



Superoxide Dismutase Assay Kit

MANUAL

(for reference TBK0527)



Tiaris Biosciences

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1 MATERIAL PROVIDED

The kit includes the following components

Components	Item N°	TBK0527
Radical Detector (WST-1)	TBR0277	1.2 mL
SOD Reaction Buffer	TBB0568	25 mL
SOD Dilution Buffer	TBB0569	12 mL
XO Enzyme Solution	TBZ0328	30 µL
SOD Standard (3000 U/mg)	TBZ0329	1 mg

Store all components at -20°C.

See Section 9 before its use.

2 STABILITY

Check the expiration date on the kit label. Used it before the expiration date outlined.

The kit will perform as specified if stored at the temperature indicated in previous section.

3 SAFETY DATA SHEETS

Before use the kit, the user must review the Safety Data Sheet available online.

4 MATERIAL REQUIRED (not supplied)

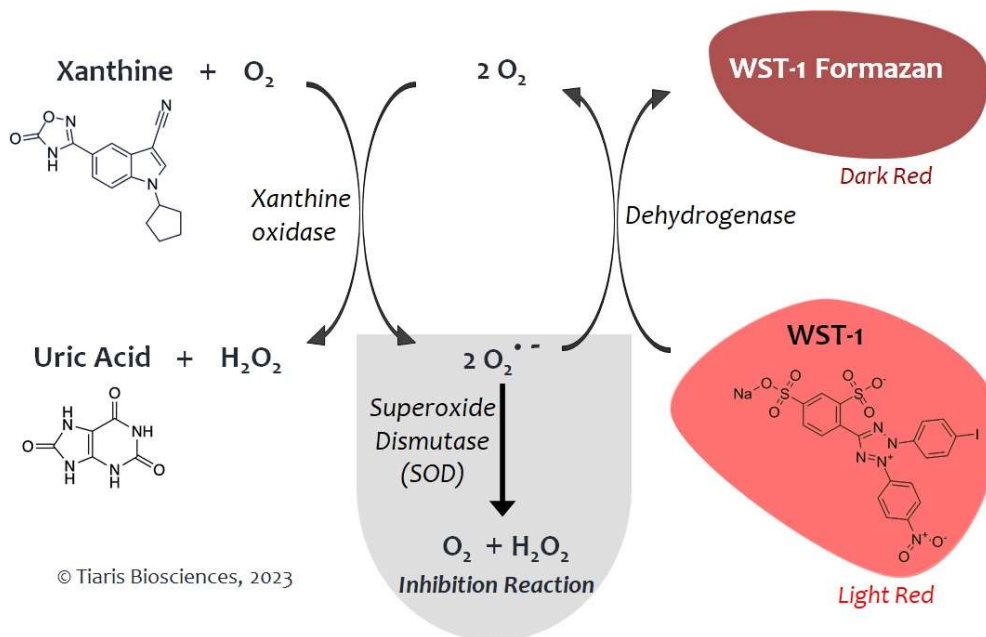
The kit does not include:

- 96-well flat bottom plate (transparent)
- 96-well plate reader
- Plate centrifuge
- Tubes
- Chloroform (CAS 67-66-3)
- Ethanol (CAS 64-17-5)
- KCN (CAS 151-50-8)
- PBS 1x pH=7.2

5 DESCRIPTION

Superoxide Dismutase Assay Kit is an excellent and efficient assay to detect superoxide dismutase (SOD) activity. SODs are one of the most important antioxidant enzymes being the first line of defense against oxygen free radicals. It catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide.

Tiaris Biosciences superoxide dismutase activity determination is based on the generation of superoxide radical ($O_2^{\bullet-}$) by xanthine oxidase action and reduction of WST-1 tetrazolium salt as an indicator of $O_2^{\bullet-}$ production. Both WST-1 and SOD enzyme will compete for $O_2^{\bullet-}$. WST-1 is converted into a water-soluble formazan dye by cellular dehydrogenases, while SOD catalyzes an inhibition reaction for this one. The presence of SOD reduces the amount of WST-1 formazan produced which is spectrophotometrically detected at 450 nm. The percent inhibition of WST-1 reduction is a measure of the amount of SOD present.



The rate of WST-1 reduction by superoxide anion is linearly related to the xanthine oxidase activity and the inhibition by SOD, showing a wide dynamic range from 0.005 to 0.5 U SOD/mL.

Superoxide dismutases are widely present in all aerobic eukaryotes and prokaryotes organisms to protect the cells from oxidative damage. These metalloproteins, according to the metal attached to their active site, can be classified into at least four types, copper-zinc (Cu/Zn SOD), manganese (Mn-SOD), iron (Fe-SOD), and nickel (Ni-SOD). This assay is designed to detect all superoxide dismutase activities, although could be adapted to detect specific SOD activities.

6 GENERAL CONSIDERATIONS

- The kit is suitable to differentiate SOD activities in different types of samples.
- Dilute the samples properly for the assay.
- Samples should be normalized using a protein quantitation assay.
- **SOD Unit Definition:** One unit is defined as the amount of SOD that inhibits 50% (IC_{50} (μg)) of xanthine oxidase activity under the assay conditions.

$$\text{SOD Activity (U/mg)} = \frac{1000}{IC_{50} (\mu\text{g})}$$

7 SAMPLE PREPARATION

Serum

1. Collect blood without anticoagulant.
2. Incubate at room temperature for 30 minutes.
3. Centrifuge at 2,000 g for 15 minutes at 4 °C.
4. Transfer the top layer (serum) to a clean tube. The sample will be stable at -80°C for at least one month. Serum should be diluted ~ 1/5 in PBS 1x before to SOD assay.

Plasma

1. Collect blood using heparin, citrate, or EDTA as anticoagulant.
2. Centrifuge at 1,000 g for 15 minutes at 4 °C.
3. Transfer the plasma layer (top yellow layer) to a clean tube without disturbing the white buffy layer (leukocytes).
4. The sample will be stable at -80°C for at least one month. Plasma should be diluted ~ 1/3 – 1/10 in PBS 1x before to SOD assay.

Erythrocytes

1. Collect blood using heparin, citrate, or EDTA as anticoagulant.
2. Centrifuge at 1,000 g for 15 minutes at 4 °C.
3. Discard the supernatant and wash the pellet with 10 volumes of PBS 1x pH=7.4.
4. Resuspend the pellet in 5 volumes of ice-cold water.
5. Incubate 15 minutes on ice.
6. Centrifuge at 14,000 g for 5 minutes at 4 °C.
7. Transfer the supernatant to a clean tube. The sample will be stable at -80°C for at least one month. Dilute the lysate ~1/100 in PBS 1x prior to SOD assay.

Urine

1. Collect fresh urine.
2. Centrifuge at 10,000 g for 15 minutes at 4°C.
3. Transfer the supernatant to a clean tube. The sample will be stable at -80°C for at least one month.

Saliva

1. With a clean mouth, collect fresh saliva.
2. Centrifuge at 10,000 g for 5 minutes at 4°C
3. Transfer the supernatant to a clean tube. The sample will be stable at -80°C for at least one month.

Tissue

4. Wash the tissue with saline or PBS 1x pH 7.2 to remove as much blood as possible.
5. Blot the tissue with paper towels and then measure its weight.
6. Homogenize 10 mg tissue in 5 mL ice-cold Lysis Buffer (50 mM Potassium Phosphate Buffer pH=7.2; 0.1 mM EDTA; 0.5% Triton X-100).
7. Centrifuge the homogenate at 14,000 g for 5 minutes at 4°C.
8. Transfer the supernatant to a clean tube. The sample will be stable at -80°C for at least one month.

Culture Cells (suspension or adherent)

1. Harvest the cells (10^6 – 10^7 cells) as usual and wash them with 1 mL ice-cold PBS 1x pH 7.2.
2. Centrifuge at 2,000 g for 10 minutes at 4°C.

3. Break cells by sonication (60 W, 0.5 sec for 15 minutes), freeze-thaw method (-20°C for 20 minutes and 37°C bath 10 minutes, repeat twice) or resuspending in 5 mL ice-cold Lysis Buffer.
4. Centrifuge at 10,000 g for 15 minutes at 4°C and transfer the cell lysate (supernatant) to a clean tube.

8 REAGENT PREPARATION

Prepare the following reagents/ Solutions before proceed with the assay.

- **Radical Detection Solution:** Dilute 1.2 mL Radical Detector with 22.8 mL SOD Reaction Buffer. Stable 2 months at 4°C.
- **Enzyme Working Solution:** Spin XO Enzyme Solution tube. Transfer 25 µL XO Enzyme Solution in a tube containing 2.5 mL SOD Dilution Buffer.
- **SOD Standard Solution (5 U/ µL):** Spin SOD Standard tube and add 600 µL SOD Dilution Buffer. This solution is stable for two days at 4 °C and is long term stable at -20 °C.

9 ASSAY PROTOCOL

9.1. ASSAY SETUP

All samples must run at least in duplicate, preferably triplicate. Keep the samples and standard on ice. Equilibrate at room temperature the rest of components.

The assay requires three blanks, sample dilutions and a standard curve. SOD activity can be measured either as percent inhibition activity, which does not require a standard curve, or by a direct comparison to the provided SOD Standard, which is used to prepare a standard curve.

- **Standard curve:** In microcentrifuges tubes, prepare the standard curve by serial dilutions of the SOD Standard in Dilution buffer in a range of 5 U/mL to 0.001 U/mL. lineal range is in 0.05 – 3 U/ mL.
- **Blanks:** In clean tubes, prepare the blanks.

	BLANK 1 without inhibition	BLANK 2 sample blank	BLANK 3 reagent blank
Sample		80 µL*	
Double Distilled Water	80 µL		80 µL
Dilution Buffer		80 µL	80 µL

* If the color of BLANK 2 is strong, use a more diluted sample to prepare it.

- **Sample Dilutions:** Using Dilution Buffer, make sample dilutions to fit SOD activity detected to the linear range of quantitation. In some cases, the samples should be concentrated with an Amicon MW 10 kDa.
- **Plate setup:** In a flat bottom 96-well plate, add

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard Curve			Sample 1		Sample 8		Sample 15				
B				Sample 2		Sample 9		Sample 16				
C				Sample 3		Sample 10		Sample 17				
D				Sample 4		Sample 11		Sample 18				
E				Sample 5		Sample 12		Sample 19				
F				Sample 6		Sample 13		Sample 20				
G				Sample 7		Sample 14		Sample 21				
H	BLANK 1		BLANK 2		BLANK 3		Sample 21					

9.2. SOD ASSAY

- Following the plate setup:
 - Add **20 µL** of dilution prepared for **Standard Curve**.
 - Add **20 µL of sample** dilution.
 - Distribute in the Blank 1 wells, 20 µL of Blank 1 previously prepared.
 - Distribute in the Blank 2 wells, 40 µL of Blank 2 previously prepared.
 - Distribute in the Blank 3 wells, 40 µL of Blank 3 previously prepared.
- Add **200 µL Radical Detection Solution** to each well.
- Add **20 µL Enzyme Working Solution** to each well, excepting Blank 2 and 3.

Since the reaction is extremely fast (turnover $2 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$), use a multichannel pipette to add the Enzyme Working Solution.

4. Shake the plate carefully.
5. Incubate the plate at 37 °C for 20 minutes.
6. Read the absorbance at 450 nm.

Avoid the presence of burbles in the wells before reading.

9.3. DIFFERENTIATION OF SOD ACTIVITIES

Cu/Zn SOD

Cu/Zn SOD comprises approximately 90% of total SOD activity in an eukaryotic cell. They are present mainly in cytosol although Cu/Zn SOD has been found in other cellular locations. To detect Cu/Zn SOD must be inactivated Mn and Fe SOD activities.

1. Add **400 µL ice-cold chloroform/ ethanol (37.5 : 62.5 (v:v))** to 200 µL erythrocytes or 800 µL to 500 µL cell/ tissue lysate and shake for 30 seconds.
2. Centrifuge at 2,500 g, 10 minutes.
3. Transfer the aqueous phase (upper) to a fresh tube
The samples will be stable at -80°C for at least one month.
4. Centrifuge at 10,000 g for 15 minutes at 4°C. The supernatant will contain cytosolic Cu/Zn SOD.
5. Proceed with SOD Assay previously described.

Extracellular Cu/Zn SOD

Extracellular Cu/Zn SODs are isolated from the extracellular matrix of tissues. These types of enzymes have been found in cerebrospinal, ascitic, and synovial fluid and also in serum.

1. To remove all cells from the extracellular fluid, centrifuge at 2,500 g for 10 minutes at 4°C.
2. Proceed with SOD Assay.

Mn SOD

Mn SOD is a mitochondrial enzyme localized at the mitochondrial matrix and inner membrane. Mn SOD are unaffected by cyanide, so its addition is suitable to inhibit Cu/Zn (> 90%) and Fe superoxide dismutase and measure Mn SOD activity.

1. Once the cell lysate is obtained, centrifuge at 10,000 g for 15 minutes at 4°C.
2. Resuspend the pellet in cold Reaction Buffer with KCN at a final concentration of 2 mM.
3. Proceed with SOD assay previously described.

Fe SOD

Iron containing SODs are found in some bacteria and plants. They are sensitive to H₂O₂ and azide.

9.4. SOD ACTIVITY DETERMINATION

SOD activity can be calculated through determination of inhibition rate which does not require a standard curve or by using the standard curve.

A. By inhibition activity

Calculate SOD activity as follow

$$\text{Inhibition Rate (\%)} = \frac{(A_{\text{BLANK 1}} - A_{\text{BLANK 3}}) - (A_{\text{SAMPLE}} - A_{\text{BLANK 2}})}{A_{\text{BLANK 1}} - A_{\text{BLANK 3}}} \times 100\%$$

B. Using the standard curve

1. Graph the standard curve using the medium value of each point.
2. Calculate the trendline equation based on your standard curve.
3. Determine SOD activity by substitution of sample readings in the equation obtained or by interpolation in the standard curve plotted.

10 TROUBLESHOOTINGS

PROBLEMS	POSIBLE REASONS	SOLUTIONS
Cero Absorbance Values		
	Radical detector was not added	Repeat the assay carefully, checking all the components have been added.
	SOD concentration to high	Use more diluted SOD samples
	Improper preservation of the samples	Tested the samples preferably in the same day that you processed them. Otherwise, store samples at -80°C and test them within 1 month.
Low Activity Detection		
	Sample too diluted	- Reduce the dilution factor and repeat the assay. - Concentrate the samples using Amicon MW10 kDa.
	Samples not homogenate completely	Check that lysis has occurred
	Low SOD activity	Concentrate the samples with an Amicon (membrane cut off 10 kDa) and repeat the assay
	Improperly thawed components	Thaw completely each component and mix well before use it.
	Presence of interference compounds in your samples	See Appendix 1
High Variability Between Replicates		
	Presence of bubbles	Centrifuge the plate 10 minutes at 600g or break the bubbles with a needle.
	Pipetting errors	Use a multichannel pipette

11 FREQUENTLY ASKED QUESTIONS

Q1. How does Tiaris Biosciences Superoxide Dismutase Assay Kit work?

Superoxide dismutase activity determination is based on the reduction of WST-1 by superoxide radicals generated by xanthine oxidase and hypoxanthine. The reduction of WST-1 produces a colored formazan dye that absorbs light at 450 nm. SOD activity reduces the amount of superoxide radicals, resulting in a decrease in the reduction of WST-1 and the formation of formazan dye.

Q2. Is it possible determine Mn-SOD or Cu/Zn SOD using this kit?

Yes. There are compounds that inhibit differentially SOD enzymes. For example, to measure Mn-SOD activity, it is necessary to block the Cu/Zn-SOD activity using potassium cyanide (KCN). Addition of 2 mM KCN to samples 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.

Q3. Why is important to determine protein concentration in the samples?

Protein concentration is a normalizer of your samples. In tissues and cells that should be disrupted and homogenized first, the protein released is different. Protein quantitation allows to correct the degree of homogenate breakage. When the samples are from body fluids, protein titration is not so necessary.

Q4. Can SOD assay be used *in vivo*?

SOD assay is typically performed *in vitro* on cell cultures or tissue homogenates. However, it is possible to measure SOD activity as a biomarker of oxidative stress *in vivo*. This can be done by measuring the activity of SOD directly in body fluids.

Q5. Is superoxide dismutase the only enzyme used as oxidative stress biomarker?

No. SODs are the only enzymes that interact with superoxide ($O_2^{\bullet-}$) but there are many other enzymatic activities that can be used as oxidative stress biomarkers, including catalase, glutathione peroxidase, glutathione reductase, myeloperoxidase, etc. They act on other Reactive Oxygen Species (ROS) or Reactive Nitrogen Species (RNS) compounds, and can be measured in various biological samples, such as blood, urine, and tissue homogenates, providing a valuable information about the oxidative stress status of cells or organisms.

Q6. Can I use the WST-1 assay for SOD activity determination in the presence of other antioxidants?

Yes. SOD activity determination using WST-1 can be used in the presence of other antioxidants. However, it is important to optimize the reaction conditions and use appropriate controls to ensure that the other antioxidants do not interfere with the SOD activity determination.

Q7. Can I use a kinetic method to determine SOD activity?

Yes. 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.

APPENDIX 1. INTERFERING COMPOUNDS IN SOD ASSAY

Compounds	SOD Assay Interference	
	Yes	No
DTT (3 mM)	✓	
DMSO	✓	
PMSF	✓	
SDS (0.1%)	✓	
CHAPS (0.1%)	✓	
Tween-20 (0.1%)	✓	
Tritón x-100 (0.1%)		✓
Borate Buffer	✓	
Tris-HCl Buffer		✓
PBS Buffer		✓
HEPES Buffer		✓
EDTA (0.8 mM)		✓
EGTA (0.8 mM)		✓
Glycerol (<1%)		✓
Trypsin (0.1 mg/ml)	✓	

REFERENCES

- Fridovich, I. (1975) Superoxide dismutases. *Annu. Rev. Biochem.* 44: 147–159.
<https://doi.org/10.1146/annurev.bi.44.070175.001051>.

