

Superoxide Dismutase Assay

PROTOCOL

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CELL TECHNOLOGY

I. Introduction

Superoxide dismutase (SOD) are metalloenzymes that catalyze the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) + molecular oxygen (O_2) and consequently provide an important defense mechanism against superoxide radical toxicity (1).

Oxidative stress dependent upon superoxide radical can account for a number of acute and chronic disease states, which include inflammation and ischemia-reperfusion (2,3). SOD protects murine peritoneal macrophages from apoptosis induced by adriamycin (4). Furthermore over expression of SOD in fibrosarcoma cells protect against apoptosis and promote cell differentiation (5).

Assay Principle:

To determine SOD activity, several direct and indirect methods have been developed. A common and convenient indirect method utilizes nitroblue tetrazolium (NBT) conversion to NBT-diformazan (formazan dye) via superoxide radical. However, there are several disadvantages to the NBT method, such as poor water solubility of the formazan dye and the interaction with the reduced form of xanthine oxidase. Though cytochrome C is also commonly used for SOD activity detection, its reactivity with superoxide is too high to determine low levels of SOD activity.

Cell Technology's SOD kit utilizes a highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion (6). The rate of the reduction with $O_2^{\cdot -}$ is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, as shown in Figure 1. Therefore, the IC₅₀ (50% inhibition activity of SOD or SOD-like materials) can be determined by this colorimetric method. Absorbance can be measured at 440nm.

Figure 1
Inhibition Curve Prepared Using SOD from Bovine Liver

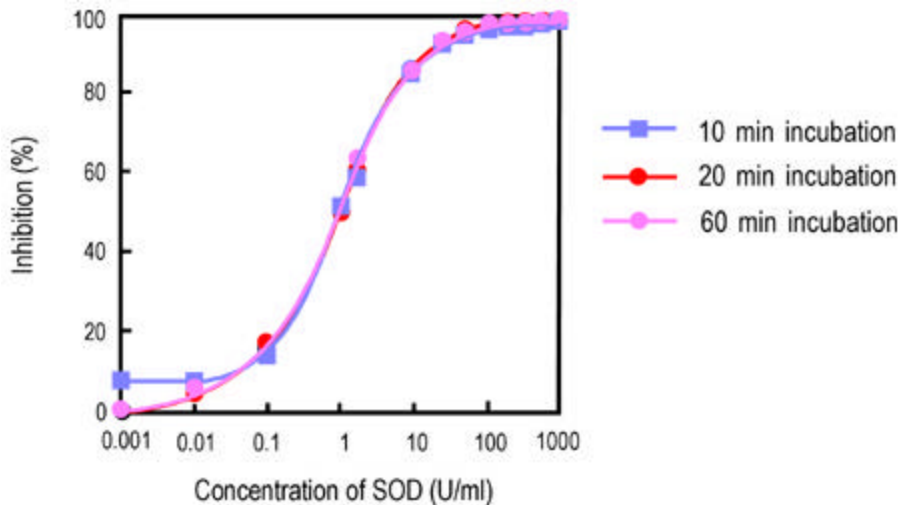
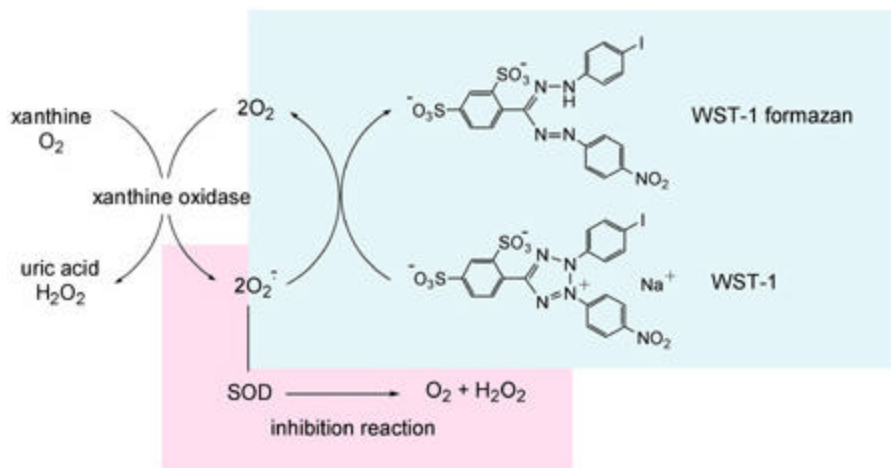


Figure 2
SOD ASSAY Reaction:



II. Kit Storage:

1. The kit should be stored at 4-8 °C and away from light. Stable for 12 months.

III. Warnings and Precautions:

1. For Research use only. Not for use in diagnostic procedures.
2. Practice safe laboratory procedures by wearing protective clothing and eyewear.
3. Reducing agents, such as ascorbic acids and reduced forms of glutathiones, interfere with the SOD assay. Please note that since the increase in the O.D. values can be subtracted as the O.D. of blank 2, these materials do not interfere with the actual SOD assay.

The following are the concentrations of materials that cause 10% increase in the O.D. value:

Ascorbic acid:	0.1mM.
Glutathione, reduced form:	5mM.
BSA showed no increase in OD readings:	5%.

IV. Part # 5016. Kit contents (for 100 assays):

1. Part # 4014. 20X WST-1 Solution: 1 ml.
2. Part # 6010. Xanthine oxidase solution (XO): 15uL.
3. Part # 3029. Assay Buffer: 20 mL.
4. Part # 3030. Xanthine oxidase Dilution Buffer: 10mL.
(XO dilution Buffer)
5. Part# 6011. SOD Enzyme: 30uL. See vial for activity.
6. Part# 9001. 96 well ELISA Plate: 1 plate
7. Part# 9002. Adhesive Plate Cover: 2.

Materials required but not supplied:

1. Plate Reader (450nm Filter).
2. Pipettors

3. Incubator
4. PBS.
5. double distilled water (ddH₂O)

V. Preparation of reagent working solutions and stability:

1. WST-1 1X Working Stock Solution.
Make a 1X working solution from the 20X WST-1 Solution (Part#4014) by diluting it 1:20 with Assay Buffer (part#3029).
Storage: This 1X solution is stable for 2 months when stored at 4°C and protected from light.
2. Working stock of Xanthine oxidase.
Centrifuge the tube for a few seconds to collect any enzyme trapped in the cap of the tube. Next vortex the tube to ensure the contents are thoroughly mixed (the enzyme is separated into two layers). Dilute the Xanthine oxidase 1:166 in Xanthine oxidase dilution buffer (part# 3030). For example after vortexing and right before removing 5uL of the enzyme, mix the contents of the tube by pipeting up and down a few times. Remove 5uL of the enzyme and add it to 825uL of Xanthine oxidase dilution buffer.
Storage: This working stock enzyme solution is stable for 3 weeks when stored at 4°C and protected from light. The enzyme maybe frozen in aliquots for long-term storage.
3. Standard Curve of SOD.
See V11: Assay Set up SOD standard curve.
Storage: Do not store the diluted standard curve material.

VI. Assay Protocol

1. Cell Lysate Preparation

1. Detach adherent cells by gentle trypsinization or cell scraping. Count the cells and wash cells two times in ice cold 1X PBS by centrifugation for 10 minutes at 250 x g at 2 - 8° C.

Note: For trypsinization of adherent cells, use 0.5% (w/v) trypsin, 0.2% (w/v) EDTA. Cell lysis buffer: You can use either Triton X-100 or NP-40 up to 1% v/v.

2. After the final wash suspend the cell pellet with 500 µL of cell lysis buffer per 1 - 5 x 10⁶ cells. Vortex and pipette up and down to ensure complete lysis.

Note: Each investigator should determine optimal cell concentration.

3. Transfer the suspension to a 1.5 mL tube and centrifuge for 5 minutes at 12,000 - 14,000 x g at 2 - 8° C. Place the supernate into a clean 1.5 mL tube. Store on ice and assay for SOD immediately or store at -80° C.

2. Tissue Lysate Preparation

1. Tissue samples should be perfused with saline or PBS containing an anticogluant(heparin at 0.17 mg/mL). After perfusion, wash the tissue in PBS and blot dry. Subsequent tissue preparations can be carried out in a ice cold sucrose buffer (50mM sucrose, 200mM mannitol, 1mM EDTA in 10mM TRIS buffer pH 7.4).

2. Measure the weight of the sample, mince and homogenize the tissue using a Teflon pestle in a Potter-Elvehjem homogenizer. If necessary, sonicate the homogenized sample on an ice bath for 15 minutes (60W with 0.5 second intervals).

3. Centrifuge the homogenized sample at 10,000 g for 15 minutes at 4°C and transfer the supernatant into a new tube. Store processed samples on ice and assay for SOD immediately or aliquot and store at -80°C.

3. Erythrocyte Lysate Preparation

1. Collect blood with heparin or EDTA as an anticoagulant and centrifuge at 2500 x g at 2 - 8° C for 5 - 10 minutes. Remove the plasma supernate and the upper layer of the red blood cell pellet which contains the buffy coat. Suspend the erythrocytes in 4 volumes of ice-cold water and mix thoroughly. Allow 5 minutes for lysis to occur. Assay for SOD immediately or aliquot and store at -80° C.

4. Differentiation of Mn/Fe -SOD from Cu/Zn-SOD

1. Mn- and Fe-SOD can be inactivated by adding 400 µL or 800 µL of ice-cold chloroform/ethanol (37.5/62.5 (v/v)) to 250 µL of erythrocyte lysate or 500 µL of tissue lysate, respectively, shaking for 30 seconds and centrifuging at 2500 x g for 10 minutes (7). Assay the aqueous phase for Cu/Zn-SOD immediately or aliquot and store at -80° C.
2. The addition of cyanide ion to a final concentration of 2 mM inhibits Cu/Zn-SOD by over 90%. Mn-SOD is unaffected by cyanide.

VII. Assay Set up.

1. Controls Sample and SOD Standard Curve.

1. Controls and Sample.

Refer to Table 1 for the amount of solutions to add to each well.

If you are using a SOD standard curve, set up wells for it in the same manner as the sample.

A. Add 20 µl of sample solution to each sample and Control 2 well (control 2 is optional and only needed if the samples have a visible color), and add 20 µl of ddH₂O (double distilled water) to each control 1 and control 3 well.

B. Add 200 µl of 1X WST Working Solution to all the sample and control wells and mix.

C. Add 20 µl of Xanthine Oxidase Dilution Buffer to each control 2 and control 3 well.

D. Add 20 µl of Enzyme Working Solution to each sample or SOD standard curve and control 1 well, and then mix thoroughly*.

E. Incubate the plate at 37°C for 20 min.

F. Read the absorbance at 450 nm using a microplate reader.

G. Calculate the SOD activity (inhibition rate %) using the following equation. SOD activity (inhibition rate %) =

$$\left\{ \frac{[(A_{\text{control 1}} - A_{\text{control 3}}) - (A_{\text{sample}} - A_{\text{control 2}})]}{(A_{\text{control 1}} - A_{\text{control 3}})} \right\} \times 100$$

(Optional)

Table 1.

	Sample or SOD standard curve	Control 1	Control 2* Optional	Control 3
Sample solution	20µL	—	20µL	—
Double distilled H ₂ O	—	20µL	—	20µL
WST 1X working Solution	200µL	200µL	200µL	200µL
XO working solution	20µL	20µL	—	—
XO dilution buffer	—	—	20µL	20µL

* **Optional:** If the sample has visible color set up this control 2.

2. SOD Standard Curve.

1. SOD 9600 units/mL Stock solution suspension in ammonium sulfate (3.8M). Centrifuge the tube for a few seconds to collect any enzyme trapped in the cap of the tube. Next vortex the tube to ensure the contents are thoroughly mixed (the enzyme is a crystallized suspension). Dilute the SOD 1:48 in Xanthine Oxidase dilution buffer (part# 3030). For example after vortexing and right before removing 10uL of the enzyme, mix the contents of the tube by pipeting up and down a few times. Remove 10uL of the enzyme and add it to 470uL of Xanthine Oxidase dilution buffer. Follow the instruction below to construct the SOD standard cuve:

- A. Obtain and label, 1-11, suitable size tubes.
- B. To tube 1 add 470 uL of Xanthine Oxidase dilution buffer + 10uL of SOD enzyme (as mentioned above). This is the first standard: 200 Units/mL.
- C. Next to tubes 2-11 add 200 uL of Xanthine Oxidase dilution buffer.
- D. Remove 200uL from tube 1 (SOD at 200 Units /mL) and add it to tube 2.
- E. Gently pipette up and down several times and vortex tube 2. This is standard 2: 100 Units/mL.
- E. Next remove 200 uL from tube 2 and add it to tube 3. Continue this serial dilution down to tube 11.

Standatd Curve.

Standard	SOD Units/mL.
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1	200 Units/mL.
2	100 Units/mL.
3	50 Units/mL.
4	20 Units/mL.
5	10 Units/mL.
6	5 Units/mL.
7	1 Units/mL.
8	0.1 Units/mL.
9	0.05 Units/mL.
10	0.01 Units/mL.
11	0.001 Units/mL.

2. Assay set up.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1	STD 1	STD 1	CTR 1	CTR 1	CTR 1	CTR 2	CTR 2	CTR 2	CTR 3	CTR 3	CTR 3
B	STD 2	STD 2	STD 2	STD 9	STD 9	STD 9	STD 10	STD 10	STD 10	STD 11	STD 11	STD 11
C	STD 3	STD 3	STD 3	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	STD 4	STD 4	STD 4	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	STD 5	STD 5	STD 5	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	STD 6	STD 6	STD 6	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	STD 7	STD 7	STD 7	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	STD 8	STD 8	STD 8	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample

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