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Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™ Superoxide Dismutase Activity Assay

Product NWK-SOD02

For Research Use Only

Simple assay kit for quantitative measurement of superoxide dismutatse enzyme activity in biological samples.

nwksod02rev010907

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Statement of Limited Warranty:

Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

Notes:

Performance Details:

Stability All unopened reagents are stable until the expiration date stated on the package label when stored at 2-8°C.

Sensitivity: The lower limit of detection is 0.2 U SOD/mL in the reaction mixture

or,

5 U SOD/mL sample added to reaction mixture.

Linearity: Linearity is maintained up to 60 U SOD/mL sample.

Assay Precision:

Coefficient of variation (CV) at 40 U SOD/mL sample	Intra	Inter
	12%	8%

Recovery: Recovery of spiked SOD in RBC human seminal plasma was calculated as 90% using this assay.

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 Vascular 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).

4. Landmesser U., Merten R., Spiekermann S., Büttner K., Drexler H. and Hornig B., Vascular Extracellular Superoxide Dismutase Activity in Patients With Coronary Artery Disease—Relation to Endothelium-Dependent Vasodilation, *Circulation* **101(19)**:2264-2270 (2000).

Introduction:

Superoxide radicals are involved in many physiological and pathophysiological processes. They are produced as a by-product of respiratory electron transport and cytochome P450 reactions. Activated neutrophils and macrophages can also produce a large amount during oxidative burst. Superoxide radical can react with NO at very fast rate to form peroxynitrite ($ONOO^-$) a very powerful oxidant that has been shown to damage DNA, protein and other biological molecules. Removal of superoxide is a necessary step in cellular defense against these damages.

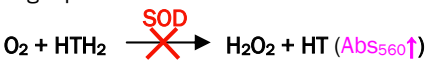
The enzyme, superoxide dismutase (SOD), rapidly decomposes superoxide anion into hydrogen peroxide and oxygen. Since its initial discovery by McCord and Fridovich in 1969, SOD has been found to be ubiquitous in all aerobic organisms from microbes to humans. Four types of SOD have been identified on the basis of their metal cofactors and distribution. The copper zinc form (Cu/ZnSOD) is most common with a primary distribution in the cytoplasm of eukaryotic cells. Another copper zinc form, extra-cellular (ecSOD), has more recently been identified. This SOD is often associated with vascular tissues but can also be found in plasma, lymph, synovial fluid, cerebrospinal fluid (CSF) and elsewhere. The manganese form (MnSOD) is generally associated with the mitochondria of aerobic organisms. The iron form (FeSOD) is found predominantly in prokaryotes. The relationship between SOD and various human diseases has now been well documented and measurement of SOD activity has become commonplace in many research model systems. One unit SOD activity is defined as the amount of enzyme that will inhibit the rate of cytochrome *c* reduction by half under specific conditions.

Intended Use:

The NWLSS™ Superoxide Dismutase Activity Assay provides a simple, rate method for determining SOD activity in tissue homogenates, cell lysates & other biological fluids or extracts where forms of SOD might be present.

Test Principle:

The NWLSS™ NWK-SOD02 method is based on monitoring the auto-oxidation rate of hematoxylin as originally described by Martin J. P., Jr et al 1987, with modifications to increase robustness and reliability. Briefly, in the presence of SOD enzyme at specific assay pH, the rate of auto-oxidation is inhibited and the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. Sample SOD activity is determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample and expressed as traditional McCord Fridovich "cytochrome c" units. The basic principal of the assay is shown schematically by the following equation:



General Specifications:

Format:	Cuvette or 96 Well Microplate
Number of Tests:	30 Cuvette or 96 Microplate
Specificity:	Cu/Zn, Mn and Fe Superoxide Dismutase
Sensitivity:	LLD = 0.3 U/mL in Reaction Mix 5 U/mL in Sample Added to Reaction Mix

Kit Contents:

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

Required Materials Not Provided:

Disposable semi-micro cuvettes (1.0 mL) or transparent microplate strips.
Microcentrifuge tubes.
Plastic or glass bottles.
Pipettors, adjustable 0.0 – 1.0 mL.
Disposable pippette tips.

Required Instrumentation:

Spectrophotometer (cuvette spectrophotometer, multi-cuvette changer with temperature control module recommended but not absolutely required) or plate reader.

Note:
The assay is sensitive to temperature. When a cuvette spectrophotometer without a temperature module is used, the experiment must be carried out in an environment where the temperature is constant (22 – 27°C). When there is a noticeable change of temperature, it is necessary to run samples and a blank (Assay Buffer) back-to-back to minimize temperature effect. For plate readers, this requirement is less restrictive since assays are carried out in parallel.

Warnings, Limitations, Precautions:

Individual components may be harmful if swallowed, inhaled or absorbed through the skin. Contact should be minimized through the use of gloves and standard good laboratory practices. If contact with skin or eyes occurs, rinse the site immediately with water and consult a physician

Storage Instructions:

Upon receipt, store the reagents at 2-8°C. Do not use components beyond the expiration date printed on the label.

Data Analysis:

1. Determine auto-oxidation rates as illustrated in Figure 2. The rate of reaction can be obtained by regression of the uninhibited linear portion of the rate curves, as shown in Figure 2. The equation for this linear regression is:

$$Y = aX + b,$$

where a is the decomposition rate or (ΔAbs_{560nm}/min).

A less preferred way to obtain the rate is to select two data points in the rate curve and to calculate rates according to following equation:

$$Rate\ (\Delta Abs_{560nm}/min) = (Y_2 - Y_1)/(X_2 - X_1).$$

Calculate average rates from duplicate or triplicate assays.

2. Obtain the ratio of sample rate and blank rate using the equation

$$Ratio_{s/b} = Rate_s/Rate_b$$

where $Rate_s$ is the average auto-oxidation rate of a sample, and $Rate_b$ is the average autooxidation rate of the blank.

3. Calculate percentage for SOD inhibition of baseline (blank) reaction rate as:

$$\%\ Inhibition = (1 - Ratio_{s/b}) * 100\%$$

4. SOD activity measured using the classical "cytochrome c reduction" method was found to be approximately 1.25 times measurable percent inhibition of hematoxylin auto-oxidation as established through in-house trials. The SOD concentration in classical McCord Fridovich "cytochrome c reduction" unit activity is therefore calculated as:

$$[SOD\ U/mL]_s = 1.25 * (\%\ Inhibition)$$

This value is representative of the SOD activity in the sample added to the reaction mixture not the final concentration in the reaction mixture.

5. The SOD concentration in the original sample ("cytochrome c reduction" U/mL) is calculated using the equation:

$$[SOD\ U/mL]_{os} = [SOD\ U/mL]_s * (Sample\ Dilution\ Factor)$$

Distinguishing types of SOD (continued):

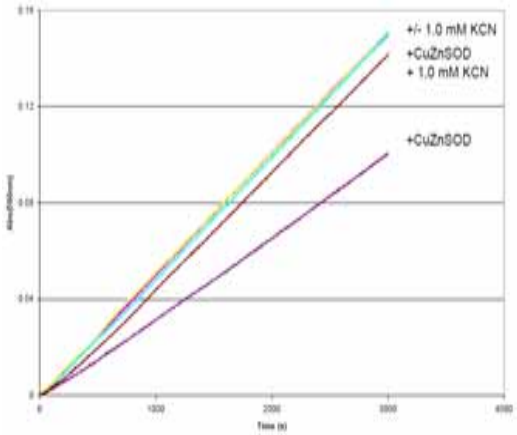


Figure 1 illustrates the effect of KCN on measurable Cu/Zn SOD activity using the hematoxylin assay method.

Addition of 1 mM KCN is shown to block Cu/Zn mediated inhibition of hematoxylin auto-oxidation.

Data Analysis:

Typical rate curves of standards with 0 U/mL and 40 U/mL are shown below in Fig. 2.

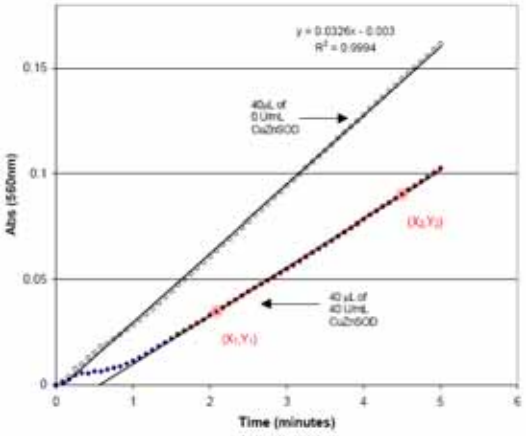


Figure 2: Rate curves of hematoxylin auto-oxidation in the absence and presence of CuZnSOD. SOD units as shown represent the concentration of enzyme added to the reaction mixture. The experiment was performed at room temperature (25°C) without a constant temperature cuvette holder.

In the 40 U/mL sample shown above, there is a slight inhibition of the reaction during the initial minute of recording. This is normal and is often observed with samples of higher SOD concentration or when interfering substances might be present as previously described in Notes 1 & 2 in the sample preparation section.

Assay/Instrument Preparation:

Spectrophotometer (Cuvette Assay) Setup

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 dH2O.

Plate Reader (Microplate Assay) Setup

1. Set measurement temperature at 25°C. Otherwise, carry out experiment at room temperature.

Reagent Preparation:

Allow the kit warm up to room temperature completely before using. It is acceptable to place reagents at room temperature the night before the experiment. However, do not reconstitute the Hematoxylin reagent until actual day of use since the reagent in liquid form is only stable for 6 hours at room temperature.

Assay Buffer:

Supplied Ready to Use. However, please shake or invert the bottle, open to air and repeat four (4) more times before use to saturate with O_2.

Hematoxylin Solution:

Reconstitute hematoxylin as supplied with 1.2 mL (1200 µL) of dH2O. 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°C (frozen) for future use.

Sample Dilution Buffer:

Supplied Ready to Use.

Sample Handling/Preparation:

Purification & Extraction:

Some samples may need to be dialyzed, extracted, or purified through chromatography to remove interfering 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. Samples typically need to be diluted with Sample Dilution Buffer before assay. For example, seminal plasma samples can be assayed directly after 1/10 dilution.

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Sample Handling/Preparation (continued):

Sample Dilution:

The following are general dilution guidelines in order to achieve the most sensitive linear assay range between 0 and 50 U/mL:

Tissues (10% homogenates):	1/2
Red Blood Cell lysate: (after chloroform/ethanol extraction to remove hemoglobin)	1/2
Seminal Plasma:	1/10

Blood Plasma or Serum:
Normal human plasma and serum SOD activities are typically quite low (< 5 U/mL) and are therefore below the level of detection of this assay. However, concentrations may be elevated to detectable levels under various experimental or treatment conditions.

Samples from at-risk patient groups or from abnormal experimental conditions may require a different dilution factor.

Note 1:
Linearity of the assay is lost at high SOD concentration. Therefore, when the auto-oxidation rate (A_{560} nm/min) of a sample is less than 50% of the auto-oxidation rate at zero SOD concentration (blank rate), it is important not to use the rate to calculate SOD concentration in the sample. Instead, adjust the dilution factor, make a new dilution and repeat the assay.

Note 2:
The auto-oxidation of hematoxylin is a self-catalyzed reaction and the rate is linear for about 10 minutes. This reaction can be inhibited by thiols (including protein thiols), ascorbic acid, and other antioxidants. In most cases however, the inhibition lasts only several minutes, especially at 1/5 or higher dilution factors. In these cases, samples can be assayed directly by the rate assay without prior treatment. In cases where excessive inhibition is noted and/or it is not possible to dilute the sample prior to assay, samples may be treated by dialysis, extraction and/or chromatography to remove interfering substances before the assay.

Sample Stability

Tissues:
We recommend storage of whole tissues at <-70°C with homogenization taking place the day of assay.

Cells:
For best analyte stability, we recommend storage of whole cell pellets at <-70°C with disruption via homogenization or sonication taking place on the day of assay.

Assay Protocol:

Cuvette Assay

1. Add 920 µL of **Assay Buffer** to each cuvette for assay.
2. Add 40 µL of **Assay Buffer** (for blank) or 40 µL of **Sample**. Mix and incubate for two (2) minutes.
3. Add 40 µL **Hematoxylin Reagent** to start the auto-oxidation reaction.
4. Mix quickly and immediately begin recording the absorbance at 560 nm every 10 seconds or smaller time interval for at least 5 minutes.

Note:
Reaction rate should be linear for approximately 10 minutes. Undialyzed samples may need longer recording time.

Microplate Assay

1. Record sample/standard layout.
2. To each well used for testing, add 230 µL of **Assay Buffer**.
3. Next add 10 µL of **Assay Buffer** (for blank) or 10 µL Sample. Shake to mix and incubate for 2 minutes.
4. Add 10 µL of **Hematoxylin Reagent** to begin reaction. Preferably, use a multi-channel pipette.
5. Mix quickly using the instrument's shaker function and immediately begin recording the absorbance at 560 nm every 10 seconds or smaller time interval for at least 5 minutes.

Note:
The reaction rate should be linear for approximately 10 minutes. Undialyzed samples may need longer recording time.

Employing a Control

To minimize inter-assay variation in ongoing studies, 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.

Distinguishing types of SOD

EC-SOD activity can be determined specifically after chromatography to eliminate other forms of SOD's.

To differentiate CuZnSOD and MnSOD activity separately in samples containing both enzymes, potassium cyanide (KCN at 1 to 2 mM) can be used to inhibit CuZnSOD but not MnSOD. Simply add the appropriate amount of KCN (under a hood to avoid possible HCN gas exposure) to a specific volume of assay buffer. The activity of a sample measured using the original assay buffer represents total SOD activity, and the result when using KCN containing assay buffer represents only MnSOD activity.