Superoxide Dismutase Assay Kit

Item No. 706002

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 Well Quantity/Size	480 Well Quantity/Size
706001	Assay Buffer (10X)	1 vial/5 ml	1 vial/20 ml
706003	Sample Buffer (10X)	1 vial/5 ml	1 vial/10 ml
706004	Radical Detector	1 vial/250 µl	2 vials/250 µl
706005	SOD Standard	1 vial/100 µl	2 vials/100 µl
706006	Xanthine Oxidase	3 vials/50 µl	6 vials/50 µl
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	5 plates
400012	96-Well Cover Sheet	1 cover	5 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring an absorbance at 440-460 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism.[1]

$$2O_2^{\bullet -} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

Three types of SODs have been characterized according to their metal content: copper/zinc (Cu/Zn), manganese (Mn), and iron (Fe). SOD is widely distributed in both plants and animals. It occurs in high concentrations in brain, liver, heart, erythrocytes, and kidney. In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial MnSOD, and extracellular SOD.[2] Extracellular SOD is found in the interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph, and synovial fluid.[3,4]

The amount of SOD present in cellular and extracellular environments is crucial for the prevention of diseases linked to oxidative stress. Mutations in SOD account for approximately 20% of familial amyotrophic lateral sclerosis (ALS) cases. SOD also appears to be important in the prevention of other neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases.[5,6] The reaction catalyzed by SOD is extremely fast, having a turnover of 2×10^9 $M^{-1}sec^{-1}$ and the presence of sufficient amounts of the enzyme in cells and tissues typically keeps the concentration of superoxide (O_2^-) very low.[1] However, in a competing reaction, nitric oxide (NO) reacts with O_2^- with a rate constant of 6.7×10^9 $M^{-1}sec^{-1}$ to form the powerful oxidizing and nitrating agent, peroxynitrite.[7] Under conditions in which SOD activity is low or absent (*i.e.*, SOD mutation) or which favor the synthesis of µM concentrations of NO (*i.e.*, ischemia/reperfusion, iNOS upregulation, etc.), NO out-competes SOD for superoxide, resulting in the formation of peroxynitrite. The presence of nitrotyrosine as a "footprint" for peroxynitrite, and hence the prior co-existence of both O_2^- and NO, has been observed in a variety of medical conditions, including atherosclerosis, sepsis, and ALS.[7]

About This Assay

Cayman's Superoxide Dismutase Assay Kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (see scheme 1, below). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical measured in change in absorbance per minute at 25°C and pH 8.0. The SOD assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). The assay provides a simple, reproducible, and fast tool for assaying SOD activity in plasma, serum, erythrocyte lysates, tissue homogenates, and cell lysates. Mitochondrial MnSOD can be assayed separately following the procedure outlined under **Sample Preparation** (see page 8).

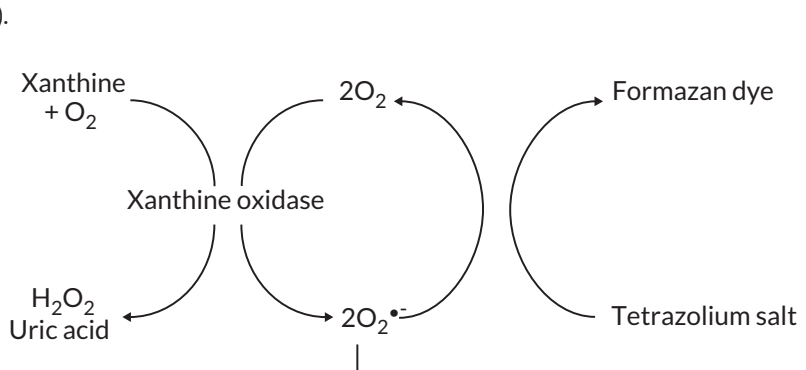


Figure 1. Scheme of the Superoxide Dismutase Assay

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer (10X) - (Item No. 706001)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water for assaying 96 wells. Prepare additional Assay Buffer as needed. This final Assay Buffer (50 mM Tris-HCl, pH 8.0, containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 0.1 mM hypoxanthine) should be used to dilute the radical detector. When stored at 4°C, this diluted Assay Buffer is stable for at least two months.

2. **Sample Buffer (10X) - (Item No. 706003)**

   Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water for assaying 96 wells. Prepare additional Sample Buffer as needed. This final Sample Buffer (50 mM Tris-HCl, pH 8.0) should be used to prepare the SOD standards and dilute the xanthine oxidase and SOD samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least six months.

3. **Radical Detector - (Item No. 706004)**

   The vials contain 250 µl of a tetrazolium salt solution. Prior to use, transfer 50 µl of the supplied solution to another vial and dilute with 19.95 ml of diluted Assay Buffer. Cover with tin foil. The diluted Radical Detector is stable for two hours. This is enough Radical Detector for 96 wells. Prepare additional detector as needed. Store unused Radical Detector at -20°C.

4. **SOD Standard - (Item No. 706005)**

   The vials contain 100 µl of bovine erythrocyte SOD (Cu/Zn). Store the thawed enzyme on ice and see **Standard Preparation** on page 14 for preparing the standard curve. Store unused enzyme at -20°C. The enzyme is stable for at least two freeze/thaw cycles.

5. **Xanthine Oxidase - (Item No. 706006)**

   These vials contain 50 µl of Xanthine Oxidase. Prior to use, thaw one vial and add 1.95 ml of diluted Sample Buffer to the vial. Mix gently, do not vortex. Store the diluted xanthine oxidase on ice. The diluted enzyme is stable for one hour. This is enough Xanthine Oxidase for 96 wells. Prepare additional Xanthine Oxidase as needed. Do not refreeze the thawed enzyme. Any unused enzyme should be thrown away.

Sample Preparation

The procedures listed below for tissue homogenates and cell lysates will result in assaying total SOD activity (cytosolic and mitochondrial). To separate the two enzymes, centrifuge the 1,500 x g supernatant at 10,000 x g for 15 minutes at 4°C. The resulting 10,000 x g supernatant will contain cytosolic SOD and the pellet will contain mitochondrial SOD.[8] Homogenize the mitochondrial pellet in cold buffer (*i.e.*, 20 mM Hepes, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). If not assaying on the same day, freeze the samples at -80°C. The samples will be stable for at least one month.

The addition of potassium cyanide to a final concentration of 1-3 mM in the assay will inhibit both the Cu/Zn-SOD and extracellular SOD, resulting in the detection of only Mn-SOD activity.[3,9]

Samples can be assayed in the absence of Xanthine Oxidase to generate a sample background. This sample background absorbance should be subtracted from the sample absorbance generated in the presence of Xanthine Oxidase thus correcting for non-SOD generated absorbance.

Tissue Homogenate

1. Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per gram tissue.
3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation at 1,000-2,000 x g for 10 minutes at 4°C. For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize or sonicate the cell pellet in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose.
3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month. Plasma should be diluted 1:5 with Sample Buffer before assaying for SOD activity.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month. The erythrocyte lysate should be diluted 1:100 with Sample Buffer before assaying for SOD activity.

Serum

1. Collect blood without using an anticoagulant such as heparin, citrate, or EDTA. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
3. Serum should be diluted 1:5 with Sample Buffer before assaying for SOD activity.

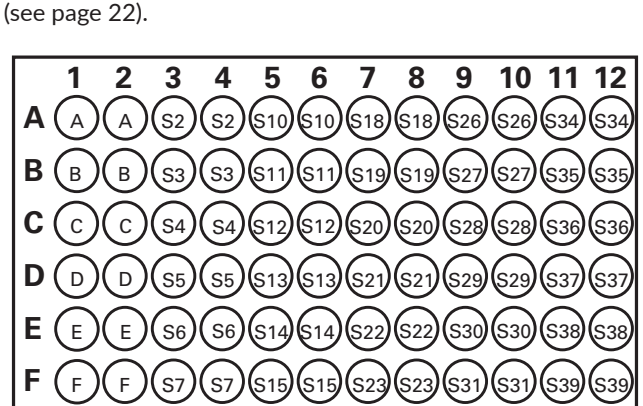
Tissue Homogenization using the Precellys 24 Homogenizer

- Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of issues in liquid nitrogen is preferred.
- Add cold 20 mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210 mM mannitol, and 70mM sucrose.
- Homogenize the tissue sample using the Precellys 24 according to appropriate settings:
- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of SOD standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 22).



A-G = Standards
S1-S41 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 230 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- All reagents except samples and Xanthine Oxidase must be equilibrated to room temperature before beginning the assay.
- It is recommended that the samples and SOD standards be assayed at least in duplicate.
- Monitor the absorbance between 440-460 nm using a plate reader.

Standard Preparation

Dilute 20 µl of the SOD Standard (Item No. 706005) with 1.98 ml of Sample Buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of SOD stock and Sample Buffer (dilute) to each tube as described in Table 1 below.

Tube	SOD Stock (µl)	Sample Buffer (µl)	Final SOD Activity (U/ml) in Well
A	0	1,000	0
B	20	980	0.005
C	40	960	0.010
D	80	920	0.020
E	120	880	0.030
F	160	840	0.040
G	200	800	0.050

Table 1. Superoxide Dismutase standards

Performing the Assay

1. **SOD Standard Wells -** add 200 µl of the diluted Radical Detector and 10 µl of Standard (tubes A-G) per well in the designated wells on the plate (see **Sample plate format**, Figure 2, page 12).
2. **Sample Wells -** add 200 µl of the diluted Radical Detector and 10 µl of sample to the wells. *NOTE: If using an inhibitor, add 190 µl of the diluted Radical Detector, 10 µl of inhibitor, and 10 µl of sample to the wells. The amount of sample added to the well should always be 10 µl. Samples should be diluted with Sample Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to fall within the standard curve range.*
3. Initiate the reactions by adding 20 µl of diluted Xanthine Oxidase to all the wells you are using. Make sure to note the precise time you started and add the Xanthine Oxidase as quickly as possible. *NOTE: If assaying sample backgrounds, add 20 µl of Sample Buffer instead of Xanthine Oxidase.*
4. Carefully shake the 96-well plate for a few seconds to mix. Cover with the plate cover.
5. Incubate the plate on a shaker for 30 minutes at room temperature. Read the absorbance at 440-460 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample. If assayed, subtract sample background absorbance from the sample.
2. Divide standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR) (*i.e.*, LR for Std A = Abs Std A/Abs Std A; LR for Std B = Abs Std A/Abs Std B).
3. Plot the linearized SOD standard rate (LR) (from step 2 above) as a function of final SOD Activity (U/ml) from Table 1. See Figure 3 (on page 18) for a typical standard curve.
4. Calculate the SOD activity of the samples using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical measured in change in absorbance per minute at 25°C and pH 8.0. SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay.

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR - y-intercept}}{\text{slope}}\right) \times \frac{0.23\ \text{ml}}{0.01\ \text{ml}}\right] \times \text{sample dilution}$$

NOTE: 0.23/0.01 is a factor for converting from U/ml in well to U/ml in 10 µl added to 230 µl well volume

Performance Characteristics

Precision:

When a series of 60 SOD standard measurements were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of 60 SOD standard measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.7%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0.005-0.050 units/ml SOD.

Representative Superoxide Dismutase Standard Curve

The standard curve presented here is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use this one to determine the values of your samples.

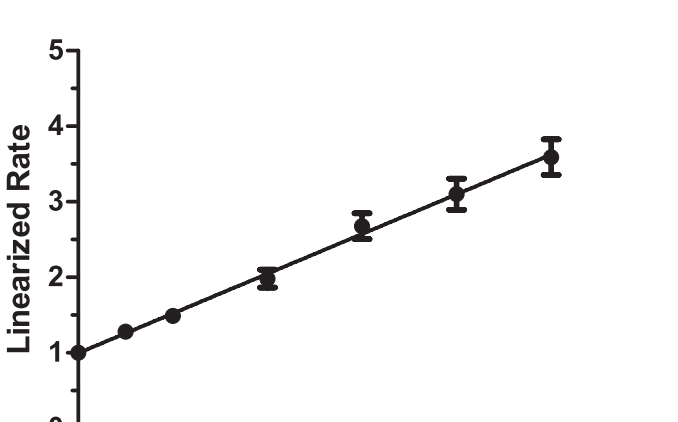


Figure 3. Superoxide Dismutase standard curve

RESOURCES

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	No
	Borate	Yes
	HEPES	No
	Phosphate	No
Detergents:	SDS (0.1%)	Yes
	Triton X-100 (≤0.1%)	No
	Polysorbate 20 (0.1%)	Yes
	CHAPS (0.1%)	Yes
Proteases/Inhibitors/ Chelators:	Antipain (0.1 mg/ml)	Yes
	PMSF (1 mM)	Yes
	Leupeptin (1 mg/ml)	Yes
	Trypsin (0.1 mg/ml)	Yes
	Chymostatin (0.1 mg/ml)	Yes
	EGTA (≤1 mM)	No
	EDTA (≤1 mM)	No
Solvents:	Ethanol (10 µl)	No
	Methanol (10 µl)	Yes
	Dimethyl Sulfoxide (10 µl)	Yes
Others:	Glutathione (≤1.5 mM)	No
	Glycerol (≤1%)	No
	BSA (≤1%)	No
	Dithiothreitol (3 mM)	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No activity was detected in the sample	SOD activity was too low or sample was too dilute	Concentrate the samples using an Amicon concentrator with a molecular weight cut-off of 10,000 and re-assay
No color development in any of the wells	The radical detector was not added to the diluted Assay Buffer	Dilute the radical detector with diluted Assay Buffer and re-assay

References

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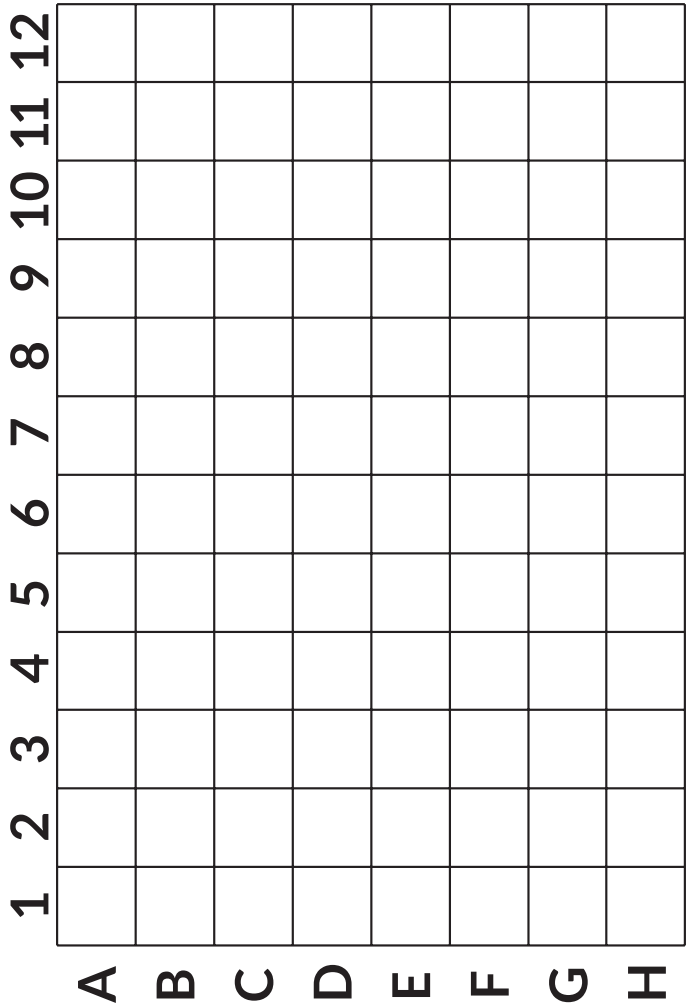

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.