling concentration before formal experiments); (see **Appendix II** for the pretreatment of hyperlipidemic serum).

Animal tissue samples: accurately weigh the tissue, add 9 volumes of homogenization medium according to the ratio of weight (g): volume (mL) = 1:9 (recommended normal saline or 0.1 mol/L PBS), mechanically homogenize in an ice-water bath to prepare a 10% homogenate, centrifuge at 3500 rpm for 10 minutes, collect the supernatant, and first perform a pretest to determine the optimal sampling concentration, then conduct the formal experiment according to the operation table. **(Protein concentration of the homogenate supernatant must be determined)**

Plant tissue samples: accurately weigh plant tissue, add 4 volumes of homogenization medium according to the ratio of weight (g): volume (mL) = 1:4 (recommended 0.1 mol/L phosphate buffer, pH 7.0–7.4), mechanically homogenize in an ice-water bath to prepare a 20% homogenate, centrifuge at 3500–4000 rpm for 10 minutes, collect the supernatant, perform a pretest to determine the optimal sampling concentration, and then carry out the formal experiment according to the operation table (some medicinal materials or plant powders can be treated using the same method).

Cell samples: collect cells, add 0.2–0.3 mL normal saline (or 0.1 mol/L PBS) to each sample (cell number should preferably not be less than 10^6; the more, the better), disrupt cells by ultrasonication in an ice-water bath (power 100–300 W, run for 5 seconds with 15-second intervals, repeat for 2–5 minutes), centrifuge at 4000 rpm for 10 minutes, and collect the supernatant for testing (first perform a pretest to determine the optimal sample concentration, then conduct formal experiments in batches; protein concentration of the supernatant must be determined).

Red blood cell samples: processing and operation methods are shown in **Appendix III**.

II. Operation table:

Reagent	Test tube	Control tube
Sample to be tested (mL)	0.05	
Reagent 1 working solution (mL)	1.0	1.0
Distilled water (mL)		0.05
Reagent 2 (mL)	0.1	0.1
Reagent 3 (mL)	0.1	0.1
Reagent 4 working solution (mL)	0.1	0.1
Mix thoroughly using a vortex mixer, incubate at 37 ℃ in a constant-temperature water bath or air incubator for 40 minutes		
Chromogenic reagent (mL)	2	2
Mix well, stand at room temperature for 10 minutes, measure at 550 nm with a 1 cm optical path, zero with distilled water using a spectrophotometer (or transfer 200 μL from each tube into a 96-well plate and read at 550 nm using a microplate reader; at the same time set one well with 200 μL distilled water, subtract the OD value of the distilled water well from all readings).		

Note 1: The optimal sampling concentration varies among different sample types due to different SOD activities. According to the parabolic relationship between enzyme percentage inhibition and enzyme activity (Appendix: SOD standard curve), sampling concentrations differ among samples. A pretest is recommended before testing each new sample type to select an optimal sampling concentration.

Note 2: Add reagents strictly in the order listed in the operation table. Reagent 1 can be mixed with Reagent 2, or Reagent 1 with Reagent 3; the mixed reagent can be added as 1 mL without affecting results. However, Reagents 1, 2, and 3 must not be mixed together, nor should Reagents 1, 2, 3, and 4 be mixed together, as this will affect results.

Determination of optimal sampling concentration

Sample dilution: When using this kit to test a new sample type for the first time, it is recommended to select two samples with relatively large differences. For each sample, prepare three test tubes with different dilution factors, using the sampling concentration given in our example as the middle concentration, doubling the concentration upward and halving it downward (if no reference information is provided for your sample, you may prepare dilutions such as 5-fold, 10-fold, 20-fold, etc.). For two samples, prepare a total of six dilution tubes plus one control tube and perform a pretest according to the operation table to determine the optimal sampling concentration.

Calculation of inhibition rate: (Absorbance of control tube − absorbance of test tube) ÷ absorbance of control tube × 100%

Selection of optimal sampling concentration: select the sample concentration corresponding to an inhibition rate between 45% and 50% as the optimal sampling concentration.

Inhibition rate range: for large-scale experiments, an effective inhibition rate within 15%–55% is acceptable.

Adjustment of optimal sampling concentration: if the percentage inhibition rate is greater than 60%, dilute the sample and retest; if the percentage inhibition rate is less than 15%, increase the sample amount or test at a higher concentration. **This adjustment is helpful for scientific data analysis and evaluation; when the percentage inhibition rate is greater than 60% or less than 10%, results among different groups often show no significant difference during assay runs.**

Note 4: Reference values for optimal sampling concentration:

① Red blood cell extracts generally need to be further diluted 5-fold;
② Rat red blood cell extracts should be diluted approximately 10-fold;
③ Human plasma (serum) is generally diluted 2-fold or not diluted;
④ Mouse plasma (serum) is diluted approximately 2.5-fold;
⑤ 1% tissue homogenate is diluted 2-fold or not diluted;
⑥ Cytoplasmic fraction is diluted 2-fold or not diluted;
⑦ Myocardial perfusate or renal dialysate should be tested as original solution with increased sampling volume to 100–200 μL;
⑧ Leukocyte suspension should be tested as original solution with increased sampling volume to 100–200 μL;
⑨ Cell culture medium should be tested as original solution with increased sampling volume to 100–200 μL.

III. Calculation:

(I). Calculation for liquid samples such as serum (plasma), myocardial perfusate, renal dialysate, cell culture medium:

1. Definition: One unit of SOD activity (U) is defined as the amount of SOD corresponding to a 50% inhibition rate of SOD in 1 mL reaction solution per milliliter of sample.

2. Calculation formula:

$$\text{Total SOD activity (U/ml)} = \frac{A_{contrast} - A_{sample}}{A_{contrast}} \div 50\% \times \frac{\text{Reaction system}}{\text{dilution times}} \times \frac{\text{Sample dilution times}}{\text{before test}}$$

(II). Calculation of total SOD activity in animal tissues (or cells):

1. Definition: One unit of SOD activity (U) is defined as the amount of SOD corresponding to a 50% inhibition rate of SOD in 1 mL reaction solution per milligram of tissue protein.

2. Calculation formula:

$$\text{Total SOD activity (U/mgprot)} = \frac{A_{contrast} - A_{sample}}{A_{contrast}} \div 50\% \times \frac{\text{Total volume of reaction solution (ml)}}{\text{Sample volume (ml)}} \times \text{Protein content (mgprot/ml) at the same homogenate concentration}$$

[Note]: *mgprot refers to milligrams of protein.

(III). Calculation of total SOD activity in plant tissue homogenates:

1. Method 1 (calculated based on tissue homogenate concentration):

Definition: One unit of SOD activity (U) is defined as the amount of SOD corresponding to a 50% inhibition rate of SOD in 1 mL reaction solution per gram of tissue.

Calculation formula:

$$\text{Total SOD activity (U/g wet weight of tissue)} = \frac{A_{contrast} - A_{sample}}{A_{contrast}} \div 50\% \times \frac{\text{Total volume of reaction solution (ml)}}{\text{Sample volume (ml)}} \times \text{Homogenate concentration (g/ml)}$$

[Note]: $\text{Homogenate concentration (g/mL)} = \frac{\text{wet weight of tissue (g)}}{\text{Homogenization medium volume (mL)}}$

2. Method 2 (calculated based on protein concentration):

Definition: One unit of SOD activity (U) is defined as the amount of SOD corresponding to a 50% inhibition rate of SOD in 1 mL reaction solution per milligram of tissue protein.

Calculation formula:

$$\text{Total SOD activity (U/mgprot)} = \frac{A_{contrast} - A_{sample}}{A_{contrast}} \div 50\% \times \frac{\text{Total volume of reaction solution (ml)}}{\text{Sample volume (ml)}} \times \text{Protein content (mgprot/ml) at the same homogenate concentration}$$

IV. Calculation examples:

Example 1: Determination of total SOD activity in human serum. The serum was diluted 2-fold, and 50 μL was sampled. The absorbance of the control tube was 0.476, and the absorbance of the test tube was 0.292. Substitute the data into the calculation formula:

$$\text{Total SOD activity (U/ml)} = \frac{0.476 - 0.292}{0.476} \div 50\% \times \frac{3.35}{0.05} \times 2$$
$$= 103.6 U/\text{ml}$$

Example 2: 100 μL of cell culture medium was taken directly for SOD determination. The absorbance of the control tube was 0.473, and the absorbance of the test tube was 0.312. Substitute the data into the calculation formula:

$$\text{Total SOD activity (U/ml)} = \frac{0.473 - 0.312}{0.473} \div 50\% \times \frac{3.4}{0.1} \times 1$$
$$= 23.146 U/\text{ml}$$

Example 3: 50 μL of 0.2% liver tissue homogenate was taken to determine total SOD activity. The absorbance of the control tube was 0.512, and the absorbance of the test tube was 0.243. The tissue protein concentration was 0.215 mg/mL. The calculation result is:

$$\text{Total SOD activity (U/mgprot)} = \frac{0.512 - 0.243}{0.512} \div 50\% \times \frac{3.35}{0.05} \div 0.215$$
$$= 327.45 U/\text{mgprot}$$

Example 4: 50 μL of 10% earthworm homogenate was taken to determine total SOD activity. The absorbance of the control tube was 0.546, and the absorbance of the test tube was

0.320. The protein concentration of the 10% earthworm homogenate was 5.932 mg/mL. The calculation result is:

$$\begin{aligned}\text{Total SOD activity} \\ (U/\text{mgprot})\end{aligned} = \frac{0.546-0.320}{0.546} \div 50\% \times \frac{3.35}{0.05} \div 5.932$$
$$= 9.35U/\text{mgprot}$$

Example 5: 50 μL of 5% rabbit retinal homogenate was taken to determine total SOD activity. The absorbance of the control tube was 0.473, and the absorbance of the test tube was 0.271. The protein concentration of the 5% rabbit retinal homogenate was 0.3378 mg/mL. The calculation result is:

$$\begin{aligned}\text{Total SOD activity} \\ (U/\text{mgprot})\end{aligned} = \frac{0.473-0.271}{0.473} \div 50\% \times \frac{3.35}{0.05} \div 0.3378$$
$$= 169.41U/\text{mgprot}$$

Example 6: 50 μL of 0.5% Chinese sturgeon liver tissue homogenate was taken to determine total SOD activity. The absorbance of the control tube was 0.506, and the absorbance of the test tube was 0.326. The protein concentration of the 0.5% Chinese sturgeon liver tissue homogenate was 0.4512 mg/mL. The calculation result is:

$$\begin{aligned}\text{Total SOD activity} \\ (U/\text{mgprot})\end{aligned} = \frac{0.506-0.326}{0.506} \div 50\% \times \frac{3.35}{0.05} \div 0.4512$$
$$= 105.65U/\text{mgprot}$$

Example 7: A certain amount of fish eggs was collected by centrifugation, then 500 μL normal saline was accurately added for homogenization. After centrifugation at 2500 rpm for 10 minutes, the supernatant was collected, diluted 5-fold with normal saline, and 50 μL was taken for total SOD determination. The absorbance of the control tube was 0.561, and the absorbance of the test tube was 0.309. The protein concentration of the original supernatant was 3.1062 mg/mL. The calculation result is:

$$\begin{aligned}\text{Total SOD activity} \\ (U/\text{mgprot})\end{aligned} = \frac{0.561-0.309}{0.561} \div 50\% \times \frac{3.35}{0.05} \div (3.1062 \div 5)$$
$$= 96.89U/\text{mgprot}$$

Example 8: 50 μL of 10% Arabidopsis leaf homogenate supernatant was taken to determine total SOD activity. The absorbance of the control tube was 0.540, and the absorbance of the test tube was 0.274. The tissue protein concentration was 1.3428 mg/mL. The calculation results are:

①. Calculated by Method 1:

$$\begin{aligned}\text{SOD activity} \\ (U/\text{g } T\text{issue})\end{aligned} = \frac{0.540-0.274}{0.540} \div 50\% \times \frac{3.35}{0.05} \div \frac{0.1}{0.9}$$
$$= 594.07U/\text{g } T\text{issue}$$

②. Calculated by Method 2:

$$\begin{aligned}SOD\ \text{activity} \\ (U/\text{mgprot})\end{aligned} = \frac{0.540-0.274}{0.540} \div 50\% \times \frac{3.35}{0.05} \div 1.3428$$
$$= 49.16U/\text{mgprot}$$

Example 9: 50 μL of 5% rice leaf homogenate was taken to determine total SOD activity. The absorbance of the control tube was 0.555, and the absorbance of the test tube was 0.273. The protein concentration of the 5% rice leaf homogenate was 0.9833 mg/mL. The calculation results are:

①. Calculated by Method 1:

$$\begin{aligned}SOD\ \text{activity} \\ (U/\text{g tissue})\end{aligned} = \frac{0.555-0.273}{0.555} \div 50\% \times \frac{3.35}{0.05} \div \frac{0.1}{1.9}$$
$$= 1293.64U/\text{g tissue}$$

②. Calculated by Method 2:

$$\frac{SOD\ \text{activity}}{(U/\text{mgprot})} = \frac{0.555-0.273}{0.555} \div 50\% \times \frac{3.35}{0.05} \div 0.9833$$
$$= 69.24U/\text{mgprot}$$

Example 10: Accurately weigh 0.05 g of extract powder, dilute to 10 mL to dissolve, and take 50 μL to determine total SOD activity. The absorbance of the control tube was 0.537, and the absorbance of the test tube was 0.296. The calculation result is:

$$\frac{SOD\ \text{activity}}{(U/\text{g Tissue})} = \frac{0.537-0.296}{0.537} \div 50\% \times \frac{3.35}{0.05} \div \frac{0.05}{10}$$
$$= 12027.56U/\text{g Tissue}$$

[Note]: * mgprot refers to milligrams of protein.

Sample reference values

○ **Mouse**

● **Serum (plasma)** T-SOD activity: 110.446±21.325 U/mL (2.5-fold dilution)

● **Liver tissue** T-SOD activity: 269.274±23.448 U/mgprot (diluted to 0.25% tissue homogenate concentration)

● **Brain tissue** T-SOD activity: 108.790±13.494 U/mgprot (diluted to 1% tissue homogenate concentration)

● **Kidney tissue** T-SOD activity: 154.277±15.646 U/mgprot (diluted to 0.5% tissue homogenate concentration)

● **Myocardial tissue** T-SOD activity: 174.330±19.961 U/mgprot (diluted to 1% tissue homogenate concentration)

●**Skin tissue** T-SOD activity: 69.01+19.95 U/mgprot (diluted to 1% tissue homogenate concentration)

● **Skeletal muscle** T-SOD activity: 101.717±12.190 U/mgprot (diluted to 1% tissue homogenate concentration)

○ **Rat**

● **Serum (plasma)** T-SOD activity: 262.786±23.240 U/mL (10-fold dilution)

● **Whole blood** T-SOD activity: 21.554±2.116 U/mgHb

● **Liver tissue** T-SOD activity: 214.689±38.803 U/mgprot (diluted to 0.25% tissue homogenate concentration)

● **Kidney tissue** T-SOD activity: 136.825±24.763 U/mgprot (diluted to 0.5% tissue homogenate concentration)

● **Intestinal tissue** T-SOD activity: 74.738±11.351 U/mgprot (diluted to 1% tissue homogenate concentration)

● **Lung tissue** T-SOD activity: 35.542±15.465 U/mgprot (diluted to 2% tissue homogenate concentration)

● **Brain tissue** T-SOD activity: 140.177±26.878 U/mgprot (diluted to 1% tissue homogenate concentration)

● **Cerebral cortex tissue** T-SOD activity: 79.037±3.996 U/mgprot (diluted to 1% tissue homogenate concentration)

● **Hippocampal tissue** T-SOD activity: 136.863±36.472 U/mgprot (diluted to 1% tissue homogenate concentration)

● **Myocardial tissue** T-SOD activity: 128.292±9.219 U/mgprot (diluted to 0.5% tissue homogenate concentration)

○ **Rabbit**

● **Serum** T-SOD activity: 429.04+31.60 U/mL (5-fold dilution)

○ **Chicken**

● **Serum** T-SOD activity: 213.208±73.368 U/mL

○ **Bovine**

● **Serum** T-SOD activity: 123.691±20.008 U/mL

● **Gastric juice** T-SOD activity: 27.880±8.076 U/mL

○ **Human**

● **Serum** T-SOD activity: 104.2+18.8 U/mL (n=100, 2-fold dilution);
Mn-SOD activity: 41.21+3.2 U/mL (n=100, 2-fold dilution);
● **Red blood cell** T-SOD activity: 19246+132 U/gHb (n=40, 5-fold dilution);
● **Whole blood** T-SOD activity: 21.554±2.117 U/mgHb

All values are expressed as mean $\overline{X}$ ±SD.
It is recommended that each laboratory establish its own normal reference range.

8. Notes

1. During measurement, **disposable plastic test tubes or centrifuge tubes can be used.**
2. Preparation of all reagents (except Reagent 4) can be carried out one day before testing to ensure complete dissolution. After preparation, reagents can be stored at 4℃ for 3–6 months. Before testing, reagents and samples taken out from refrigeration should be equilibrated at room temperature for 30 minutes before use.
3. To avoid testing errors, discard the first aspirated reagent as a rinse for the pipette. If liquid adheres to the outer surface of the pipette tip, gently wipe it dry with filter paper. Add samples and reagents vertically into the tube, not along the tube wall (as the volumes of reagents and samples are small). Before water bath or air bath incubation, mix thoroughly using a vortex mixer.
4. Each incubation time is 40 minutes. When room temperature is below 20℃, the incubation time may be appropriately extended to 45 minutes. The reaction time should be kept constant for experiments of the same type.
5. For each batch of experiments, it is recommended to prepare two control tubes and place them among all test tubes, and take their average value. Alternatively, prepare one control tube for every nine test tubes.
6. Perform a preliminary test before formal testing to determine the optimal sample concentration: when using this kit for the first time to test a new tissue sample, it is recommended to prepare three test tubes with different concentrations. For example, when testing a 10% brain tissue homogenate, take the sampling volume in the example as the middle value, and prepare two additional concentrations at twofold higher and twofold lower, together with one control tube. Conduct a preliminary test according to the operation table to determine the optimal sample concentration. The $\frac{\text{Control } OD - T\text{set } OD}{\text{Control } OD}$ result should fall between 0.15 and 0.55, corresponding to a percentage inhibition rate of 15–55%. Then select the sample concentration of the tube with an inhibition rate around 45% or 48% as the

optimal sampling concentration, because the percentage inhibition rate of the enzyme shows a parabolic relationship with enzyme activity. If the percentage inhibition rate is greater than 60%, the sample should be diluted and retested. If the percentage inhibition rate is less than 20%, the sample amount should be increased and retested.

7. EDTA chelates metal ions and inhibits SOD activity, which may result in reduced or undetectable SOD activity. Therefore, **EDTA must not be used as an anticoagulant** when collecting plasma samples.

9. Advantages of this method

8. 1. Rapid: The entire procedure takes about 50 minutes, during which more than 100 samples can be measured. This short-time, high-throughput testing method is well received by operators.
9. 2. Extremely small sample volume: Using this method, SOD in red blood cells can be measured with only 5–10 μL of fingertip or earlobe peripheral blood after dilution; SOD in leukocytes and platelets can be measured with only 1 mL of venous blood; tissue homogenates and cytosol can be measured with only 6 mg of tissue; SOD in mitochondria and microsomes can be measured with 0.2 g of tissue. Therefore, this method is favored by researchers.
10. 3. High sensitivity: IC50 = 0.05 g/mL, which is 18 times more sensitive than the pyrogallol method. Therefore, it can be used to determine SOD in myocardial perfusate, cerebrospinal fluid, pleural and ascitic fluid, renal dialysate, myocardial cultured cells, and tumor cultured cells. This method is the most sensitive.
11. 4. Good stability: Reagents can be stored in a refrigerator at 0℃–4℃ for 6 months. Mixed serum stored at 4℃ shows no change in SOD activity within three days.
12. 5. Good reproducibility: The coefficient of variation (CV) is 1.7%. Results obtained from the same sample show little difference when measured again after several days.
13. 6. Recovery test: X±SD=103.3±2.63%
14. 7. Minimal interference from external factors: This method has been used by hundreds to thousands of research institutions and universities, and is widely recognized as a domestic first, superior to other chemical detection methods and to radioimmunoassay.
15. 8. Wide application range: This method can measure T-SOD, Mn-SOD, and CuZn-SOD. It can be used to test red blood cells, white blood cells, platelets, serum (plasma), myocardial tissue, lung, liver, kidney, spleen, tympanic membrane, adrenal gland, and dozens of other tissue homogenates, as well as mitochondria, microsomes, isolated myocardial perfusate, renal dialysate, ascitic fluid, pleural fluid, cerebrospinal fluid, myocardial cultured cells, tumor cultured cells, with excellent results. It can also be used to measure SOD in plant leaves, roots, stems, aquatic products, cosmetics, nutritional products, wine, and beverages.
16. 9. Low cost: The main raw materials of this kit are imported from the United States, but the price is comparable to that of the domestic pyrogallol method.
17. 10. No expensive or special instruments are required; only simple instruments such as a constant-temperature incubator and a visible-light spectrophotometer or microplate reader are needed to obtain high-quality results.

Appendix I: Preparation of the SOD Standard Curve

1. Pretreatment:

Preparation of 1 μg/mL standard solution: Weigh 1 mg of SOD standard (activity: 5000 U/mg), dissolve and make up to 10 mL in a volumetric flask to obtain a 100 μg/mL standard stock solution. Take 1 mL of this stock solution and dilute to 100 mL in a volumetric flask to prepare a 1 μg/mL standard working solution (5 U/mL).

2. Operation table:

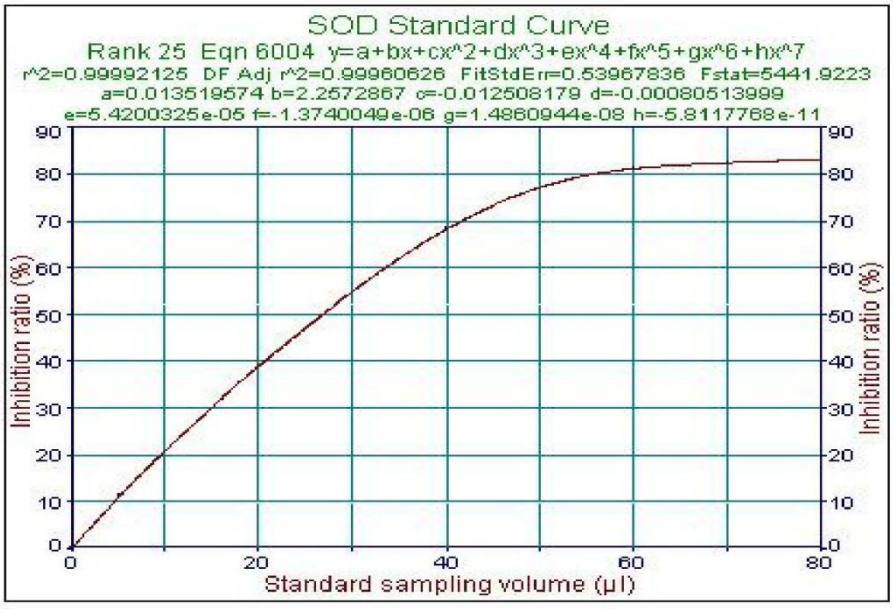
Tube No.	0	1	2	3	4	5	6	7	8	9	10
Reagent I working solution (mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1 μg/mL standard (mL)	0	0.0025	0.005	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08
Distilled water (mL)	0.08	0.0775	0.075	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.0
Reagent II (mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Reagent III (mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Reagent IV working solution (mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Mix thoroughly with a vortex mixer and incubate in a 37℃ water bath for 40 minutes											
Chromogenic reagent (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Mix well, after 10 minutes, measure at 550 nm with a 1 cm optical path, zero with distilled water, and perform colorimetric measurement

3. Determination results:

Tube No.	1	2	3	4	5	6	7	8	9	10
OD value	0.520	0.488	0.437	0.334	0.251	0.173	0.127	0.103	0.097	0.094
Inhibition rate %	5.45	11.27	20.55	39.27	54.36	68.55	76.91	81.27	82.36	82.91
Equivalent to U	0.0125	0.025	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40
Equivalent to U/mL	0.15625	0.3125	0.625	1.25	1.875	2.5	3.125	3.75	4.375	5.0

4. The curve is plotted as follows:



Appendix II: Determination of T-SOD in Hyperlipidemic Serum (Plasma)

1. Pretreatment of hyperlipidemic serum (plasma) samples:

Mild hyperlipidemia: The appearance is slightly turbid. Before testing, simply dilute the serum with an equal volume of physiological saline and then test.

Moderate hyperlipidemia: The appearance is obviously turbid. Before testing, add an equal volume of physiological saline, then add an amount of absolute ethanol equal to half the serum volume (volume ratio), mix well, and then test.

Severe hyperlipidemia: Take 50 μL of hyperlipidemic serum (plasma), add 200 μL of physiological saline and mix well, then add 150 μL of absolute ethanol and mix thoroughly for 1 minute, then add 150 μL of chloroform, mix thoroughly for 1 minute, centrifuge at 3500 rpm (bench-top centrifuge) for 10 minutes, and take the supernatant for testing.

The supernatant should first be subjected to optimal sampling concentration exploration ("Determination of optimal sampling concentration"). After determining the optimal sampling concentration, perform the formal experiment according to the table below.

2. Operation table:

Reagent	Test tube	Control tube
Supernatant (mL)	0.15	
Reagent I working solution (mL)	1.0	1.0
Reagent II (mL)	0.1	0.1
Reagent III (mL)	0.1	0.1
Reagent IV working solution (mL)	0.1	0.1
Mix thoroughly with a vortex mixer and incubate in a 37℃ constant-temperature water bath or air bath for 40 minutes		
Chromogenic reagent (mL)	2	2
Supernatant (mL)		0.15
Mix well, stand at room temperature for 10 minutes, measure at 550 nm with a 1 cm optical path, zero with distilled water, and perform spectrophotometric colorimetry (or take 200 μL from each tube, with one well containing 200 μL distilled water for zeroing, and read at 550 nm using a microplate reader).		

3. Calculation of SOD activity in hyperlipidemic serum (plasma):

Definition: The amount of SOD corresponding to a 50% inhibition rate in 1 mL of reaction solution is defined as one unit (U) of SOD activity.

$$\text{SOD activity (U/ml)} = \frac{A_{contrast} - A_{sample}}{A_{contrast}} \div 50\% \times \frac{\text{Reaction system dilution times}^{*}}{\text{Sample dilution times before test}^{**}}$$

[Note]:*

$$\frac{\text{Reaction system dilution times}}{} = \frac{\text{Total reaction volume}}{\text{Sample volume}}$$

$$= \frac{\text{Reagent volume } 3.3\,ml + \text{Sample volume in the reaction system}}{\text{Sample volume in the reaction system}}$$

**

$$\text{Dilution factor before serum (plasma) testing for severe hyperlipidemia} = \frac{\text{Sample volume } 50\mu l + \text{Normal Saline } 200\mu l + \text{Anhydrous Ethanol } 150\mu l}{\text{Sample volume } 50\mu l} = \frac{400\mu l}{50\mu l}$$

4. Calculation example:

To determine total SOD in hyperlipidemic rabbit serum, take 50 μL of hyperlipidemic serum, add 200 μL of physiological saline and mix well, then add 150 μL of absolute ethanol and mix thoroughly for 1 minute, then add 150 μL of chloroform and mix thoroughly for 1 minute, centrifuge at 3500 rpm for 10 minutes, and take 150 μL of supernatant for testing. The absorbance of the control tube is 0.434, and that of the test tube is 0.262. Substitute the data into the calculation formula:

$$SOD \text{ activity } (U/\text{ml}) = \frac{0.434 - 0.262}{0.434} \div 50\% \times \frac{3.45}{0.15} \times \frac{400}{50} = 145.84 U/\text{ml}$$

Appendix III: Determination of T-SOD in Red Blood Cells

1. Sample pretreatment (preparation of red blood cell extract):

① Collect 50 μL of fresh blood, or heparin-anticoagulated venous or arterial blood, add it to a graduated centrifuge tube containing 1–2 mL of physiological saline, and centrifuge at 500–1000 rpm for 10 minutes.

② Use a micropipette or a syringe fitted with an injection needle to aspirate and discard the supernatant completely, leaving the sedimented red blood cells.

③ Add 0.2 mL of cold distilled water to the sedimented red blood cells and mix well (to fully lyse the red blood cells; **the hemolysate appears transparent when observed against light if lysis is complete**).

④ Add 0.1 mL of 95% ethanol and shake for 30 seconds.

⑤ Add 0.1 mL of chloroform and extract thoroughly by vortex mixing for 1 minute.

⑥ Centrifuge at 3500 rpm for 8 minutes. At this time, the liquid separates into three layers: the upper layer is the SOD extract, the middle layer is hemoglobin precipitate, and the lower layer is chloroform. If the supernatant is turbid, add a small amount of solid NaCl and centrifuge again for 5 minutes to clarify. Record the volume of the supernatant, take 5–20 μL, and refer to "**Exploration of Optimal Sample Concentration**" **to determine the optimal sampling volume. After determining the optimal sampling volume, perform the formal experiment according to the table below. (The remaining red blood cell extract can be stored frozen for later use.)**

[Note] For Mouse and Rat, blood can be collected from the inner canthus using a glass capillary tube inserted obliquely toward the pharynx, or by tail cutting. Drop the blood onto a heparinized and dried glass slide (drying temperature should be below 60℃), then use a pipette to take 50 μL of blood for SOD extraction. When the blood volume is small, 10–20 μL of anticoagulated whole blood can be used. (In chronic animal experiments, blood can be collected repeatedly 5–6 times during the feeding period.)

2. Operation procedure:

Reagent	Test tube	Control tube
Reagent I working solution (mL)	1.0	1.0
Sample (mL)	a*	
Distilled water (mL)		a*
Reagent II (mL)	0.1	0.1
Reagent III (mL)	0.1	0.1
Reagent IV working solution (mL)	0.1	0.1

Mix thoroughly with a vortex mixer and incubate in a 37℃ constant-temperature water bath or air bath for 40 minutes

Chromogenic reagent (mL)	2	2
Mix well, stand at room temperature for 10 minutes, measure at 550 nm with a 1 cm optical path, zero with distilled water, and perform spectrophotometric colorimetry (or take 200 μL from each tube, with one well containing 200 μL distilled water for zeroing, and read at 550 nm using a microplate reader).		

3. Calculation formula for SOD activity in red blood cells:

Definition: The amount of SOD corresponding to a 50% inhibition rate in 1 mL of reaction solution per gram of hemoglobin is defined as one unit (U) of SOD activity.

$$\frac{\text{SOD activity in}}{\text{Red blood cells (U/ml)}} = \frac{A_{contrast} - A_{sample}}{A_{contrast}} \div 50\% \times \text{Reaction liquid volume} \times \frac{\text{Total Extraction Volume}}{\text{Measured Extraction Volume}} \times \frac{1ml}{\text{Blood sample volume}} \div \frac{\text{Hemoglobin content}}{(gHb/ml)}$$

4. Calculation exam le:
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Example 1: Take 10 μL of red blood cell extract to measure SOD activity. The absorbance of the control tube is 0.465, and that of the test tube is 0.293. The hemoglobin concentration in whole blood is 105 g/L (i.e., 0.105 g/mL).

$$\frac{\text{SOD activity in Red blood cells}}{(U/gHb)} = \frac{0.465-0.293}{0.465} + 50\% \times 3.31 \times \frac{0.3**}{0.01***} \times \frac{1}{0.05*} \times 0.105$$
$$= 1469.21\ U/ml + 0.105 gHb/ml = 13992.50\ U/gHb$$

[Note]:
The blood collection volume is 50 μL;
50 μL of whole blood is used for red blood cell SOD extraction, yielding 300 μL of extract;
10 μL of red blood cell extract is used for determination.