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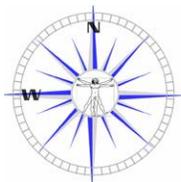
NWLSTM
Superoxide Dismutase
ELISA

Product NWK-SOD01
For Research Use Only



****Revised Protocol: TMB Substrate Now Supplied Ready to Use**

Simple ELISA kit for quantification of human Cu/Zn
Superoxide Dismutase in biological samples.



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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:

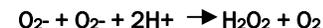
Procedure Checklist

- ___ Predilute serum or plasma samples with Working PBS 1/20
- ___ Wash Microwell Strips twice with **Diluted Wash Buffer**
- ___ Add 100 µL **Working PBS** to standard wells except the 5ng/mL wells.
- ___ Pipette 200 µL **Cu/ZnSOD Standard as supplied** into the first standard wells to create standard dilutions ranging from 5 to 0.08 ng/mL by serial dilution directly on the plate. Discard 100µL from the last (0.8 ng/mL) wells
- ___ Add 100 µL **Working PBS** to the blank wells
- ___ Add 90 µL **Working PBS** to the sample wells
- ___ Add 10 µL 1/20 **Prediluted Sample** to designated wells
- ___ Prepare **Working HRP-Conjugate**
- ___ Add 50 µL **Working HRP-Conjugate** to all wells
- ___ Cover microwell strips and incubate 1 hour at room temperature (18° to 25° C)
- ___ Prepare **Working TMB Substrate Solution** a few minutes prior to use
- ___ Empty and wash microwell strips 3 times with **Diluted Wash Buffer**
- ___ Add 100 µL of mixed **Working TMB Substrate Solution** to all wells including blank wells
- ___ Incubate the microwell strips for about 10 to 20 minutes at room temperature (18°-25° C)
- ___ Add 100 µL **Stop Solution** to all wells including blank wells
- ___ Blank microwell reader and measure color intensity at 450 nm

Note: For samples that diluted according to the instructions given in this manual (1/200), the concentration read from the standard curve must be multiplied by the dilution factor (x200). Calculation of samples with an ABS₄₅₀ exceeding 2.0 may result in underestimation of Cu/ZnSOD levels. Such samples require additional dilution with Working PBS in order to quantitate the Cu/ZnSOD level accurately.

Introduction:

Superoxide Dismutases (SODs) are a unique family of metalloproteins that catalyze the dismutation of superoxide anion radicals (O₂⁻) to oxygen (O₂) and hydrogen peroxide (H₂O₂).



SOD protects cells against oxygen-mediated free radical damage. Four types of SOD have been defined on the basis of their metal cofactors and distribution. The Cu/Zn form is the most common, with a primary distribution in the cytoplasm of eukaryotic cells. The manganese form (MnSOD) is generally associated with the mitochondria of aerobic organisms. The iron (FeSOD) form is found predominantly in prokaryotes. The most recently discovered form, extra-cellular (ecSOD) is found in the extra-cellular fluids of mammalian species, often associated with vascular tissues.

Human Cu/ZnSOD is a dimeric protein composed of 2 subunits of 153 amino acid residues with a molecular weight of 16 kDa each. Dissociation of the subunits is facilitated by alkylation of the two sulfhydryl groups in the protein or by removal of the copper and zinc ions.

The human Cu/ZnSOD gene has been localized to chromosome 21q22.1. Cu/Zn SOD gene expression can be induced by mediators of oxidative stress such as sulfhydryl antioxidants, interleukin-1 and tumor necrosis factor. Constitutive expression of Cu/ZnSOD mRNA is highest in dividing cells.

Intended Use:

The NWLSS™ Cu/ZnSOD ELISA is intended for the quantitative detection of human Cu/ZnSOD in human plasma, serum cell culture supernatants, erythrocyte (RBC) or other cell lysates and other biological fluids where SOD may be present.

Test Principle:

The NWLSS™ Cu/ZnSOD Assay is a simple “sandwich” ELISA using a plate bound capture antibody (anti hu Cu/ZnSOD) and a horseradish peroxidase conjugated secondary tracer antibody. Subsequent addition of an tetramethylbenzidine (TMB) substrate solution facilitates blue color development directly proportional to the nitrotyrosine present in the sample. The reaction is stopped using a phosphoric acid solution causing a color change to yellow that is read on a plate reader at 450 nm. Sample Cu/ZnSOD concentration is determined by comparing sample absorbance at 450 nm with those of a seven point standard curve created by diluting the assay calibrator supplied.

Specifications:

Format:	1 X 96 well ELISA	
Number of tests:	Triplicate =	24
	Duplicate =	40
Specificity:	Human Cu/Zn Superoxide Dismutase	
Sensitivity:	0.4 ng/mL	
Range:	0.4 ng/mL–5ng/mL	

Kit Contents:

1 Foil Pouch	Microwell plate precoated with murine derived monoclonal anti- human Cu/ZnSOD.	
2 vials	Anti-Cu/ZnSOD-HRP Conjugate: 20 µl	
2 vials	Cu/ZnSOD Standard (5 ng/ml): 0.5 mL	
1 bottle	20X Concentrated Wash Buffer Concentrate containing 1% Tween 20: 50 mL	
1 vial	20X Concentrated Assay Buffer containing 1% Tween 20 and 10% BSA: 5 mL	
1 vial	20X Concentrated Phosphate Buffered Saline: 5mL	
1 vial	TMB Substrate Solution: 15 mL	
1 vial	Phosphoric Acid Stop Solution (1M)	
2	Adhesive Plate Covers	

Required Materials Not Provided:

Adjustable micropipettes with disposable tips (5-1000 µL). Multi-channel pipettes are useful and help to reduce intra-sample variability.

Serological pipettes.

Deionized water.

Automatic plate washer or other aspiration devices are optional.

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- 7) Niwa Y., O. Iizawa, K. Ishimoto, H. Akamatsu, and F. Kanoh. (1993). Age-dependent basal level and induction capacity of copper/zinc and manganese superoxide dismutase and other scavenging enzyme activities in leukocytes from young and elderly adults. *Am. J. Pathol.* 143, 312-320.
- 8) Porstmann T., R. Wietschke, S. Jahn, R. Grunow, H. Schmechter, B. Porstmann, S. Kießing, M. Bergande, R. Bleiber, and R. von Baehr. (1989). Aufbau eines superschnellen Enzym Immunoassays für humane Cu/Zn Superoxid-Dismutase mit monoklonalen Antikörpern und Beispiele für seine klinische Anwendung. *Monoklonale Antikörper, Springer Verlag Wien, New York.* Ed.: R. von Baehr, H. P. Ferber, T. Porstmann.
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Required Instrumentation:

Plate reader with **450 nm** capability (650 nm is required for optional monitoring of color development prior to stopping the reaction).

Warnings, Precautions & Limitations:

Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.

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.

Substrate solutions must be at room temperature prior to use. Avoid contact of substrate solutions with oxidizing agents and metal.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Storage Instructions:

Store kit reagents between 2° and 8°C. Immediately after use reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

Prolonged exposure of kit components to light should be avoided.

Assay Preparation

1. Determine the number of wells required to assay standards, samples and controls for the appropriate number of replicates. It is recommended that testing be performed in duplicate or triplicate if possible.
2. Create an assay template showing positioning of standards, controls and samples.
3. Bring all samples and reagents to room temperature before use.
4. To avoid condensation, do not open foil pouches containing the micro-titer strips until after they have reached room temperature. Next remove the required number of strips and place in the frame supplied.

Return unused wells to the storage bag with desiccant, seal and store at 2-8°C.

Reagent Preparation:**Wash Buffer:**

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

For each 6 strips to be assayed, transfer 25 ml of the Wash Buffer Concentrate into a clean 1000 ml graduated cylinder. Bring final volume to 500 ml with deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle, label as **Diluted Wash Buffer** and store at 2° to 25°C. The Diluted Wash Buffer is stable for 30 days.

Assay Buffer

For each 6 strips to be assayed, mix the contents of the bottle well. Add 2.5 mL Assay Buffer Concentrate to 47.5 ml deionized water and mix gently to avoid foaming. Label as **Working Assay Buffer** and store at 2° to 8°C. The Working Assay Buffer is stable for 30 days.

Phosphate Buffered Saline

Mix the contents of the bottle well. For each 6 strips to be assayed add 2.5 mL PBS Concentrate to 47.5 mL deionized water. Label as **Working PBS**. Working PBS is stable for 30 days after mixing.

HRP-Conjugate

Dilute the HRP Conjugate Concentrate 1:5 just prior to use by adding 80 µL *Working Assay Buffer* to the original tube. For each 6 strips to be assayed, additionally dilute the **Diluted HRP-Conjugate** 1:100 by adding 30 µL *Diluted HRP-Conjugate* in 2.97 mL *Working Assay Buffer* in a clean tube labeled as **Working HRP-Conjugate**. Working HRP-Conjugate must be used within **30 minutes** after final dilution.

TMB Substrate Solution

Note: The protocol for this product has been modified. A single bottle of ready to use TMB Substrate Solution is now supplied in place of the two bottles (Substrate I and Substrate II) previously supplied.

Performance Details:**Specificity**

The interference of human MnSOD was evaluated by spiking this protein at physiologically relevant concentrations into a Cu/ZnSOD positive serum. There was no detectable cross reactivity.

Sensitivity

The limit of detection for Cu/ZnSOD, defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 0.04 ng/mL (mean of 10 independent assays).

Precision

Intra-assay = 5.1 %

Inter-assay = 5.8 %

Accuracy

	Spiked amounts of Cu/Zn SOD in ng			
	<u>4</u>	<u>2</u>	<u>1</u>	<u>0.5</u>
Average Recovery (% Spike)	90	93	103	105

Overall mean recovery = 98%

Dilutional recovery:

Four serum samples with different levels of Cu/ZnSOD were assayed at four serial two-fold dilutions (1:200 - 1:1600) covering the working range of the standard curve. Recoveries ranged from 80% to 107% with an overall mean recovery of 90% on dilution.

Expected and example experimental values

Typical OD readings for this assay run from approximately 0.025 for blanks to around 1.9 for the Highest (5 ng/mL) standard.

A panel of 22 sera from apparently healthy blood donors (male and female) was tested for Cu/ZnSOD. The detected Cu/ZnSOD levels ranged between 22.5 and 102.9 ng/ml with a mean level of 56.5 ng/mL and a standard deviation of 20.0 ng/mL.

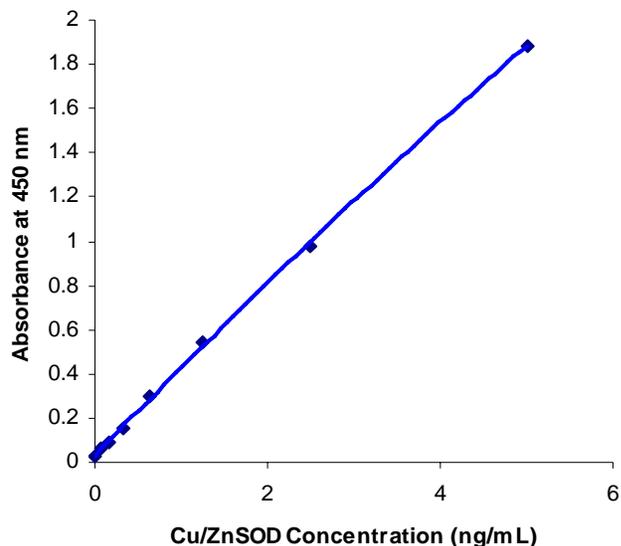
Measurement of Cu/ZnSOD from erythrocytes of fetal umbilical vein blood resulted in Cu/ZnSOD levels of 11-16 ng SOD/million fetal erythrocytes for normals and > 20 ng SOD/million fetal erythrocytes for fetuses with Down's Syndrome (5, 10, 11).

Data Analysis:

1. Plot the mean absorbance at 450 nm for each standard replicate versus the Cu/ZnSOD concentrations. A 2nd order polynomial curve fit is recommended. This can typically be done using the software provided with most plate readers or using Microsoft Excel. An example curve is shown below.

2. Unknown Cu/ZnSOD concentrations are determined by comparing their absorbance measurements at 450 nm with those of the standard curve.

Sample Standard Curve



3. Samples data as read from the standard curve must be multiplied by the dilution factor used (X 200 if following standard plasma method as described in this manual).

Note: Samples with an ABS₄₅₀ exceeding 2.0 should be additionally diluted with Working PBS and re-assayed in order to avoid erroneous results.

Standard Curve Preparation::

The standard curve is created by performing a serial dilution directly in the microplate as indicated in steps 4 & 5 of the *Assay Protocol* section.

Sample Handling/Preparation**Plasma & Serum**

Predilute serum or plasma samples 1/20 with *Working PBS* according to the following dilution scheme: 10 μ L Sample + 190 μ L PBS

Freeze/Thaw Stability

Aliquots of serum samples (unspiked or spiked with Cu/ZnSOD) were stored at -20°C and thawed several times, and the Cu/ZnSOD level determined. There was no significant loss of Cu/ZnSOD concentrations between 0 and 5 freeze-thaw cycles.

Storage Stability

Aliquots of a serum sample (unspiked or spiked with Cu/ZnSOD) were stored at -20°C, 2-8°C, room temperature and at 37°C and the Cu/ZnSOD level determined after 24, 48 and 96 hours. No significant loss of Cu/ZnSOD immunoreactivity was noted during storage under these conditions.

Comparison of Serum and Plasma

Serum, EDTA, citrate and heparinized plasma from 22 subjects obtained at the same time point were evaluated. All of these preparations are suitable for Cu/ZnSOD determinations with no significant differences noted. For comparison purposes however, it is recommended that all samples are harvested and treated in the same manner.

Erythrocytes

Collect blood in suitable tubes containing anticoagulant then centrifuge tubes at 1500 x g for 10 minutes at 4°C. Pipette off the plasma and buffy coat fractions. Wash remaining RBC pellet in cold saline then centrifuge again and remove wash saline. RBC's can be lysed by addition of ice cold deionized water. Centrifuge lysate to remove debris then harvest lysate to clean tubes for future assay. If assaying same day, RBC lysate may be stored on ice at 4°C else frozen at -70°C for later assay. Samples may be normalized to hemoglobin, protein or RBC cell count.

Tissue samples

Testing of tissue homogenates may be possible with this assay but has not yet been validated.

Assay Protocol:

1. Before use, assure that all reagents are mixed thoroughly without foaming.
2. Wash the microwell strips to be used twice with 300 μ L *Diluted Wash Buffer* per well waiting 1 minute between aspirations.
3. After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess *Diluted Wash Buffer*. Washed microwell strips should be used immediately after washing or placed upside down on a wet absorbent paper for not longer than 15 minutes being careful not to allow wells to dry.
4. Add 100 μ L of **Working PBS** to each standard well from 2.5 ng/mL to 0.08 ng/mL. Leave the high standard wells at 5 ng/ml empty.
5. Add **200 μ L of Cu/ZnSOD Standard**, as supplied to each of the 5 ng/mL standard wells. Serially dilute the standards by transferring **100 μ L from each 5 ng/mL standard well to the 2.5ng/mL standard wells and so on** to create full range of standards down to 0.08 ng/mL. Be careful not to scratch the inner surface of the microwells.

Discard 100 μ L of the contents from the last microwells (e.g. G1, G2 for duplicates corresponding to 0.08 ng/mL) such that each standard well contains 100 μ L of the appropriate standard.
6. Add **100 μ L of Working PBS** to blank wells.
7. Add **90 μ L Working PBS** to all wells designated for samples.
8. Add **10 μ L of each Pre-diluted Sample** to the appropriate sample wells according to assay template.
9. Add 50 μ L of **Working HRP Conjugate** to all wells.
10. Cover plate and incubate at Room Temperature (18° to 25°C) for 1 hour.
11. Remove Plate Cover and empty wells. Wash microwells 3 times with 300 μ L *Diluted Wash Buffer* per well waiting 1 minute between aspirations. Proceed immediately to the next step.

Assay Protocol: (continued):

12. Pipette 100 μ L of **Working TMB Solution** to all wells, including the blank wells. Best results will be obtained using a multi-channel pipette such that all replicate wells receive Working TMB Solution at the same time.
13. Incubate the microwell strips at room temperature (18° to 25°C) for Approximately 10 minutes in the dark on a rotator/shaker, if one is available.

Color development should be monitored and the reaction stopped before the highest standard (5 ng/mL) wells are no longer properly recordable. It is recommended to add the stop solution when the highest standard has developed a dark blue color or, if monitoring using a plate reader, the reaction should be stopped when the OD at 650 nm reaches 0.6 – 0.65.

Note: Incubation without shaking may yield lower absorbance values than expected or as indicated on the sample standard curve. Slightly lower absorbance values will not affect the validity of data obtained.

14. Stop the reaction by pipetting 100 μ L of **Stop Solution** into each well including blank wells. It is important that the Stop Solution is added quickly and uniformly throughout the microwells to completely inactivate the enzyme. Best results will be obtained using a multi-channel pipette such that all replicate wells receive stop solution at the same time. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
15. Read absorbance at 450 nm of each microwell on a plate reader. Blank the plate reader according to the manufacturer's instructions using the blank wells as set-up on the assay template. Determine the net absorbance of all samples and CU/ZnSOD standards.