コスモ・バイオ株式会社

Applied Bioanalytical Labs

SOD-560

A Colorimetric Assay Kit for Superoxide Dismutase Activity

(For 30 test tube assays or 100 microplate assays)

Store at 2-8°C

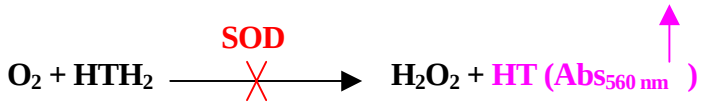
For *In-Vitro* Research Use Only

Introduction

The discovery of enzyme superoxide dismutase (SOD) by McCord and Fridovich in 1969 started a new era of research on the role of free radicals in biology and medicine. Now it has been found that SOD is ubiquitous in every aerobic organism from microbes to human. In animal cells, there are two kinds of SODs, a cytosolic SOD containing a CuZn active site and a mitochondria SOD containing a Mn active site. An extracellular CuZn-SOD (EC-SOD) is also found in mammalian extracellular fluids such as plasma, lymph, synovial fluid, cerebrospinal fluid and seminal plasma. The EC-SOD is probably bound to heparan sulfate proteoglycans on cell surfaces, in basal membranes and in connective tissue matrix. Prokaryotic SODs are more diverse in active site composition consisting of CuZn, or Mn, or Fe, or Ni metal centers.

SOD decomposes superoxide anion into hydrogen peroxide and oxygen at close to highest reaction rate possible. Superoxide radical is involved in diverse physiological and pathophysiological processes. It is produced in respiratory and cytochome P450 electron transport chain reactions as a by-product. An intense amount is also produced during oxidative burst by activated neutrophils and macrophages. A very interesting chemistry involves interaction between superoxide with nitric oxide (NO) that is a vasodilator and a cellular signal molecule. Superoxide can react with NO at very fast rate to form peroxynitrite – a very powerful oxidant that cause damages to DNA, protein and other biological molecules. Removal of superoxide provides a denfense against these damages. The relationship between SOD and various human diseases has been well documented in literature. One SOD activity unit is define as the amount of SOD that inhibits the rate of cytochrome *c* reduction by half at pH 7.8 at 25^{o}C under specific conditions.

The "standard" method of SOD activity assay is the inhibition of cytochrome *c* reduction coupled with superoxide generation by xanthine oxidase.[1] Adjusting amounts of cytochrome *c*, xanthine oxidase and samples are tedious. There has been no specific superoxide indicator to date, xanthine oxidase coupled assays such as NBT are generally not satisfactory.[2] A "direct" method by monitoring UV absorbance of superoxide has been described, the problem is that a fresh potassium superoxide solution must be prepared before each single assay and that assay pH is alkaline where MnSOD activity is adversely affected. The method used in our kit as essentially that described by Martin J. P., Jr etc, with modifications to increase robustness and reliability. Briefly, auto-oxidation of hematoxylin is inhibited by SOD at the assay pH, the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range.[3] The amount of SOD in a sample is determined with our kit in the "standard cytochrome c" SOD unit, by measuring ratios of auto-oxidation rates in the presence and absence of the sample. One unique feature of our assay kit is its ability to determine CuSOD and MnSOD activity distinctively in a single sample, by simply adding cyanide to inhibit CuSOD activity but not MnSOD activity. The hematoxylin auto-oxidation rate is not affected by cyanide. The principal of the assay is described schematically (simplified) in the following equation:



Reagents

Materials Provided (for 30 test tube test or 100 microplate test):

Assay Buffer	30 mL
Hematoxylin	1 vial
Sample Dilution Buffer	30 mL

Items Required but Not Provided:

Spectrophotometer (cuvette spectrophotometer, multi-cuvette changer with temperature control module recommended but not absolutely required) or plate reader
Disposable semi-micro cuvettes (1.0 mL) or transparent microplates
Microcentrifuge tubes
Plastic or glass bottles
Pipettors, adjustable 0.0 – 1.0 mL
Disposable pippette tips

Storage and Handling

The kit should be stored as a whole in the original box in a refrigerator (2-8^{o}C). Dispose solution after kit's expiration date.

Preparation of solutions

The assay is sensitive to temperature. The experiment can only be carried out in an environment where temperature is constant (22 – 27^{o}C) when a cuvette spectrophotometer without temperature module is used. When there is a noticeable change of temperature, it is necessary to run sample and a blank (Assay Buffer) back-to-back to minimize temperature effect. This requirement for plate reader is less restrictive since assays are carried out in parallel.

Let the kit warm up to room temperature completely. It takes a long time for buffers to reach equilibrium with room temperature, therefore they should be placed at room temperature the night before the experiment. Hematoxylin Solution must be reconstituted with 1200.0 µL of dH_2O. At room temperature, it should be used within 6 hours. If only a portion of the reconstituted hematoxylin is needed in one experiment, the excess solution should be immediately stored at –20^{o}C (frozen) for future use.

Assay Buffer:

Shake or invert the bottle, open to air. Repeat 4 more times.

Hematoxylin Solution:

Add 1200.0 µL of dH_2O to the hematoxylin vial, shake to dissolve. Incubate 30 minutes before use.

Samples:
Some samples may need to be dialyzed, or extracted, or purified through chromatograph to remove interference substances that completely inhibit hematoxylin auto-oxidation. These purification steps are not necessary when inhibition time is less than 7 minutes because hematoxylin auto-oxidation rate is linear for the first 10 minutes. For example, semen plasma sample can be assayed directly after 1/10x dilution. The samples typically need to be diluted with Sample Dilution Buffer before assay. Following are the general guidelines of dilution (samples from patients or abnormal conditions may require a different dilution factor) to adjust their concentration to the most sensitive linear assay range between 0 and 50 U/mL:
Human Semen Plasma: 1/10x (e.g., add 50 µL to 450 µL of Sample Dilution Buffer)
Tissues (10% homogenates): 1/2x
Red Blood Cell lysate (after chloroform/ethanol extraction to remove hemoglobin): 1/2x

If auto-oxidation rate (A_{560nm}/min) with a sample is less than 50% auto-oxidation rate at zero SOD concentration (blank rate), it is important not to use the rate to calculate SOD concentration in the sample. Linearity of the assay is lost sharply at high SOD concentration. Adjust the dilution factor and make a new dilution, repeat the assay. It is also possible by adding a smaller volume of the diluted sample and making up the total sample volume required with the Sample Dilution Buffer directly in the assay mixture specified in the Procedures. For example, instead of adding 40.0 µL of a secondary 1/4x dilution of a diluted sample, one may add 10.0 µL of the diluted sample and 30.0 µL of Sample Dilution Buffer).

Procedure

The SOD activity is determined from the ratio of auto-oxidation rate of a sample and that of a blank (Assay Buffer). Duplicate or triplicate assays per sample are recommended. It is necessary for the blank since all the ratios is dependent on accuracy of its rate.

For cuvette spectrophotometers:
1. Set measurement temperature at 25°C. Otherwise, carry out the experiment at room temperature. When there is a noticable change of temperature, it is necessary to run sample and a blank (Assay Buffer) back-to-back to minimize temperature effect.
2. Zero absorbance at 560 nm using dH_2O.
3. To a clean cuvette, add 920.0 µL of Assay Buffer. Add 40.0 µL of Assay Buffer or sample. Mix and incubate for 2 minutes.
4. Add 40.0 µL of hematoxylin to start auto-oxidation reaction that yields increase in absorbance at 560 nm. Mix quickly and start recording absorbance at 560 nm immediately every 10 seconds or at a smaller time interval for at least 5 minutes. Reaction rate is linear for about 10 minutes, undialyzed samples may need longer recording time – see Typical Results and Calculation Section for detail.

For plate readers:
1. Set measurement temperature at 25°C. Otherwise, carry out experiment at room temperature.
2. Use a clean 96-well plate. Record sample/standard layout. Be careful to avoid forming bubbles on the surface when pipetting solutions (bubbles form easily when pippeting air into

the solution, reverse pippeting technique overcomes bubble formation). To each well used for test, add 230.0 µL of Assay Buffer. Add 10.0 µL of Assay Buffer (blank) or sample solution. Shake to mix, and incubate for 2 minutes.
3. Add 10.0 µL of hematoxylin (also avoid bubble formation) to start auto-oxidation reaction that yields increase in absorbance at 560 nm. Preferably, use a multi-channel pipette. Mix quickly by shaking and start recording absorbance at 560 nm immediately every 10 seconds or at the smallest time interval for at least 5 minutes. Reaction rate is linear for about 10 minutes, undialyzed samples need longer time – see Typical Results and Calculation Section for detail.

Typical Results and Calculations:

The auto-oxidation of hematoxylin is a self-catalyzed reaction. The rate is linear for about 10 minutes. This reaction is inhibited by thiols (including protein thiols), ascorbic acid, and other antioxidants. It is recommended to treat biological samples by dialysis, extraction and/or chromatography to remove these interfering substances before the assay. But in most cases, the inhibition lasts only several minutes, especially with 1/5x or higher dilution of biological samples. In these cases, samples without prior treatment can be assayed directly by the rate assay. Typical rate curves of standards with 0 U/mL and 40 U/mL are shown below in Fig. 1. In the 40 U/mL sample, there is a slight inhibition for the initial 1 minutes of reaction. This inhibition is often observed for high concentration of SOD or for biological samples. The rate of reaction can be obtained by regression of the linear (un-inhibited) portion of the rate curves, as shown in Figure 1. In the regression equation, $y = ax + b$, a is the rate ($\Delta A560_{nm}$/min). A less preferred way to obtain the rate is to select two data points in the linear portion of the curve and to calculate rate according to following equation: Rate($\Delta A560_{nm}$/min) = (Y2 – Y1)/(X2 – X1), as shown in Fig. 1 for the 40 U/mL SOD curve.

If auto-oxidation rate (A_{560nm}/min) with a sample is less than 50% of auto-oxidation rate at zero SOD concentration (blank rate), it is important not to use the rate to calculate SOD concentration in the sample. Linearity of the assay is lost sharply at high SOD concentration. The sample has to be diluted further and assayed again.

Calculations:

1. Determine auto-oxidation rates as illustrated in Figure 1. Calculate average rates from duplicate or triplicate assays. Obtain the ratio of rate of a sample and rate of the blank:

$$\mathbf{r_i} = Rate_i/Rate_{blank}$$

where $Rate_i$ is the average auto-oxidation rate of sample i, and $Rate_{blank}$ is the average auto-oxidation rate of the blank solution. Percentage of SOD inhibition of reaction rate is 100%-$\mathbf{r_i}$.
2. The SOD concentration in the sample i added to the reaction mixture (not concentration in the reaction mixture) is:

$$C_i \text{ (SOD U/mL)} = 125*(100\% - \mathbf{r_i})$$

where Ci is the SOD concentration of sample i in the "standard cytochrome c" U/mL.
3. The SOD concentration in the original sample ("standard cytochrome c" U/mL) is

$$C^O_i = C_i * (\text{Dilution Factor}_i)$$

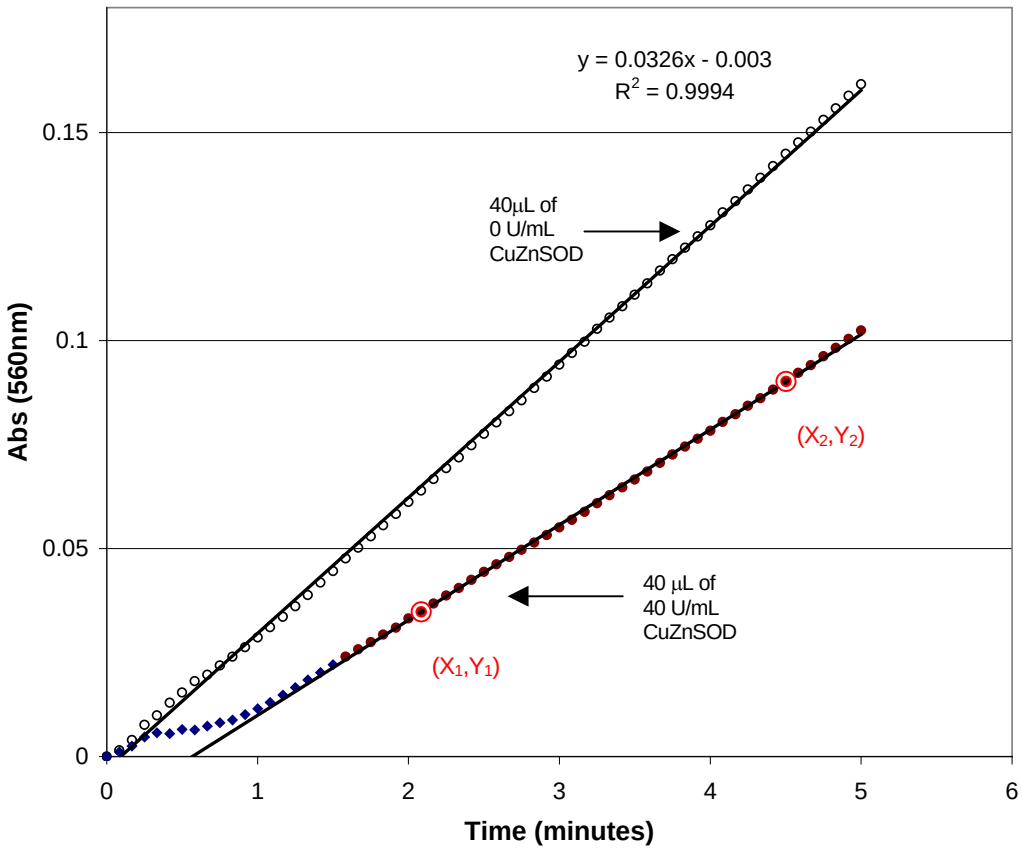


Figure 1: Rate curves of hematoxylin auto-oxidation in the absence and presence of CuZnSOD. SOD unit represents concentration in sample added, not in final assay mixture. The experiment was performed at 25.2°C room temperature without a constant temperature cuvette holder.

Note:

1. To minimize inter-assay variation such as in a large-scale study, it is recommended that a control sample is prepared, aliquoted and frozen at –20°C or below. This control sample should be assayed each time along with other samples. Final results should be normalized with the control sample.
2. To determine CuZnSOD and MnSOD activity separately in samples containing both enzymes, cyanide (1 to 2 mM) can be used to inhibit CuZnSOD but not MnSOD. Simply add appropriate amount of KCN (under a hood to avoid possible HCN gas exposure) to specific volume of assay buffer. The activity in the original assay buffer represents total SOD activity, and the result from KCN-containing assay buffer represents only MnSOD activity.
3. EC-SOD activity is usually determined specifically after chromatography that eliminates other forms of SOD's.

Specifications (Performance Characteristics for Rate Assay without a Constant Temperature Module):

LLD: The lower limit of detection is 5 U/mL SOD in the samples added to the reaction mixture (0.2 U/mL in the reaction mixture).
Linearity: Linearity is maintained up to 60 U/mL of SOD (in samples).
Assay Precision: The intra-assay and inter-assay coefficient of variation (CV) are 8% and 12% for the 40 U/mL standard.
Recoveries: The recovery for human semen plasma is 90%.

References:

1. Flohe L. & Otting F., "Superoxide Dismutase Assays", *Methods in Enzymology 105*, 93-104 (1984).
2. Tarpey M. M. & Fridovich I., "Methods of Detection of Vasular Reactive Species – Nitric Oxide, Superoxide, Hydrogen Peroxide and Peroxynitrite", *Circulation Research 89*, 224-236 (2001).
3. Martin J. P. Jr., Dailey M. & Sugarman E., "Negative and Positive Assays of Superoxide Dismutase Based on Hematoxylin Autoxidation", *Arch. Biochem. Biophys. 255*, 329-336 (1987).

Revision 2.02, 5/2007

Applied Bioanalytical Labs
4211 Saint Charles Drive
Sarasota, FL 34243
USA
941-355-4933
info2@appliedbiolabs.com
www.appliedbiolabs.com