*Oxis*Research™

A Division of OXIS Health Products, Inc.

BIOXYTECH^{O} SOD-525™

Spectrophotometric Assay for Superoxide Dismutase
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21010

INTRODUCTION

The Analyte

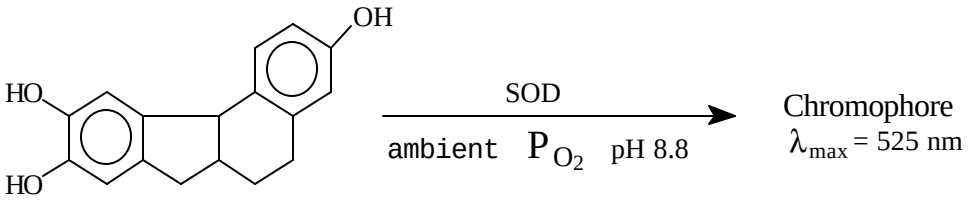
Superoxide dismutases (SOD) are metalloenzymes that catalyze the dismutation of superoxide anion into oxygen and hydrogen peroxide, according to the following reaction:

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

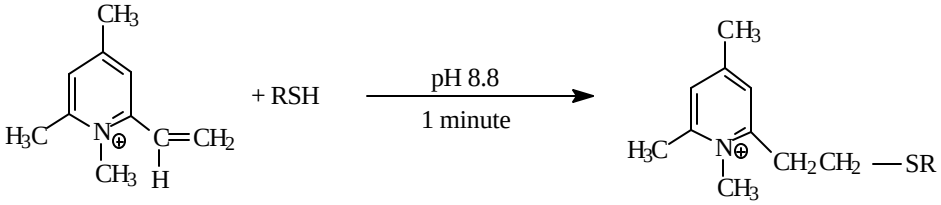
Different types of SOD have been described. The three most discussed types are characterized by the redox-active metals at the catalytic active site, *i.e.*, Cu/Zn-SOD, Mn-SOD, and Fe-SOD. Cu/Zn-SOD is found primarily in eukaryotes, localized mainly in the cytosol and nucleus, as a dimer of identical 16,000 MW subunits. Fe-SOD, a dimer of identical 20,000 MW subunits, is present mainly in prokaryotes. Mn-SOD is found in both prokaryotes and eukaryotes; most abundantly in the mitochondria, and has been isolated as both dimers and tetramers of 21,000 MW identical subunits.[1] It has been widely recognized that such enzymes provide a defense system that is essential for the survival of aerobic organisms.[2]

PRINCIPLES OF THE PROCEDURE

The BIOXYTECH® SOD-525™ method[3] is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene R1 in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. The chromophore has not been isolated or characterized.



Interference due to mercaptans (RSH) such as reduced glutathione, is controlled by pretreating samples with 1,4,6-trimethyl-2-vinylpyridinium R2, which directly eliminates mercaptans by means of a fast alkylation reaction.



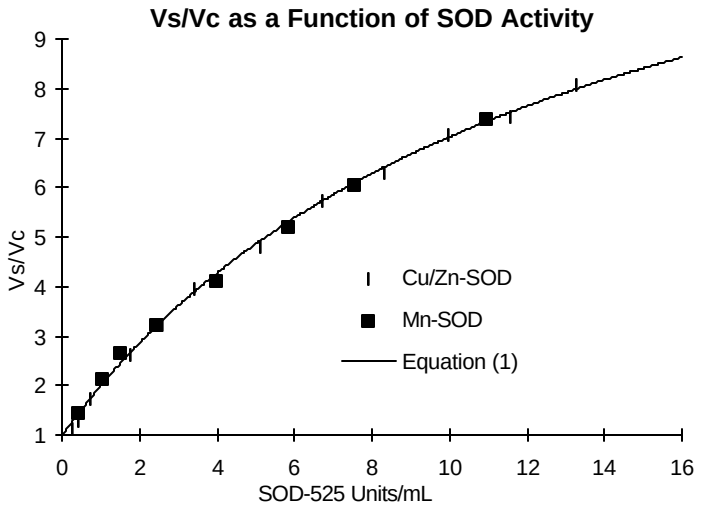
The kinetic measurement of the 525 nm absorbance change is performed after the addition of R1. The SOD activity is determined from the ratio of the autoxidation rates in the presence (Vs) and in the absence (Vc) of SOD. The Vs/Vc ratio as a function of SOD activity is independent of the type of SOD (Cu/Zn-SOD, Mn-SOD, Fe-SOD) being measured.[3] One SOD-525 activity unit is defined as

the activity that doubles the autoxidation rate of the control blank (Vs/Vc = 2). The relationship between the Vs/Vc ratio and SOD activity is described by Equation (1).

$$\frac{Vs}{Vc} = 1 + \frac{[SOD]}{\alpha \bullet [SOD] + \beta} \qquad \text{Equation (1)}$$

Where: Vs = Rate of sample containing SOD
Vc = Average rate of blank (SOD = 0) samples
SOD = the SOD activity of the sample in SOD-525 units
α = dimensionless coefficient
β = coefficient in SOD-525 units

One SOD-525 activity unit has a Vs/Vc ratio of 2; therefore, $\beta = 1 - \alpha$. The determination of α and β by experimental measurement of a range of SOD samples results in $\alpha = 0.073$, and $\beta = 0.93$ SOD-525 units. Representative results are demonstrated in the following plot.



REAGENTS

Materials Provided (for 100 tests)

- Reagent R1 — 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene, in HCl containing diethylenetriaminepentaacetic acid (DTPA) and ethanol, 1 x 3.3 mL.
- Reagent R2 — 1,4,6-trimethyl-2-vinylpyridinium trifluoromethanesulfonate, in HCl, 1 x 3.3 mL.
- Buffer — 2-amino-2-methyl-1,3-propanediol, containing boric acid and DTPA, pH = 8.8 (at 37°C), 1 x 100 mL.

Materials Required But Not Provided

- Spectrophotometer set up to measure absorbance at 525 nm; preferably equipped with a 37°C temperature controlled cuvette holder and the ability to record the increase in absorbance in over time.
- Spectrophotometric cuvette, 1 mL semi-micro cuvette with a 1 cm optical path length.
- Water bath or heat block at 37 ± 1°C.
- Vortex mixer.
- Pipettes with disposable tips.
- Disposable test tubes.

- Optional Chloroform/Ethanol Extraction Reagent: absolute ethanol/chloroform, 62.5/37.5 (v/v), for removal of hemoglobin interference (will only determine activity specific to Cu/Zn-SOD.
- Fresh deionized water for Blank.

Warnings and Precautions

Use established laboratory precautions when handling or disposing any chemicals contained in this product. Refer to the Material Safety Data Sheet for risk, hazard, and safety information.

Reagent Storage and Stability

Store the reagents tightly closed at 2-8°C in the dark. Do not freeze. Keep R1 and R2 on ice during use. Stored properly, reagents are stable until expiration date printed on the label. Do not reuse the Buffer after warming to 37°C.

PROCEDURE

Reagent Preparation

The reagents are ready to use.

Buffer Preparation

- Place the required amount of Buffer in an open container at 37°C and allow to equilibrate with air. Repeatedly drawing and expelling Buffer into the container with a pipet will speed the oxygen saturation of the Buffer.
- A minimum of 4 blank controls with each set of sample data is recommended.
- The first sample assayed may be used as a guide to determine the desired time range for the linear portion of the reaction.

Sample Preparation

Note: Please read the appropriate **NOTES** sections before starting sample preparation procedure.

Assay

1. Zero the spectrophotometer at 525 ± 2 nm with deionized water.
2. Add 900 µL Buffer to a test tube for each blank or sample.
3. Add 40 µL Blank or Sample to the test tube.
4. Add 30 µL of R2 to the test tube and vortex.
5. Incubate at 37°C for 1 minute.
6. Add 30 µL of R1 to the test tube and vortex briefly.
7. Immediately transfer to a spectrophotometric cuvette and measure the absorbance over time.

Details of Calibration

No calibration is required. The activity is calculated directly from the rate of sample *versus* the average rate of blank control.

Quality Control Procedures and Materials

If desired, assay a control sample of known activity to verify the accuracy of results.

CALCULATIONS

Computerized Rate Determinations

Computer data collection and calculations are preferred. However, care must be taken in choosing the calculation interval. Because there is only a short pseudo-linear range about the inflection point,

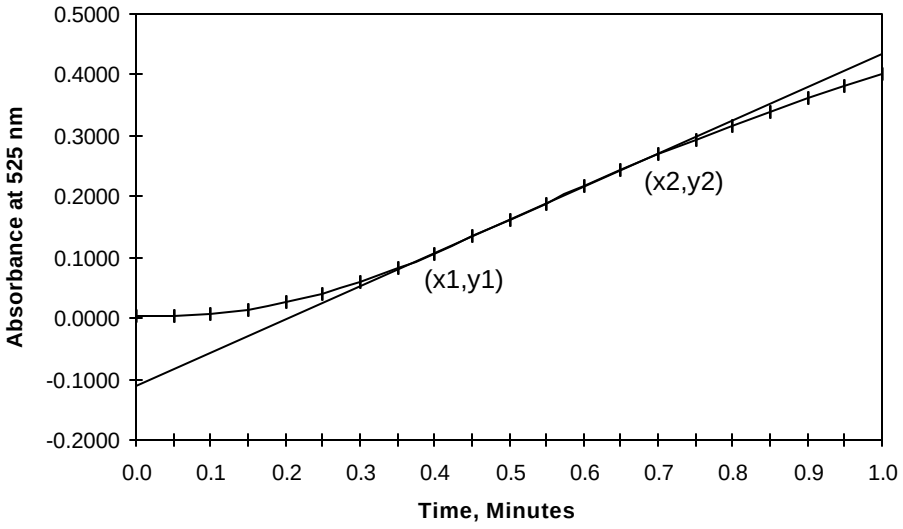
linear regression calculations will underestimate the slope if a wide interval is covered. Noise in the absorbance measurements is magnified greatly in the rate calculations if short intervals are used. Choose the shortest interval compatible with the precision and noise of the absorbance measurements. Confirm that the automated procedure used gives reproducible rates that are consistent with the manual method.

Rate Calculation

Calculate the rate (ΔA/min) from the slope of the linear range of the curve about the inflection point.

1. Plot the curve of absorbance *versus* time.
2. Select the two time points between which the curve has maximum slope and is approximately linear (this time range may be different for EACH sample).
3. Calculate the slope of the line in that region, either by linear regression, or a two-point slope calculation:
   Slope = $(y_2 - y_1)/(x_2 - x_1)$.

Determining Rate from Linear Range



Determine the Vs/Vc Ratio

1. Average a minimum of 4 blank rate determinations and use for Vc.
2. Divide each sample rate (Vs) by the Vc.
3. NOTE: The Vs/Vc ratio must be greater than 1. A ratio of less than 1 indicates the presence of an interferent. The precision of the SOD-525 method is greatest in the Vs/Vc region between 3 and 8. If Vs/Vc ≥ 8, the sample should be diluted and reassayed.

Determining SOD Activity

There are two ways to determine the SOD activity from the Vs/Vc ratio, either use the provided Ratio Table or calculate directly from Equation (2).

- **Using the Ratio Table to Calculate SOD Activity**

Given the Vs/Vc ratio, the SOD activity in SOD-525 units is found in the Ratio Table by finding the nearest Vs/Vc value in the Vs/Vc column and reading the SOD activity from the adjacent units column.

Vs/Vc RATIO TABLE

Vs/Vc	Units	Vs/Vc	Units	Vs/Vc	Units	Vs/Vc	Units	Vs/Vc	Units	Vs/Vc	Units	Vs/Vc	Units
1.00	0.00	2.00	1.00	3.00	2.18	4.00	3.57	5.00	5.25	6.00	7.32	7.00	9.93
1.05	0.05	2.05	1.06	3.05	2.24	4.05	3.65	5.05	5.35	6.05	7.44	7.05	10.08
1.10	0.09	2.10	1.11	3.10	2.31	4.10	3.73	5.10	5.44	6.10	7.56	7.10	10.23
1.15	0.14	2.15	1.17	3.15	2.37	4.15	3.80	5.15	5.54	6.15	7.68	7.15	10.38
1.20	0.19	2.20	1.22	3.20	2.44	4.20	3.88	5.20	5.63	6.20	7.80	7.20	10.53
1.25	0.24	2.25	1.28	3.25	2.50	4.25	3.96	5.25	5.73	6.25	7.92	7.25	10.69
1.30	0.29	2.30	1.34	3.30	2.57	4.30	4.04	5.30	5.83	6.30	8.04	7.30	10.85
1.35	0.33	2.35	1.39	3.35	2.64	4.35	4.12	5.35	5.93	6.35	8.16	7.35	11.01
1.40	0.38	2.40	1.45	3.40	2.71	4.40	4.21	5.40	6.03	6.40	8.29	7.40	11.17
1.45	0.43	2.45	1.51	3.45	2.77	4.45	4.29	5.45	6.13	6.45	8.42	7.45	11.34
1.50	0.48	2.50	1.57	3.50	2.84	4.50	4.37	5.50	6.23	6.50	8.55	7.50	11.50
1.55	0.53	2.55	1.63	3.55	2.91	4.55	4.46	5.55	6.34	6.55	8.68	7.55	11.67
1.60	0.58	2.60	1.68	3.60	2.98	4.60	4.54	5.60	6.44	6.60	8.81	7.60	11.84
1.65	0.63	2.65	1.74	3.65	3.06	4.65	4.63	5.65	6.55	6.65	8.94	7.65	12.02
1.70	0.69	2.70	1.81	3.70	3.13	4.70	4.71	5.70	6.65	6.70	9.08	7.70	12.20
1.75	0.74	2.75	1.87	3.75	3.20	4.75	4.80	5.75	6.76	6.75	9.22	7.75	12.38
1.80	0.79	2.80	1.93	3.80	3.27	4.80	4.89	5.80	6.87	6.80	9.35	7.80	12.56
1.85	0.84	2.85	1.99	3.85	3.35	4.85	4.98	5.85	6.98	6.85	9.50	7.85	12.74
1.90	0.90	2.90	2.05	3.90	3.42	4.90	5.07	5.90	7.09	6.90	9.64	7.90	12.93
1.95	0.95	2.95	2.11	3.95	3.50	4.95	5.16	5.95	7.21	6.95	9.78	7.95	13.12

- **Direct Calculation**

Rearrangement of Equation (1) and substituting the values for α and β permits the direct calculation of the SOD-525 activity from the experimental Vs/Vc ratio using Equation (2).

$$[SOD] = \frac{0.93 \bullet \left(\frac{Vs}{Vc} - 1 \right)}{1.073 - 0.073 \bullet \left(\frac{Vs}{Vc} \right)} \qquad \text{Equation (2)}$$

Correction for Dilution

The resulting activity value is multiplied by the dilution factor for the sample and divided by the volume of diluted sample added to the 1 mL assay volume (e.g., 0.04 mL) to give the pre-dilution SOD activity concentration of the sample in SOD-525 units per mL.

Conversion of SOD Activity

SOD activity can be expressed relative to any other relevant parameter which has been separately determined, such as protein or hemoglobin content.

Sample Calculation

1. Rate Calculation: The autoxidation rate for the sample presented in the Rate Calculation section above was calculated by selecting the range of data from 0.4 to 0.7 minutes as the linear region of the curve. Then, a linear regression analysis was performed. The resulting slope of the line is 0.5452 = Vs. Similarly, the average slope of 4 blanks was calculated as 0.0801 = Vc.
2. Determine Vs/Vc Ratio: Vs/Vc = 0.5452/0.0801 = 6.806.

3. Determine SOD Activity:

   (a) Using the Ratio Table, the corresponding activity is:

   Vs/Vc = 6.80 is 9.35 units/mL.

   (b) Direct Calculation:

   {[0.93x(6.806-1)]/[1.073-(0.073x6.806)]} = 9.372 units/mL.

4. Correction for Dilution: Assuming the sample had been diluted 50 fold and 40 µL added to the assay mixture, the SOD activity in the original sample is calculated as:

   9.35 U/mL x (50/0.040) = 12,700 U/mL.

5. Conversion of SOD Activity: If the original sample contains 5 mg protein/mL, the SOD activity can be expressed as SOD activity per milligram protein by dividing the units/mL by the protein concentration, (12,700 U/mL) / (5 mg/mL) = 2540 U/mg.

PERFORMANCE CHARACTERISTICS

Precision

Precision of the BIOXYTECH® SOD-525™ Assay was determined by measuring the activity in three samples containing SOD of representative concentrations in the final assay mixture. The samples were assayed in replicates of 30 on 2 different days. Typical study results are shown below.

Replicates = 30		DAY 1	DAY 2
Level 1	Mean U-525 units/mL	0.82	0.81
	Standard Deviation	0.06	0.10
	%CV	7.32	12.35
Level 2	Mean U-525 units/mL	2.85	2.86
	Standard Deviation	0.25	0.23
	%CV	8.77	8.04
Level 3	Mean U-525 units/mL	13.6	13.22
	Standard Deviation	1.29	1.42
	%CV	9.48	10.74

Sensitivity

The lower limit of detection (LLD), defined as the lowest concentration which can be distinguished from zero at the 95% confidence level, was determined from the mean and standard deviation of 30 replicate Vc measurements. The LLD is 0.1 SOD-525 units per mL assay volume. This must be multiplied by the sample dilution factor specific to each type of sample.

Assay Range

The assay has the best precision in the Vs/Vc region less than 8. If the ratio is 8 or greater, the sample should be diluted and re-assayed.

Limitations

A “lag-time” with little or no absorbance change may be present for up to 90 seconds after initiation of the reaction. This is usually an indication that the sample is too concentrated. Perform a dilution and reassay the sample.

The plasma SOD activity in normal serum is below the detection limit of the SOD-525 assay. SOD has a very short half-life in plasma. The utility of the Assay in plasma is limited and not recommended.

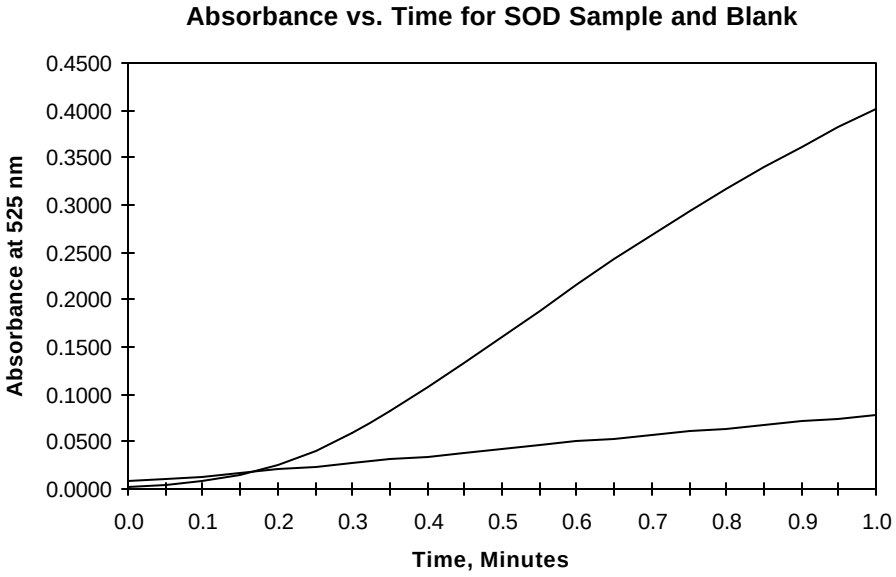
Cyanide interferes with the autoxidation of the chromogen R1 and should be avoided. Cyanide cannot be used to distinguish Cu/Zn-SOD activity from Mn-SOD activity in samples.

The ethanol-chloroform Extraction Reagent inactivates Mn-SOD and Fe-SOD.

Whole tissue specimens should be free of trapped blood to prevent sample contamination by red cell SOD. The expected concentration of SOD in erythrocytes is much greater than found in most tissues. Rinse and/or perfuse tissue with 0.9% NaCl containing 0.16 mg/mL heparin.

Expected Values

The upper curve in the following plot shows typical results demonstrating the change in 525 nm absorbance in the presence of SOD as a function of time. The increase in absorbance due to the autoxidation reaction may begin at a later time than presented. Monitor the first sample assay to determine the proper time range for collection of the linear portion of the curve about the inflection point. The lower curve is an example of the typical change in 525 nm absorbance in the absence of SOD. In laboratory experiments with a limited group of human red blood cell samples (N = 21), the average SOD concentration in the SOD-525™ Assay was 2.5 U/mg Hb, with a range of 1.3 - 5.0.



NOTES

The type of SOD activity measured can be controlled by the sample isolated for assay. Cu/Zn-SOD may be measured in the cytosol. Fe-SOD is collected from bacteria or other prokaryotes. Mn-SOD is isolated from the mitochondria.

Potentially Interfering Substances

Compounds in the sample exhibiting redox properties may interfere with the SOD-525 method. Interference can be assessed by testing linearity of the measured activity upon sample dilution or by recovery of known amounts of SOD activity added to the sample. Interferents in the sample, other than the mercaptans removed with R2, may be removed by extraction, dialysis, or gel filtration (*e.g.,* pre-packed disposable columns PD-10, Pharmacia Biotech, Uppsala, Sweden).

Samples

- **Purified SOD** can be assayed without prior sample preparation if dissolved in water, saline or non-interfering buffer.
- **Erythrocyte lysate** pellets should be resuspended in 4 packed-cell volumes of ice-cold water. Store frozen if lysate is not further processed or assayed promptly.
- **Tissues** should be washed or perfused with 0.9% NaCl containing 0.16 mg/mL heparin to remove red blood cells, followed by homogenization and centrifugation.
- **Cell Culture** SOD can be measured on clarified cell homogenates without extraction (2×10^7 cells).

Extraction to Remove Hemoglobin Interference

Ethanol-chloroform extraction inactivates Mn-SOD and Fe-SOD, therefore, extracts will be specific for Cu/Zn-SOD. The sample may be a lysate or supernatant from any cells expected to contain Cu/Zn-SOD.

1. Prepare the Extraction Reagent with ethanol/chloroform 62.5/37.5 (v/v) and store at 2-8°C.
2. Add 400 µL of ice-cold Extraction Reagent to 250 µL of sample in a test tube.
3. Vortex for a minimum of 30 seconds and centrifuge at 3000 x *g* and 4°C for 5 minutes.
4. Collect the upper aqueous layer.
5. Place extract at 2-8°C if assayed within 24 hours; otherwise, store frozen.

REFERENCES

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3. Nebot, C., et al. *(1993) Spectrophotometric Assay of Superoxide Dismutase Activity Based on the Activated Autoxidation of a Tetracyclic Catechol*. Analytical Biochemistry. **214**, 442-451.

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