Enzo Life Sciences

Enabling Discovery in Life Sciences®

> SOD activity kit

Catalog # ADI-900-157

Sufficient Reagents for 480 tests with 5 x 96-well plates
For use with mammalian cell and tissue extracts

All reagents, except Standard and Xanthine Oxidase, should be stored at 4˚C. Store Standard and Xanthine at -20˚C

Check our website for additional protocols, technical notes and FAQs.

For proper performance, use the insert provided with each individual kit received.

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Introduction

The SOD activity kit is a complete kit for the measurement of isoenzymes SOD1 (cytosolic Cu/Zn SOD), SOD2 (mitochondrial Mn SOD), and SOD3 (extracellular Cu/Zn SOD) activity in mammalian cell and tissue extracts.

Harmful reactive oxygen species (ROS) arise from environmental factors as well as from normal aerobic metabolic pathways, including oxidative phosphorylation in mitochondria, antimicrobial responses of polymorphonuclear leukocytes[1], and the detoxification reactions of the Cytochrome P450 family of monooxygenases[2]. Oxygen is an ideal terminal electron acceptor; the transfer of four electrons to oxygen leads to the formation of two molecules of H_2O, but partial reduction results in the formation of hazardous ROS. In particular, the transfer of a single electron to O_2 forms superoxide anion, whereas the transfer of two electrons yields peroxide[3]. Superoxide Dismutase (SOD) catalyzes the conversion of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Figure 1), and provides the primary cellular defense against the toxicity of superoxide anion[3]. Overexpression of SOD protects murine fibrosarcoma cells from apoptosis and promotes cell differentiation[4]. SOD also inhibits adriamycin-induced apoptosis in murine peritoneal macrophages[5]. Finally, mutant variants of SOD have been implicated in the etiology of amyotrophic lateral sclerosis[6-8].

Superoxide anions have a very short half life and, accordingly, they must be produced continuously. In this colorimetric based assay, superoxide ions are generated from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase (Figure 1). The superoxide anion then converts WST-1 to WST-1 formazan, a colored product that absorbs light at 450 nm. SOD reduces the superoxide ion concentration and thereby lowers the rate of WST-1-formazan formation[9]. Reduction in the appearance of WST-1-formazan is a measure of SOD activity present in your experimental sample. In other words, relative SOD activity of the experimental sample is determined from percent inhibition of the rate of formation of WST-1 formazan.

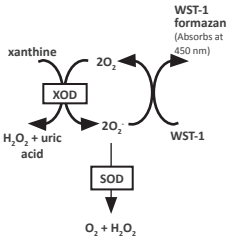


Figure 1.
SOD reaction scheme illustrating the coupled reaction between Xanthine Oxidase (XOD), SOD, the generation of superoxide radical, and the reduction of the dye WST-1 by superoxide anion to a colored WST-1 formazan product that absorbs light at 450 nm. SOD scavenges superoxide anion and thereby reduces the rate of formation of WST-1 formazan product.

Principle

1. Samples and standards are added to wells of a 96-well plate. SOD Master mix is added to the wells.
2. Xanthine solution is added to the wells to initiate the reaction.
3. The plate is transferred to a plate reader and absorbance readings are taken at 450 nm every minute for 10 minutes.

Materials Supplied

1. Clear Microtiter Plate
   Five Plates of 96 Wells, Catalog No. 80-1639
   Clear uncoated solid plates.
2. SOD Standard
   50 µL, Catalog No. 80-1642
   Superoxide Dismutase standard with an activity of 50 units/µL. One unit of SOD reduces the rate of WST-1 formazan formation by 50%.
3. 10X SOD Buffer
   20 mL, Catalog No. 80-1643
4. Xanthine Oxidase
   3 mL, Catalog No. 80-1644
5. 10X Xanthine Solution
   2 mL, Catalog No. 80-1645
6. 20% Triton X-100
   1 mL, Catalog No. 80-1641
7. WST-1 Reagent
   3 mL, Catalog No. 80-1646
8. Superoxide Dismutase Assay Layout Sheet
   1 each, Catalog No. 30-0238

Storage

The SOD Standard and Xanthine Oxidase should be stored at -20°C. All other components of this kit are stable at 4°C. All kit components are stable at their recommended storage temperatures until the kit expiration date.

Materials Needed but Not Supplied

1. PBS
2. Distilled water
3. Protease inhibitors (optional) such as phenylmethylsulfonyl fluoride (PMSF), Sigma P7626 or equivalent
4. Reagents to determine protein concentration
5. Ficoll-Hypaque™ (erythrocyte, lymphocyte and monocyte preparations)
6. Ethanol and chloroform (erythrocyte preparation)
7. Microtubes, 0.5 and 1.5 mL
8. 15 mL conical tubes (adherent and suspension cell preparation)
9. 50 mL conical tubes (tissue preparation)
10. Precision pipettes for volumes between 5-1000 µL
11. Multichannel pipettor for volumes between 1 - 50 µL and 50 µL – 200 µL
12. Microplate reader capable of reading at 450 nm and taking readings every minute for ten minutes and exporting data to an Excel spreadsheet
13. Centrifuge and microfuge for processing samples to an Excel spreadsheet.

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

The physical, chemical, and toxicological properties of the chemicals and reagents contained in this kit may not yet have been fully investigated. Therefore, we recommend the use of gloves, lab coats, and eye protection while using any of these chemical reagents.

Reagents require separate storage conditions.

Reagent Preparation

All solutions must be prepared just before use.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

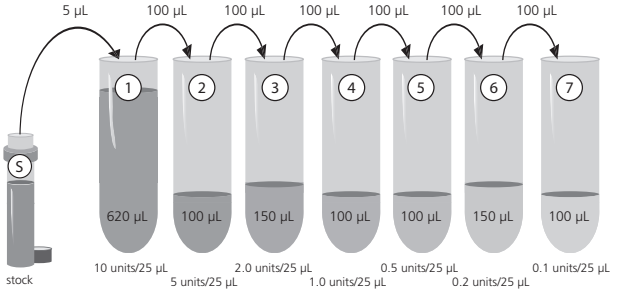
Enzyme should be diluted just before use. Store enzyme on ice. Discard unused enzyme dilutions.

The SOD standard curve is used as a positive control to verify that the assay is working. It should not be used to calculate the units of SOD in your experimental sample.

1. 1X SOD Buffer

   Dilute the 10X SOD Buffer to 1X (1:10) with distilled water. The 1X SOD Buffer is used to prepare 1X Xanthine Solution and dilutions of SOD Standard. The 10X SOD Buffer is used directly to prepare 1X Cell Extraction Buffer and Master Mix.

2. SOD Standard Curve



Thaw the 50 units/µL SOD Standard on ice. Label seven 1.5 mL microtubes #1 through #7. Pipet 620 µL of 1X SOD Buffer into tube #1. Pipet 100 µL of 1X SOD Buffer into tubes 2, 4, 5, and 7. Pipet 150 µL of 1X SOD Buffer into tubes #3 and 6. Add 5 µL of SOD Standard to tube #1 and vortex thoroughly. Add 100 µL of tube #1 to tube #2 and vortex thoroughly. Add 100 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.

Diluted standards should be used immediately. The concentrations of SOD in the tubes are labeled above.

3. 1X Cell Extraction Buffer

Prepare sufficient amount of Cell Extraction Buffer. Preparation for 10 mL is as follows:

10X SOD Buffer	1.0 mL
20% (v/v) Triton X-100	0.2 mL
Distilled water	8.8 mL
200 mM PMSF (optional)	(10 µL)

4. 1X Xanthine Solution

Count the total number of wells needed for the samples and add 24 (for the complete standard curve and Activity Control wells in triplicate). Use the following formula to calculate the volume of 1X Xanthine Solution required.

A. Total volume required
   [Total number of wells needed + 24] x 25 µL = _____ µL

B. Volume of 10X Xanthine Solution required
   Total Volume Required (from A. above)] x 0.1 = _____ µL

C. Volume of 1X SOD Buffer required
   [Total Volume required (from A. above)] x 0.9 = _____ µL

5. Master Mix

   Count the total number of wells needed for the samples and add 24 (for the complete standard curve and Activity Control wells in triplicate). Use the following formula to calculate the volume of Master Mix required.

   A. Total volume required
      [Total number of wells needed + 24] x 150 µL = _____ µL
   B. Volume of 10X SOD Buffer required
      [Total Volume Required (from A. above)] x 0.1 = _____ µL
   C. Volume of WST-1 Reagent required
      [Total Volume required (from A. above)] x 0.0333 = _____ µL
   D. Volume of Xanthine Oxidase required
      [Total Volume required (from A. above)] x 0.0333 = _____ µL
   E. Volume of distilled water required
      [Total Volume required (from A. above)] x 0.833 = _____ µL

   Prepare Master Mix by combining the appropriate reagent volumes calculated in B, C, D, and E above. For example, to prepare 150 µL of Master Mix the following volumes are combined: 15 µL 10X SOD Buffer, 5 µL WST-1 reagent, 5 µL Xanthine Oxidase, and 125 µL distilled water.

Thaw the Xanthine Oxidase on ice. Keep the stock on ice while in use.

6. Biological Extracts

   After preparing the samples as outlined in the Sample Handling section that follows, make serial dilutions of cell or tissue extracts with 1X SOD Buffer. Initial concentrations between 0.5 µg/25 µL to 50 µg/25 µL are recommended.

Sample Handling

Choose the appropriate protocol in Section A to process sample before proceeding to Section B. Keep samples on ice to maintain enzyme activity.

Samples must be kept on ice to maintain enzyme activity.

Section A. Processing Samples

Suspension cells:

1. Centrifuge 2 to 6 x 10^6 suspension cells at 250 x g for 10 minutes at 4ºC. Discard the supernatant.
2. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to a 1.5 mL microtube on ice. Centrifuge, discard supernatant, and place on ice.
3. Proceed to Section B. Preparation of Cytosolic Extracts

Adherent cells:

1. Wash 2 to 6 x 10^6 adherent cells with 1X PBS. Adherent cells may be harvested by gentle trypsinization.
2. Transfer to a 15 mL tube on ice. Centrifuge at 250 x g for 10 minutes at 4ºC and discard the supernatant.
3. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to 1.5 mL microtube on ice. Centrifuge, discard supernatant, and place on ice..
4. Proceed to Section B. Preparation of Cytosolic Extracts.

Erythrocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at 800 x g for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Remove the remaining liquid from above the red blood cell pellet. Wash the pellet with 10 cell volumes of PBS.
4. Determine the packed cell volume and add 10 cell volumes of cold distilled water. Mix well and incubate on ice for 10-15 minutes to lyse the red blood cells. Lysis occurs when the opaque solution changes to a brilliant clear red solution, indicating the release of hemoglobin.
5. Precipitate the hemoglobin by adding 0.25 volumes of ethanol and 0.15 volumes of chloroform. Shake for 1 min and centrifuge at 10,000 x g for 10 minutes at 4°C.
6. Recover the clear top layer and dialyze overnight at 4°C against 1X PBS or 50 mM potassium phosphate, pH 7.8.
7. Centrifuge the dialyzed erythrocyte extract to remove any precipitate that formed during the dialysis and place on ice.
8. Proceed to Section B. Preparation of Cytosolic Extracts.

Lymphocytes and Monocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at 800 x g for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Dilute the blood mononuclear cells with 5 volumes of PBS and centrifuge at 400 x g for 10 minutes at 4°C. Discard the supernatant
4. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to a pre-chilled 1.5 mL microtube. Centrifuge, discard supernatant, and place on ice.
5. Proceed to Section B. Preparation of Cytosolic Extracts.

Tissue

1. Remove tissue and place in cold PBS in a 50 mL conical tube. Repeatedly wash the tissue with PBS to remove blood clots and other debris.
2. Transfer the tissue to a Petri dish on ice and mince the tissue to small pieces with surgical scissors.
3. Transfer the tissue pieces to a clean stainless steel sieve. Place the sieve with the tissue pieces in a Petri dish which contains about 20 mL of cold 1X PBS.
4. Create a single cell suspension of the tissue as follows: Using a pestle or a round bottom tube, grind the tissue pieces thoroughly until the bulk of the tissue passes through the sieve.
5. Transfer the PBS containing the single cell suspension to a 50 mL conical tube. Fill with cold PBS and mix by inverting the tube several times. Let the tube stand on ice for 1 minute to allow large aggregates of tissue to settle out of solution.

6. Carefully transfer the supernatant containing the single cell suspension to a clean 50 mL conical centrifuge tube. Centrifuge at 400 x g for 10 minutes at 4ºC. Discard the supernatant. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to a pre-chilled 1.5 mL microtube on ice. Centrifuge, discard the supernatant, and place on ice.
7. Proceed to Section B. Preparation of Cytosolic Extracts.

Section B. Preparation of Cytosolic Extracts from Cells and Tissue

1. Measure the approximate volume of the cell pellets prepared above (except for erythrocytes) and suspend the cells in 5-10 volumes of cold 1X Cell Extraction Buffer. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.
2. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10 minutes at 4ºC to remove insoluble material. Recover the supernatant to a fresh tube pre-chilled on ice. Occasionally, the pellet may float and can easily be removed with a pipet tip.
3. Determine the protein concentration of the cleared cell lysate.
4. If not assaying for SOD immediately, snap-freeze the cleared cell extract in 100 µL aliquots by immersing the aliquots in liquid nitrogen and store at -80ºC. Avoid repeated freezing and thawing of the extract.
5. The detection of SOD in subcellular fractions is detailed in reference [10].

Section C. Differentiation between SOD1, SOD2, and SOD3.

1. SOD2 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 cell/tissue lysate, respectively, shaking for 30 sec, and then centrifuging at 2,500 x g for 10 min. Assay the upper aqueous phase for SOD1 immediately or freeze in aliquots at -80°C.
2. The addition of cyanide ion to a final concentration of 2 mM inhibits more than 90% of SOD1 activity. SOD2 is unaffected by cyanide.
3. SOD3 is isolated from the extracellular matrix of tissue. SOD3 has been found in serum and in cerebrospinal, ascitic, and synovial fluids. Ensure that all cells are removed from the extracellular fluid by centrifuging at 250 x g for 10 minutes at 4ºC. Assay the supernatant for SOD3 activity.

Assay Procedure

Refer to the Assay layout Sheet to determine the number of wells to be used.

All standards, controls, and samples should be run in triplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagents to the sides of the wells to avoid possible contamination.

1. Pipet 25 µL of the 1X SOD Buffer to the bottom of the Activity Control wells.
2. Pipet 25 µL of the prepared SOD Standards #1 through #7 to the bottom of the appropriate wells.
3. Pipet 25 µL of the diluted sample to the bottom of the appropriate wells.
4. Pipet 150 µL of Master Mix into each well.
5. Initiate the reaction by adding 25 µL of 1X Xanthine Solution to all the wells using a multichannel pipet.
6. Immediately transfer the plate to a microtiter plate reader and take absorbance readings at 450 nm every minute for 10 minutes at room temperature. If possible, include a 10 second orbital shake prior to the initial read.

Calculation of Results

A. Determine rate change in absorbance

1. Take the average triplicate absorbance readings for both the SOD standards and samples. Plot these absorbance values at 450 nm on the Y axis versus time in minutes on the X axis. Take the slope of each curve (change in absorbance at 450 nm per minute). The change in absorbance at 450 nm as a function of time is shown in Figure 2.

The SOD standard curve is used as a positive control to verify that the assay is working. It should not be used to calculate the units of SOD in your experimental sample.

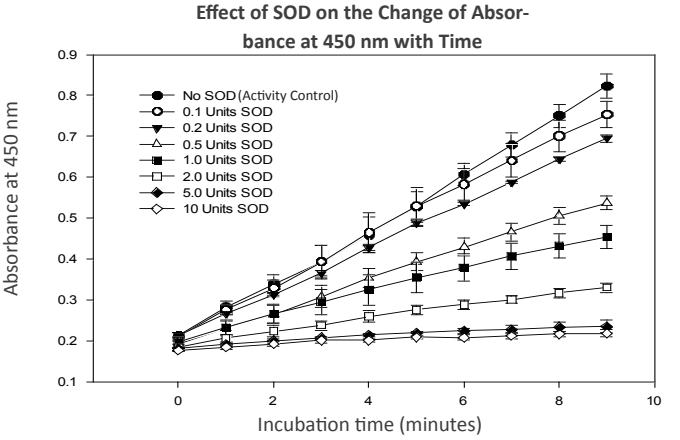


Figure 2. Change in absorbance at 450 nm with time for the SOD standard.

B. Determine % inhibition of the rate of change in absorbance at 450 nm

1. The slope obtained in the absence of SOD (the Activity Control) should be maximal and is taken as the 100% value. All other slopes generated with SOD standards or cell tissue extracts are compared to it. The % inhibition of the rate of increase in absorbance at 450 nm is calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{Slope of 1X SOD Buffer Control} - \text{Slope of Sample}) \times 100}{\text{Slope of 1X SOD Buffer Control}}$$

C. Plot % inhibition versus Log [Units/well SOD standard]

1. The SOD standard is provided at a concentration of 400 ng/µL with an activity of about 50 units/µL. Plot the % Inhibition versus Log [units/well SOD standard]. A typical inhibition curve for the SOD standard is shown in Figure 3:

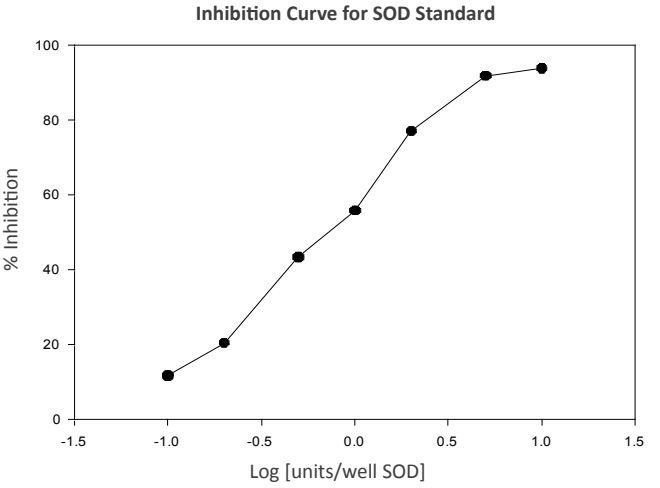


Figure 3. Inhibition curve for the SOD Standard

D. Plot % Inhibition of your cell extract versus Log [µg/well cell extract]

1. In a similar manner, plot % inhibition of your cell extract versus the Log [µg/well cell extract]. From the curve, determine the amount of protein in the cell extract which causes a 50% inhibition of the rate of increase in absorbance at 450 nm. This is shown in Figure 4 for SOD activity in Jurkat cell extracts:

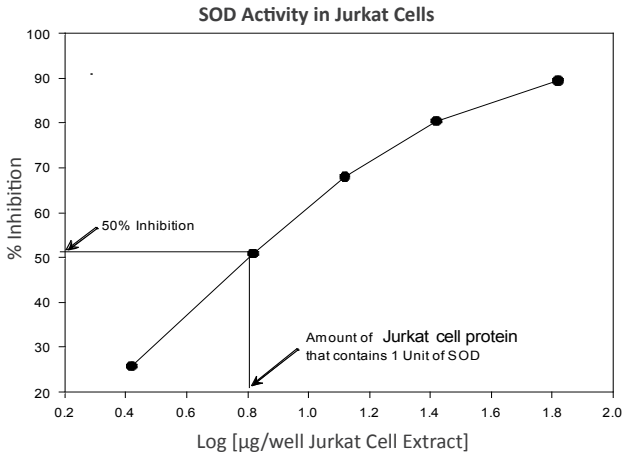


Figure 4. Inhibition curve for Jurkat cell extracts.

E. Calculation of SOD Activity in the standard and in cell/tissue extracts

1. Determine the amount of standard or cell extract protein that causes 50% inhibition (e.g., as shown in Figure 4).

Cell Extract:

Amount of extract causing 50% inhibition	=	log 0.805
Antilog 0.805	=	6.38 µg
SOD specific activity	=	1 unit/6.38 µg
	=	0.157 units/µg
Protein concentration of Jurkat cell extract	=	5.72 µg/µL
SOD concentration in extract	=	0.157 units/µg x 5.72 µg/µL
	=	0.898 units/µL

Troubleshooting

PROBLEM	CAUSE	SOLUTION
No color in wells with SOD buffer alone	Failure to add XOD or WST-1 reagents to the Master Mix	Add XOD or WST-1 reagents to the Master Mix
No color in wells with SOD alone	SOD concentration too high	Add greater dilutions of SOD to each well when making the standard curve
No inhibition of WST-1 formazan formation in wells containing cell or tissue extracts	SOD activity in cells and tissues very low	Extend reaction to 20 minutes
		Reduce the amount of distilled water in the Master Mix. Add 100 µL of this modified Master Mix and 50 µL of your extract to each well

References

1. Weiss SJ, LoBuglio AF. 1982. Phagocyte-generated oxygen metabolites and cellular injury. Laboratory Investigation, 47:5-18.

2. Scholz W, Schutze K, Kunz W, and Schwarz M. 1990. Phenobarbital enhances the formation of reactive oxygen in neoplastic rat liver nodules. Cancer Res., 50:7015-7022.

3. Berg JM, Tymoczko JL, Stryer L. 2002. Biochemistry, 5th Edition, WH Freeman and Company, New York.

4. Zhao Y, Kiningham KK, Lin SM, St. Clair DK. 2001. Overexpression of Mn-SOD protects murine fibrosarcoma cells from apoptosis and promotes a differentiation program upon treatment with 5-azacytidine. Antioxid Redox Signal. 3:375-386.

5. Dominguez-Rodriguez JR, et.al. 2001. In vivo inhibition by antioxidants of adriamycin-induced apoptosis in murine peritoneal macrophages. Anticancer Res. 21:1869-1872.

6. Nikolic-Kokic A, Stevic Z, Blagojevic D, Davidovic B, Jones DR, Spasic MB. 2006. Alterations in anti-oxidative defence enzymes in erythrocytes from sporadic amyotrophic lateral sclerosis (SALS) and familial ALS patients. Clin Chem Lab Med. 44:589-593.

7. Stathopulos PB, Rumfeldt JA, Karbassi F, Siddall CA, Lepock JR, Meiering EM. 2006. Calorimetric analysis of thermodynamic stability and aggregation for apo and holo amyotrophic lateral sclerosis-associated Gly-93 mutants of superoxide dismutase. J Biol Chem. 281:6184-6193.

8. Rishi R, Crow JP, Lepock JR, Kondejewski LH, Cashman NR, Chakrabartty A. 2004. Monomeric Cu,Zn-superoxide Dismutase Is a common misfolding intermediate in the oxidation models of sporadic and familial amyotrophic lateral sclerosis. J Biol Chem., 279:15499-15504.

9. Ukeda H, Maeda S, Ishii T, Sawamura M. 1997. Spectrophotometric assay for superoxide dismutase based on tetrazolium salt 3'--1--(phenylamino)-carbonyl--3, 4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate reduction by xanthine-xanthine oxidase. Anal Biochem. 251:206-209.

10. Okado-Matsumoto A, Fridovich I. 2001. Subcellular distribution of superoxide dismutases (SOD) in rat liver. J Biol Chem. 276:38388-38393.

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MSDS (Material Safety Data Sheet) available online

Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

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