Measurement of superoxide dismutase activity

SOD Assay Kit-WST

Product Code: S311

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In order to determine the SOD activity, several direct and indirect methods have been developed. Among these methods, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use. 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.

SOD Assay Kit-WST allows a very convenient and highly sensitive SOD assay by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with a superoxide anion (Fig. 1). The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD (see figure below). Therefore, the IC_{50} (50% inhibition concentration) of SOD or SOD-like materials can be determined using colorimetric methods.

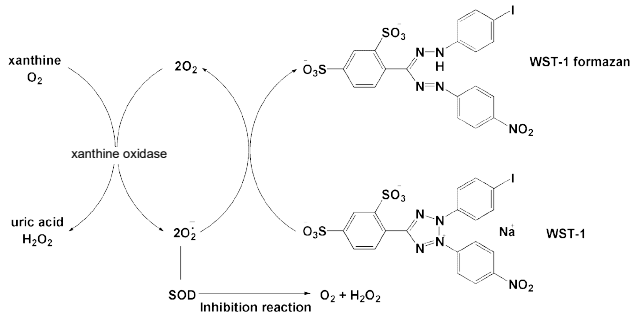

Fig.1 SOD inhibition assay mechanism

1. Preparation of Sample Solutions

► Erythrocytes or Plasma

1. Centrifuge 2-3 ml of anticoagulant-treated blood (such as heparin 10 U/ml with final concentration) at 600 xg for 10 minutes at 4°C.
2. Remove the supernatant and dilute it with saline to use as a plasma sample. Add saline to the pellet to prepare the same volume, and suspend the pellet.
3. Centrifuge the pellet suspension at 600 xg for 10 minutes at 4°C, and discard the supernatant.
4. Add the same volume of saline, and repeat Step 3 twice.
5. Suspend the pellet with 4 ml distilled water, then add 1 ml ethanol and 0.6 ml chloroform.
6. Shake the mixture vigorously with a shaker for 15 minutes at 4°C.
7. Centrifuge the mixture at 600 xg for 10 minutes at 4°C and transfer the upper water-ethanol phase to a new tube.
8. Mix 0.1 ml of the upper phase with 0.7 ml of distilled water, and dilute with 0.25% ethanol to prepare sample solution.

► Tissue(100 mg)

1. Wash the tissue with saline to remove as much blood as possible. Blot the tissue with paper towels and then measure its weight.
2. Add 400-900 µl sucrose buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4) and homogenize the sample using Teflon homogenizer. If necessary, sonicate the homogenized sample on an ice bath (60W with 0.5 second intervals for 15 minutes).
3. Centrifuge the homogenized sample at 10,000 xg for 60 minutes at 4°C, and transfer the supernatant to a new tube.
4. Dilute the supernatant with distilled water to prepare sample solution.

2. Preparations of Solutions (for one 96-well plate)

► WST working solution

Dilute 1 ml of WST Solution with 19 ml of Buffer Solution

► Enzyme working solution

Centrifuge the Enzyme Solution tube for 5 seconds. Mix by pipetting and dilute 15 µl of Enzyme Solution with 2.5 ml of Dilution Buffer.

► Sample solution

Dilute sample solution prepared with Dilution Buffer or Saline.
e.g.) dilution rate: x1(no dilution), x1/5, x1/5^2, x1/5^3, x1/5^4, x1/5^5, x1/5^6

Contents of the Kit

	500 tests
WST Solution	5 ml x 1
Enzyme Solution	100 µl x 1
Buffer Solution	100 ml x 1
Dilution Buffer	50 ml x 1

Required Equipment & Materials

Microplate Reader (450 nm filter)
96-well microplate
2-20 µl & 20-200 µl multi-channel pipettes
Incubator(37°C)

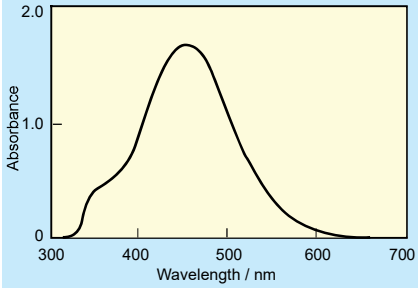

Fig. 2 Absorption spectrum of WST-1 formazan

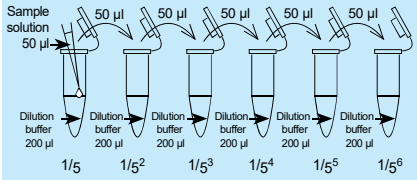

Fig. 3 Serial dilution process

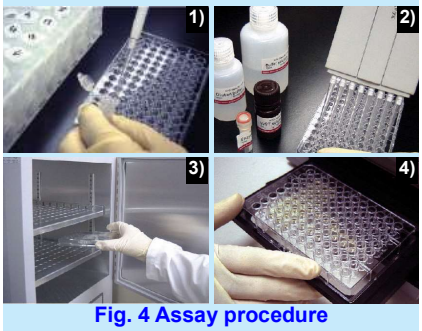

Fig. 4 Assay procedure

3. General Protocol (refer to Table 1, Fig. 4 and Fig. 5)

1. Add 20 µl of sample solution to each sample well and blank 2 well, and add 20 µl of ddH_2O(double-distilled water) to each blank 1 and blank 3 well.
2. Add 200 µl of WST Working Solution to each well, and mix by pipetting.
3. Add 20 µl of Dilution Buffer to each blank 2 and blank 3 well.
4. Add 20 µl of Enzyme Working Solution to each sample and blank 1 well.
5. Incubate the plate at 37ºC for 20 minutes.
6. Read the absorbance at 450 nm using a microplate reader.
7. Calculate the SOD activity(inhibition rate %) using the following equation.

SOD activity(inhibition rate %) = $\{[(A_{blank\ 1}-A_{blank\ 3})-(A_{sample}-A_{blank\ 2})]/(A_{blank\ 1}-A_{blank\ 3})\}$x100

Table 1 Solution and buffer volumes in each well

	sample	blank 1	blank 2	blank 3
Sample Solution	20 µl	-	20 µl	-
ddH_2O	-	20 µl	-	20 µl
WST Working Solution	200 µl	200 µl	200 µl	200 µl
Dilution Buffer	-	-	20 µl	20 µl
Enzyme Working Solution	20 µl	20 µl	-	-

4. Inhibition Curve

As Fig. 6 shows, SOD Assay Kit-WST can measure 100% inhibition because WST-1 does not react with the reduced form of xanthine oxidase(XO).

5. Definition of Unit(U)

One unit is defined as a point where a 20 µl of sample solution gives 50% inhibition of a colorimetric reaction between WST-1 and superoxide anion.

* Unit definition differ from the unit definition of Cytochrome C assay.

6. Calculate Unit(U)

1. Calculate a dilution ratio where the inhibition curve gives 50% inhibition.
2. SOD unit in original sample can be calculated by multiplying the dilution rate.

7. Example of Calculating Unit(U): Erythrocytes(x108 dilution sample)

1. Calculate a dilution ratio from the point of IC_{50} in the inhibition curve. Fig. 7 gives the dilution rate at IC_{50} of 1/1.8.
2. According to the definition of unit, 20 µl of this sample is calculated 1.8 U.
3. SOD unit per 1ml of this sample solution can be calculated by the following equation, 1.8 / 0.02 = 90.0 U/ml.
4. Original erythrocytes sample was diluted 108 times during the sample preparation. To calculate the SOD unit in the original, multiply 90.0 U/ml by 108. The SOD unit in the original sample is 9,720 U/ml of blood.

* SOD unit can be calculated as U/gram or U/mg.

8. Distinguish Mn-SOD from Cu/Zn-SOD and EC-SOD

Mn-SOD can be measured by blocking the Cu/Zn-SOD and EC-SOD activity using potassium cyanide(KCN) or Diethyldithiocarbamate(DDC).

9. Interference

Reducing agents, such as ascorbic acids and reduced forms of glutathiones, interfere with the SOD assay. Table 2 shows the concentrations of materials that cause 10% increase in the O.D. value. If sample contains these materials, please dilute the sample to avoid the interfere.

10. References

1. J. M. McCord, *et al*., An Enzymic Function for Erythrocuprein(hemocuprein). *J Biol Chem*. 1969;**244**:6049-6055.
2. B. L. Geller, *et al*., A Method for Distinguishing Cu,Zn- and Mn-Containing Superoxide Dismutases. *Anal Biochem*. 1983;**128**:86-92.
3. S. Goldstein, *et al*., Comparison Between Different Assays for Superoxide Dismutase-like Activity. *Free Rad Res Commun*. 1991;**12**:5-10.
4. R. H. Burdon, *et al*., Reduction of a Tetrazolium Salt and Superoxide Generation in Human Tumor Cells (HeLa). *Free Rad Res Commun*. 1993;**18**:369-380.
5. M. W. Sutherland, *et al*., The Tetrazolium Dyes MTS and XTT Provide New Quantitative Assays for Superoxide and Superoxide Dismutase. *Free Radic Res*. 1997;**27**:283-289.
6. H. Ukeda, *et al*., Flow-Injection Assay of Superoxide Dismutase Based on the Reduction of Highly Water-Soluble Tetrazolium. *Anal Sci*. 1999;**15**:353-357.
7. H. Ukeda, *et al*., Spectrophotometric Assay for Superoxide Dismutase Based on the Reduction of Highly Water-soluble Tetrazolium Salts by Xanthine-Xanthine Oxidase. *Biosci Biotechnol Biochem*. 1999;**63**:485-488.
8. H.Ukeda, *et al*., Spectrophotometric Assay of Superoxide Anion Formed in Maillard Reaction Based on Highly Water-soluble Tetrazolium Salt. *Anal Sci*. 2002;**18**:1151-1154.
9. N. Tsuji, *et al*., Enhancement of Tolerance to Heavy Metals and Oxidative Stress in *Dunaliella Tertiolecta* by Zn-induced Phytochelatin Synthesis. *Biochem Biophys Res Commun*. 2002;**293**:653-659.

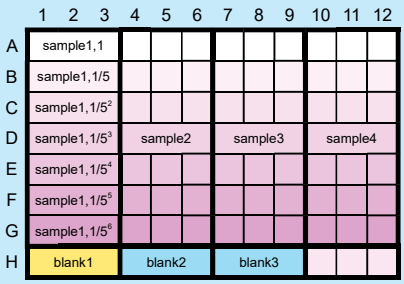

Fig. 5 Sample and blank arrangement on a 96-well microplate

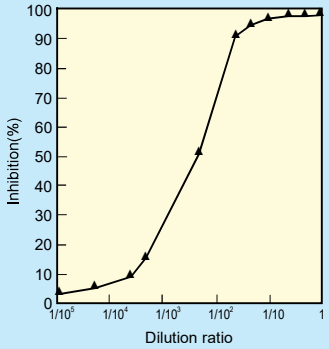

Fig. 6 Inhibition curve of Cu/Zn-SOD

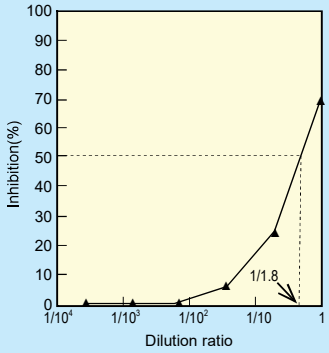

Fig. 7 Inhibition curve on erythrocytes sample

Table 2 Minimum Concentrations of Interfering Substances

Detergents	SDS	0.05%
	Tween 20	0.5%
	NP-40	0.5%
	Triton X-100	0.2%
Solvents	Ethanol	25%
	DMSO	5%
Reducing agents	Glutathione, reduced form	1.25 mmol/l
	Ascorbic acid	0.1 mmol/l
Other	EDTA	2 mmol/l
	BSA	1%w/v

Table 3 Measurement Examples

Total SOD	
erythrocyte	9,720 U/ml of blood
serum	355 U/ml of blood
heart(rat)	15,712 U/g (wet)
liver(rat)	142,907 U/g (wet)
HeLa cell	73 U/1x10^7 cells
HL60 cell	226 U/1x10^8 cells

11. Recent Publications

Title	Reference
Amyotrophic Lateral Sclerosis Model Derived from Human Embryonic Stem Cells Overexpressing Mutant Superoxide Dismutase 1	T. Wada, *et al*., *Stem Cells Trans Med*. 2012; **1**: 396
Copper chelation by tetrathiomolybdate inhibits lipopolysaccharide-induced inflammatory responses in vivo	H. Wei, *et al., Am J Physiol Heart Circ Physiol*. 2011; **301**: H712
A New Enteral Diet, MHN-02, Which Contains Abundant Antioxidants and Whey Peptide, Protects Against Carbon Tetrachloride–Induced Hepatitis	T. Takayanagi, *et al., JPEN J Parenter Enteral Nutr*. 2011; **35**: 516
Effects of dietary supplementation of methionine and its hydroxy analog DL-2-hydroxy-4-methylthiobutanoic acid on growth performance, plasma hormone levels, and the redox status of broiler chickens exposed to high temperatures	H. Willemsen, *et al., Poult. Sci*., 2011; **90**: 2311
Raf Kinase Inhibitor Protein RKIP Enhances Signaling by Glycogen Synthase Kinase-3β	F. Al-Mulla, *et al., Cancer Res*. 2011; **71**: 1334
Temporal changes in the expression of mRNA of NADPH oxidase subunits in renal epithelial cells exposed to oxalate or calcium oxalate crystals	Saeed R. Khan, *et al., Nephrol. Dial. Transplant*. 2011; **26**: 1778
Profiling of superoxide dismutase isoenzymes in compartments of the developing bovine antral follicles	C. Combelles, *et al., Reproduction*, 2010; **139**: 871
Deletion of nuclear factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice	H. Sugimoto, *et al., Am J Physiol Gastrointest Liver Physiol*. 2010; **298**: G283
Gallium Disrupts Iron Uptake by Intracellular and Extracellular Francisella Strains and Exhibits Therapeutic Efficacy in a Murine Pulmonary Infection Model	O. Olakanmi, *et al., Antimicrob. Agents Chemother.* 2010; **54**: 244
Iron restriction improves type 2 diabetes mellitus in Otsuka Long-Evans Tokushima fatty rats	Y. Minamiyama, *et al., Am J Physiol Endocrinol Metab*, 2010; **298**: E1140

12. FAQ

►What is the definition of a Unit?
One unit is defined as a point where a sample gives 50% inhibition of a colorimetric reaction between reactive dye (such as cytochrome C, WST-1, nitro-tetrazolium blue or XTT) and superoxide anion. For example, if the O.D. of "Blank 1" that does not contain any SOD is 1.0, the sample that gives 0.5 O.D. is defined as having 1 unit of SOD activity. You can use this unit to determine the SOD activity of your sample. Therefore, SOD activities determined using different dyes or methods are not comparable with each other.

►Can I use standard SOD to determine SOD activity in sample solutions?
Yes, you can. Prepare a inhibition curve (typical inhibition curve, and determine SOD activity in the sample solution. SOD bovine erythrocytes (CAS# 9054-89-1, EC 1.15.1.1) can be purchased from Sigma (catalog# S7571).

►Can I use a kinetic method to determine SOD activity?
Yes, you can use a kinetic method for SOD assay. Since the rate of the color development remains the same for up to 20 minutes, measure the slope for 5 minutes during this linear phase.

►The sample has color. Can I still use this sample?
Yes, you can still use the sample. Diluting the sample will minimize the interference. Subtract the O.D. of blank 2 from the O.D. of the sample to cancel out the background color. However, if the SOD activity in the sample is low, it may not be measurable.

►How do I prepare more Dilution Buffer?
Dilution Buffer is PBS. Please prepare the Dilution buffer with following concentrations; 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4.

►Can I determine Mn-SOD and Cu/Zn-SOD independently using this kit?
Yes. In order to measure Mn-SOD activity, it is necessary to block the Cu/Zn-SOD activity using potassium cyanide(KCN). Adding 1 mM KCN to samples can block Cu/Zn-SOD activity completely. To measure Cu/Zn-SOD activity, measure the total SOD activity with and without KCN, and then subtract the Mn- SOD activity from total SOD activity.

►How long can I store the sample?
A sample stored in a freezer at -80ºC is stable for 1 month.

►Can I measure the levels of superoxide anion using this kit?
No. However, you could simply use WST-1, instead of this kit, to measure superoxide. You would need a standard to determine the amount of superoxide in sample solution. Since superoxide is not stable and reacts with various materials, it might be difficult to determine the total amount of superoxide generated in the system. The xanthine-xanthine oxidase system in this kit can be used as a standard for measuring the relative amount of superoxide production in each sample.