TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

HT Superoxide Dismutase Assay Kit

Cat# 7501-500-K

High throughput (HT) assay for the analysis of Superoxide Dismutase in cell and tissue extracts.

Sufficient reagents for 480 tests and five 96-well plates.

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Table of Contents

I. Introduction

The production of superoxide radicals, via immune responses and normal metabolism, is a substantial contributor, if not the primary cause, of pathology associated with neurodegenerative diseases, ischemia reperfusion injury, atherosclerosis and aging.[1-3] Superoxide Dismutases (SODs) catalyze the dismutation of the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2) which diffuses into the intermembrane space or mitochondrial matrix (Fig. 1), and thus, SODs provide an important defense against the toxicity of superoxide radicals.[4]

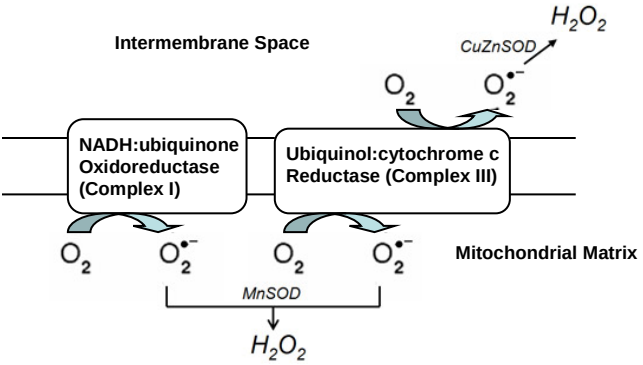

Figure 1. Hydrogen peroxide production by SODs.

In Trevigen's **HT Superoxide Dismutase Assay,** superoxide radical ($O_2^{\bullet-}$) ions, generated from the conversion of xanthine to uric acid and H_2O_2 by xanthine oxidase (XOD), convert WST-1 to WST-1 formazan. WST-1 formazan absorbs light at 450 nm. SODs reduce superoxide ion concentrations and thereby lower the rate of WST-1 formazan formation.[5,6] The extent of reduction in the appearance of WST-1 formazan is a measure of SOD activity present in your experimental sample (Fig. 2).

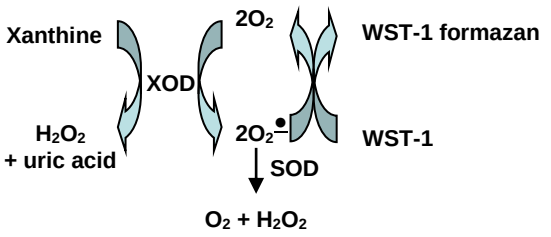

Figure 2. XOD and SOD antagonism in the generation of WST-1 formazan. Xanthine Oxidase (XOD) generates superoxide radical resulting in the reduction of WST-1 by superoxide anion to a colored WST-1 formazan product that absorbs light at 450 nm. SOD scavenges superoxide anion thereby reducing the rate of WST-1 formazan generation.

Trevigen's **HT Superoxide Dismutase Assay** is free of interference by other catalytic activities, and is ideal for assaying SODs in mammalian tissue and cell lysates in a 96 well format. Unlike some other assay kits for SOD, this system is

not greatly disturbed by trace metals. Sufficient reagents are provided for 480 tests. The assay is performed in as little as 6 minutes and relative SOD activity of the experimental sample is determined from percent inhibition of the rate of formation of WST-1 formazan. Furthermore, the assay is suitable for the assay of isozymes SOD1 (cytosolic Cu/Zn-SOD), SOD2 (mitochondrial Mn-SOD), and SOD3 (extracellular Cu/Zn-SOD).

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the chemicals and reagents contained in the HT SOD Assay Kit may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.

III. Materials Supplied

Catalog Number	Component	Amount Provided	Storage Temperature
7501-500-01	SOD Standard, 50 units/µl	50 µl	-20ºC
7501-500-02	10X SOD Buffer	20 ml	4ºC
7501-500-03	Xanthine Oxidase (XOD)	3 ml	-20ºC
7501-500-04	10X Xanthine Solution	2 ml	4ºC
7501-500-05	20% Triton™ X-100	1 ml	4ºC
7512-100-06	96-well plates	5	RT
7501-500-06	WST-1 Reagent	3 ml	4ºC

IV. Materials/Equipment Required But Not Supplied

Reagents:

1. SOD Inhibitors or cells/tissue to be tested
2. 10X PBS (Trevigen # 4870-500-6)
3. Distilled water
4. Protease inhibitors (optional) such as phenylmethylsulfonylfluoride (PMSF)
5. Reagents to determine protein concentration
6. Ficoll-Paque™ (erythrocyte, lymphocyte and monocyte preparations) (GE Healthcare cat# 17-1440-03).
7. Ethanol and Chloroform (erythrocyte preparation)

Disposables:

1. 1 - 200 µl and 100 - 1000 µl pipette tips
2. 0.5 and 1.5 ml microtubes
3. 15 ml conical (adherent and suspension cell preparation)
4. 50 ml conical (tissue preparation)

Equipment:

1. Micropipettes
2. Multichannel pipettor 1 - 50 µl and 50 µl – 200 µl
3. 96-well plate reader with 450 nm filters (capable of taking readings every minute for ten minutes and exporting data to Excel spreadsheet)
4. Centrifuge for processing samples

V. Reagent Preparation

Prepare all solutions prior to the start of the assay:

1. 1X SOD Buffer

Dilute the 10X SOD Buffer to **1X (1:10)** with dH_2O. 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. 1X Cell Extraction Buffer

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

10X SOD Buffer (Cat# 7501-500-02)	1.0 ml
20% (v/v) Triton™ X-100 (Cat# 7501-500-05)	0.2 ml
dH_20	8.8 ml
200 mM PMSF (optional)	(10 µl)

3. Master Mix (Should be at room temperature when used.)

Prepare sufficient amount of Master Mix (150 µl per well):
Preparation for 150 µl is as follows:

10X SOD Buffer (Cat# 7501-500-02)	15 µl
WST-1 Reagent (Cat# 7501-500-06)	5 µl
Xanthine Oxidase (Cat# 7501-500-03)	5 µl
dH_2O	125 µl

4. 1X Xanthine Solution (Should be at room temperature when used.)

Prepare sufficient amount of 1X Xanthine Solution (25 µl per well).
Preparation for 25 µl is as follows:

10X Xanthine Solution (Cat# 7501-500-04)	2.5 µl
1X SOD Buffer	22.5 µl

5. SOD Standard (Enzyme stored in a cold block.)

One unit of SOD reduces the rate of WST-1 formazan formation by 50%. The kit contains 50 µl of SOD Standard at a concentration of 50 units/µl. *The enzyme should be diluted appropriately with **1X** SOD Buffer just before use.* **Note: Diluted enzyme should be used immediately and any remainder discarded. Prepare samples before diluting. Store the stock SOD reagent at -20ºC.**

VI. Preparation of Cell and Tissue Extracts

Choose the appropriate protocol in Section A to Process Sample before proceeding to Section B: Please note that samples should 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 (Note: Mn- and Fe-SODs are inactivated by the recommended chloroform/EtOH extraction, however, hemoglobin or albumin can inhibit the generation of WST-1 formazan.[7]) For measurement of Cu/Zn-SOD:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Paque™ 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/leukocyte pellet. Wash the pellet with 10 cell volumes of PBS.
4. Determine the packed cell volume and add 10 cell volumes of cold dH_2O. 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, using 6-8 kDa cut-off tubing, 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-Paque™ 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 4ºC. Discard the supernatant
4. Suspend the cell pellet in 1 ml of ice-cold **1X** PBS and transfer to a prechilled 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 prechilled 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 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 [8].

Section C. Differentiation between Mn- Fe- and Cu/Zn-SOD activity

1. Mn- and Fe-SODs 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 Cu/Zn-SOD 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 (extracellular Cu/Zn-SOD) 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.

VII. SOD Assay Protocol

A. SOD Standard Curve

Note: 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. In 0.5 ml or 1.5 ml microtubes, prepare serial dilutions of the SOD standard with 1X SOD Buffer. Each level of SOD is performed in triplicate and requires 25 µl per well for a total of 75 µl. It is recommended that you make at least 100 µl of each serial dilution of SOD at the following concentrations: 10 units/25 µl, 5 units/25 µl, 2 units/25 µl, 1 unit/25 µl, 0.5 units/25 µl, 0.2 units/25 µl, and 0.1 units/25 µl.

2. Activity Controls: Triplicate wells without SOD. These wells provide the 100% activity reference point. Distribute 25 µl of **1X** SOD Buffer into wells as indicated in **Figure 3**.

3. Add 25 µl of the serial dilutions of the SOD standard to each of triplicate wells as indicated in **Figure 3**.

B. Biological Extracts

1. Make serial dilutions of your cell or tissue extracts with 1X SOD Buffer between 0.5 µg/25 µl to 50 µg/25 µl protein.

2. Add 25 µl of the extract dilutions to triplicate wells of the 96-well plate as indicated in **Figure 3**.

3. Add 150 µl of Master Mix (See Section V 4.) to all the wells.

4. The volume in each well is 175 µl:

Diluted cell/tissue extracts, SOD standards or 1X SOD Buffer:	25 µl
Master Mix:	150 µl
Total volume:	175 µl

C. Initiation of Reactions

Note: Ensure that the plate reader is ready prior to addition of the 1X Xanthine solution.

1. Initiate the reactions by adding 25 µl of 1X Xanthine solution to all the wells using a multichannel pipettor.

2. **Immediately** transfer the plate to a plate reader and take absorbance readings at 450 nm every minute for 10 minutes at room temperature.
Note: Time is critical in this assay because rates are to be measured.

VIII. Data Interpretation

For your convenience, a calculation worksheet (MS Excel required) is provided: http://www.trevigen.com/docs/1429016536.7501-500-k_calculation_worksheet.xls

A. Determine rate of change in absorbance at 450 nm

1. Take the average of your triplicate absorbance readings for both the SOD standards and your cell/tissue extracts. 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 for the SOD standard is shown in **Figure 4** below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	No SOD (Buffer)	No SOD (Buffer)	No SOD (Buffer)	No sample (Buffer)	No sample (Buffer)	No sample (Buffer)	No sample (Buffer)	No sample (Buffer)	No sample (Buffer)	No sample (Buffer)	No sample (Buffer)	No sample (Buffer)
B	0.1 Units SOD	0.1 Units SOD	0.1 Units SOD	0.5 µg protein Extract 1	0.5 µg protein Extract 1	0.5 µg protein Extract 1	0.5 µg protein Extract 2	0.5 µg protein Extract 2	0.5 µg protein Extract 2	0.5 µg protein Extract 3	0.5 µg protein Extract 3	0.5 µg protein Extract 3
C	0.2 Units SOD	0.2 Units SOD	0.2 Units SOD	1 µg protein Extract 1	1 µg protein Extract 1	1 µg protein Extract 1	1 µg protein Extract 2	1 µg protein Extract 2	1 µg protein Extract 2	1 µg protein Extract 3	1 µg protein Extract 3	1 µg protein Extract 3
D	0.5 Units SOD	0.5 Units SOD	0.5 Units SOD	2 µg protein Extract 1	2 µg protein Extract 1	2 µg protein Extract 1	2 µg protein Extract 2	2 µg protein Extract 2	2 µg protein Extract 2	2 µg protein Extract 3	2 µg protein Extract 3	2 µg protein Extract 3
E	1.0 Unit SOD	1.0 Units SOD	1.0 Units SOD	5 µg protein Extract 1	5 µg protein Extract 1	5 µg protein Extract 1	5 µg protein Extract 2	5 µg protein Extract 2	5 µg protein Extract 2	5 µg protein Extract 3	5 µg protein Extract 3	5 µg protein Extract 3
F	2.0 Units SOD	2.0 Units SOD	2.0 Units SOD	10 µg protein Extract 1	10 µg protein Extract 1	10 µg protein Extract 2	10 µg protein Extract 2	10 µg protein Extract 2	10 µg protein Extract 2	10 µg protein Extract 3	10 µg protein Extract 3	10 µg protein Extract 3
G	5.0 Units SOD	5.0 Units SOD	5.0 Units SOD	20 µg protein Extract 1	20 µg protein Extract 1	20 µg protein Extract 2	20 µg protein Extract 2	20 µg protein Extract 2	20 µg protein Extract 2	20 µg protein Extract 3	20 µg protein Extract 3	20 µg protein Extract 3
H	10 Units SOD	10 Units SOD	10 Units SOD	50 µg protein Extract 1	50 µg protein Extract 1	50 µg protein Extract 1	50 µg protein Extract 2	50 µg protein Extract 2	50 µg protein Extract 2	50 µg protein Extract 3	50 µg protein Extract 3	50 µg protein Extract 3

Figure 3: Suggested 96-well plate setup.

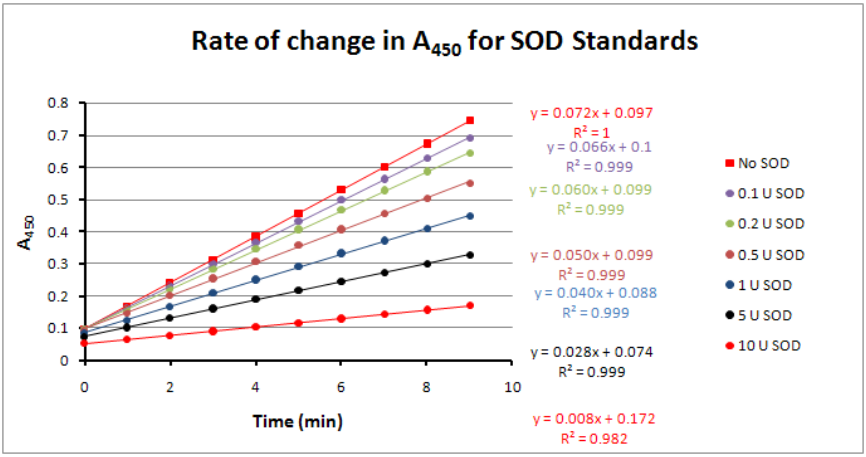

Figure 4: Change in absorbance at 450 nm with time for a SOD standard dilution series. A linear regression analysis of the reaction rates during the first 10 minutes of incubation is shown.

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

1. The slope obtained in the absence of SOD (the 1X SOD Buffer 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 5**.

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. For in house testing, we use the Bio Rad Protein Assay. Results are shown in **Figure 6** for SOD activity in Jurkat cell extracts.

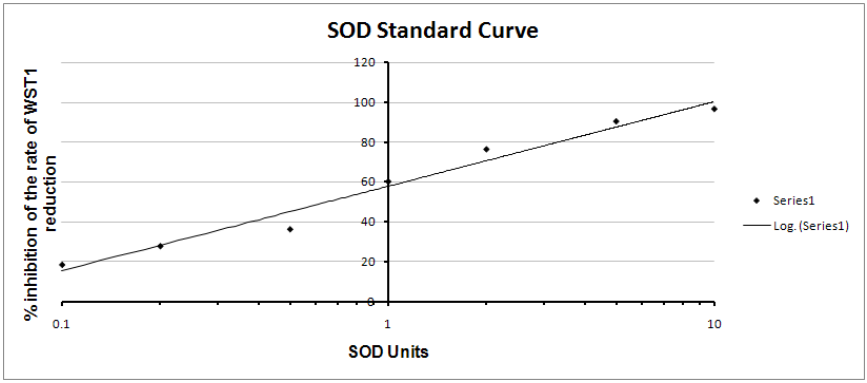

Figure 5. Inhibition curve for the SOD standard dilution series shown in figure 4.

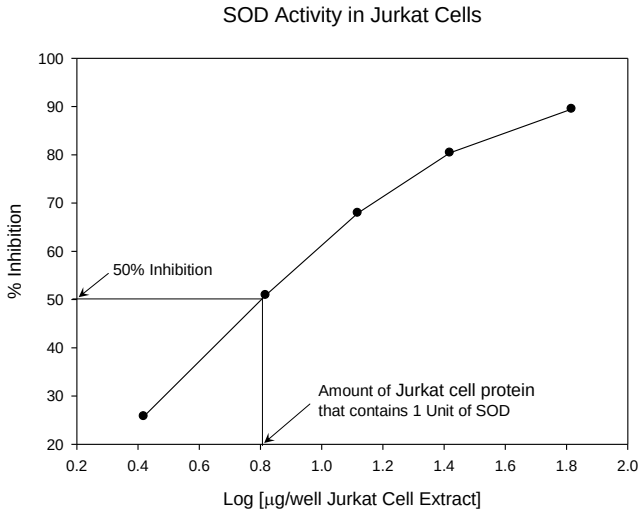

Figure 6. Inhibition curve for Jurkat cell extracts.

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.

   *I. Cell Extract **(Figure 5)**:*

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

IX. References

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8. Okado-Matsumoto A, Fridovich I. 2001. Subcellular distribution of superoxide dismutases (SOD) in rat liver. *J Biol Chem* **276**:38388-93.

X. 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 dH_2O in the Master Mix. Add 100 µl of this Master Mix and 50 µl of your extract to each well

XI. Related Products Available from Trevigen

Oxidative Damage Kits:

Catalog #	Description	Size
4418-096-K	γH2AX Pharmacodynamic Assay	96 tests
7510-100-K	Glutathione Reductase Assay	100 Reactions
7511-100-K	HT Glutathione Assay Kit	384 tests
7512-100-K	HT Glutathione Peroxidase Assay Kit	480 tests
7513-500-K	HT Glutathione Reductase Assay Kit	500 tests
7500-100-K	Superoxide Dismutase Assay Kit	100 tests

PARP Kits:

Catalog #	Description	Size
4520-096-K	PARP in vivo Pharmacodynamic Assay	96 tests
4684-096-K	HT Colorimetric PARP Apoptosis Assay	96 tests
4685-096-K	HT Chemiluminescent PARP Apoptosis Assay	96 tests
4690-096-K	HT F Homogeneous PARP Inhibition Assay Kit	96 tests

DNA Damage Antibodies:

Catalog #	Description	Size
4418-APC-100	Anti-Phosphorylated Histone H2AX polyclonal	100 µl
4354-MC-050	anti-8-oxo-dG monoclonal (clone 2E2)	50 µl

FLARE™ Assay Kits:

Catalog #	Description	Damage Recognized	Size
4040-100-FK 4040-100-FM	Fpg Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples
4045-01K-FK 4045-01K-FM	Endonuclease III Kit	Thymine Glycol, 5,6-dihydro-thymine, urea, 5-hydroxy-6-hydrothymine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6-hydrouracil, uracil glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxycytosine,5-hydroxy-uracil, methyl-tartonylurea, thymine ring saturated or fragmentation product	75 samples 100 samples
4130-100-FK 4130-100-FM	hOGG1 Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples

CometAssay®:

Catalog #	Description	Size
4250-050-ESK	CometAssay® Starter Kit	each
4250-050-ES	CometAssay® Electrophoresis System (ES)	each
4250-050-K	CometAssay®	50 samples
4251-050-K	CometAssay® Silver Kit	50 samples
4252-040-K	CometAssay® Higher Throughput Kit	40 samples
4253-096-K	CometAssay® Kit 96 Wells	96 samples
4256-010-CC	CometAssay® Alkaline Control Cells	10 assays
4257-010-NC	CometAssay® Neutral Control Cells	10 assays

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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