KAMIYA BIOMEDICAL COMPANY

Human Cu/Zn SOD ELISA

For the quantitative determination of human Cu/Zn SOD in cell culture supernatants, serum, plasma, urine, amniotic fluid, fetal umbilical vein blood and other body fluids

Cat. No. KT-034

For research use only, not for use in diagnostic procedures.

Product Information

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PRODUCT
The **K-ASSAY®** Human Cu/Zn SOD ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of human Cu/Zn SOD in cell culture supernatants, serum, plasma, urine, amniotic fluid, fetal umbilical vein blood and other body fluids. **The Human Cu/Zn SOD ELISA is for research use only. Not for use in diagnostic procedures.**

DESCRIPTION
Superoxide dismutases (SODs) are a unique family of metalloproteins that catalyze the dismutation of superoxide anion radicals (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2)

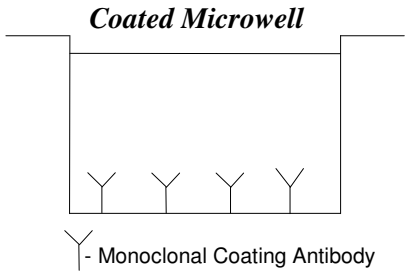
$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

SOD is ubiquitous in oxygen metabolizing cells protecting these cells against direct and indirect oxygen-mediated free radical damage. Four types of SOD have been defined on the basis of distinctions in their metal cofactors and distribution: Manganese (Mn SOD) principally located in the matrix of mitochondria of all aerobes, copper/zinc (Cu/Zn SOD) mainly present in the cytoplasm of eukaryotic cells, iron (Fe SOD), predominantly in the cytosol, chloroplasts or mitochondria of prokaryotes as well as extracellular (EC SOD), which is found in the extracellular fluids or membrane associated in mammals.

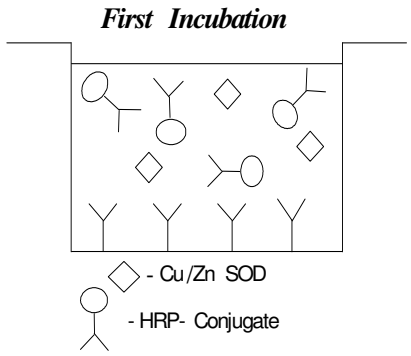
The properties of Cu/Zn superoxide dismutase are quite different from those of the manganese or iron enzymes. Sequence analysis has indicated a homology between Mn and Fe class enzymes but these have no homology with the Cu/Zn enzyme. The human Cu/Zn superoxide dismutase is a dimeric protein composed of 2 subunits of 153 amino acid residues and 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/Zn SOD gene has been localized to chromosome 21q22.1. Cu/Zn SOD gene expression is induced by mediators of oxidative stress like sulfhydryl antioxidants, interleukin-1, tumor necrosis factor. Constitutive expression of copper and zinc SOD mRNA is highest in dividing cells. Induction of Cu/Zn SOD expression resulting in elevated levels of Cu/Zn SOD in human body fluids is of value for measuring the activity of different diseases.

PRINCIPLE

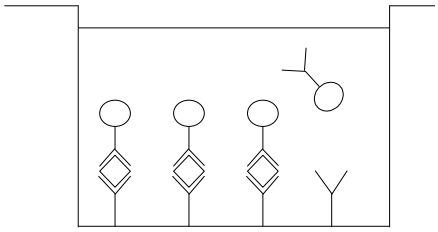
An anti-Cu/Zn SOD monoclonal coating antibody is adsorbed onto microwells.



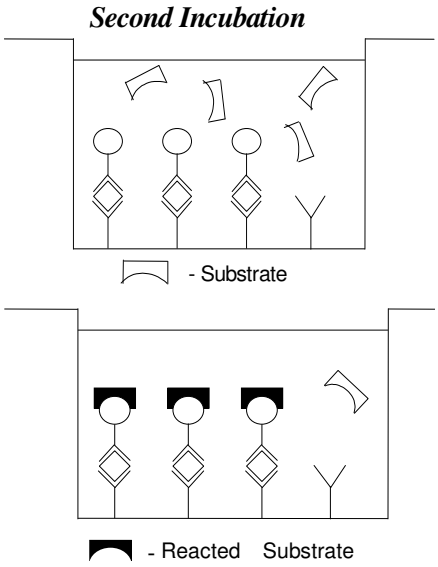
Cu/Zn SOD present in the sample or calibrator binds to antibody adsorbed to the microwells; a HRP-conjugated monoclonal anti-Cu/Zn SOD antibody is added and binds to Cu/Zn SOD captured by the first antibody.



Following incubation unbound enzyme-conjugated anti-Cu/Zn SOD is removed during a wash step and substrate solution reactive with HRP is added to the wells.



A colored product is formed in proportion to the amount of Cu/Zn SOD present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibration curve is prepared from seven Cu/Zn SOD calibrator dilutions and Cu/Zn SOD sample concentration determined.



COMPONENTS

- 1 aluminum pouch with **Microwell Plate** (12 x 8-well strips) coated with monoclonal antibody to human Cu/Zn SOD
- 2 vials (20 µL) **HRP-Conjugate** anti-Cu/Zn SOD monoclonal (mouse) antibody
- 2 vials (0.5 mL) **Cu/Zn SOD Calibrator,** 5 ng/mL
- 1 bottle (50 mL) **Wash Buffer Concentrate** 20X (PBS with 1% Tween 20)
- 1 vial (5 mL) **Assay Buffer Concentrate** 20X (PBS with 1% Tween 20 and protein stabilizer)
- 1 bottle (5 mL) **Phosphate Buffered Saline Concentrate** (PBS), 20X
- 1 vial (15 mL) **Substrate Solution**
- 1 vial (15 mL) **Stop Solution** (1M Phosphoric acid)
- 2 adhesive **Plate Seals**

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1,000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multi-channel micropipette with disposable tips
- Multi-channel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multi-channel wash bottle or automatic wash system)
- Microplate reader capable of reading at 450 nm (620 nm as optional reference wavelength)
- Distilled or de-ionized water
- Statistical calculator with program to perform linear regression analysis.

STORAGE

Store kit reagents at 4℃ as indicated. Immediately after use remaining reagents should be returned to cold storage (4℃). Expiration date of the kit and reagents is stated on labels. The expiration date of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

SPECIMEN COLLECTION

Cell culture supernatants, human serum, EDTA, citrate or heparinized plasma, urine, amniotic fluid, fetal umbilical vein blood, or other body fluids will be suitable for use in the assay. Remove serum or plasma from the clot or red cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20℃ to avoid loss of bioactive Cu/Zn SOD. If samples are to be run within 24 hours, they may be stored at 4℃. Avoid repeated freeze-thaw cycles. Prior to assay, frozen sample should be brought to room temperature slowly and mixed gently.

For sample stability, see page 11.

PRECAUTIONS FOR USE

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.

- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens, which may invalidate the assay, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acids will inactivate the conjugate.
- Distilled water or de-ionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose samples and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5℃.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

PROCEDURES

PREPARATION OF REAGENTS

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

A. Wash Buffer (1X)
Pour entire contents (50 mL) of the Wash Buffer Concentrate (20X) into a clean 1,000 mL graduated cylinder. Bring final volume to 1,000 mL with distilled or de-ionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25℃. Please note that the Wash Buffer (1X) is stable for 30 days.
Wash Buffer (1X) may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20X) (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

B. Assay Buffer (1X)
Add contents of Assay Buffer Concentrate (20X) (5.0 mL) to 95 mL distilled or de-ionized water and mix gently to avoid foaming. Store at 4℃. Please note that the Assay Buffer (1X) is stable for 30 days.
Assay Buffer (1X) may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20X) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Phosphate Buffered Saline (PBS) (1X)
Mix the contents of the bottle well. Add contents of PBS concentrate (20X) (5.0 mL) to 95 mL distilled or de-ionized water and mix gently to avoid foaming. Store at 4℃. Please note that the PBS (1X) is stable for 30 days.
PBS (1X) may be prepared as needed according to the following table:

Number of Strips	PBS Concentrate (20X) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

D. Preparation of HRP-Conjugate

Dilute the HRP-Conjugate 1:5 just prior to use by adding 80 µL Assay Buffer (1X) to the tube containing the HRP-Conjugate concentrate. Mix the contents of the tube well. Make a further 1:100 dilution with Assay Buffer (1X) in a clean plastic tube. Please note that the HRP-Conjugate should be used within 30 minutes after dilution. The HRP-Conjugate may be prepared as needed according to the following table:

Number of Strips	Prediluted (1:5) HRP-Conjugate (mL)	Assay Buffer (1X) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

ASSAY PROTOCOLS

a. Predilute serum or plasma samples 1:20 with PBS (1X) according to the following dilution scheme:

10 µL Sample + 190 µL PBS (1X)

For fetal umbilical vein blood first adjust samples to 2 x 10^7 erythrocytes/mL. Then proceed as above.

b. Determine the number of Microwell Strips required to assay the desired number of samples plus appropriate number of wells needed for running blanks and calibrators. Each sample, calibrator, blank, and optional control sample should be assayed in duplicate. Remove extra Microwell Strips from holder and store in the foil bag sealed tightly with the provided desiccant at 4°C.

c. Wash the Microwell Strips twice with approximately 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10-15 seconds before aspiration.Take care not to scratch the inner surface of the microwells.

After the last wash, empty wells and tap Microwell Strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the Microwell Strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

d. Add 100 µL of PBS (1X), in duplicate, to the calibrator wells, leaving the first wells (5 ng/mL) empty. Prepare calibrator dilutions by pipetting 200 µL of Cu/Zn SOD Calibrator, in duplicate, into well A1 and A2 (see Figure 1 and 2). Transfer 100 µL to wells B1 and B2 respectively. Mix the contents by repeated aspiration and ejection and transfer 100 µL to well C1 and C2 respectively. Take care not to scratch the inner surface of the microwells.

Continue this procedure four times, creating two rows of Cu/Zn SOD Calibrator dilutions ranging from 5 to 0.08 ng/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of Cu/Zn SOD Calibrator dilutions:

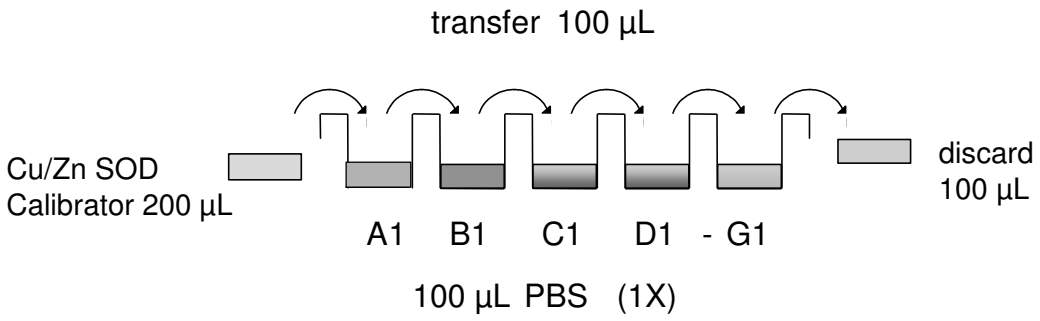


Figure 2. Diagram depicting an example of the arrangement of blanks, calibrators and samples in the Microwell Strips:

	1	2	3	4
A	Calibrator 1 (5 ng/mL)	Calibrator 1 (5 ng/mL)	Sample 1	Sample 1
B	Calibrator 2 (2.5 ng/mL)	Calibrator 2 (2.5 ng/mL)	Sample 2	Sample 2
C	Calibrator 3 (1.25 ng/mL)	Calibrator 3 (1.25 ng/mL)	Sample 3	Sample 3
D	Calibrator 4 (0.63 ng/mL)	Calibrator 4 (0.63 ng/mL)	Sample 4	Sample 4
E	Calibrator 5 (0.31 ng/mL)	Calibrator 5 (0.31 ng/mL)	Sample 5	Sample 5
F	Calibrator 6 (0.16 ng/mL)	Calibrator 6 (0.16 ng/mL)	Sample 6	Sample 6
G	Calibrator 7 (0.08 ng/mL)	Calibrator 7 (0.08 ng/mL)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

e. Add 100 µL of PBS (1X), in duplicate, to the blank wells.

f. Add 90 µL of PBS (1X) to all wells designated for samples.

g. Add 10 µL of each prediluted Sample, in duplicate, to the designated wells.

h. Prepare HRP-Conjugate. (Refer to PREPARATION OF REAGENTS).

i. Add 50 µL of diluted HRP-Conjugate to all wells, including the blank wells.

j. Cover with a Plate Seal and incubate at RT (18° to 25°C) for 1 hour, if available on a rotator set at 100 rpm.

k. Remove Plate Seal and empty wells. Wash Microwell Strips 3 times according to point c. of the Assay Protocols. Proceed immediately to the next step.

l. Pipette 100 µL of TMB Substrate Solution to all wells, including the blank wells.

m. Incubate the Microwell Strips at room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light. **The color development on the plate should be monitored and the substrate reaction stopped (see point n. of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.** It is recommended to add the stop solution when the highest calibrator has developed a dark blue color. Alternatively, the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as an OD of 0.9 – 0.95 is reached for calibrator 1.

n. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the Microwell Strips are stored at 4°C in the dark.

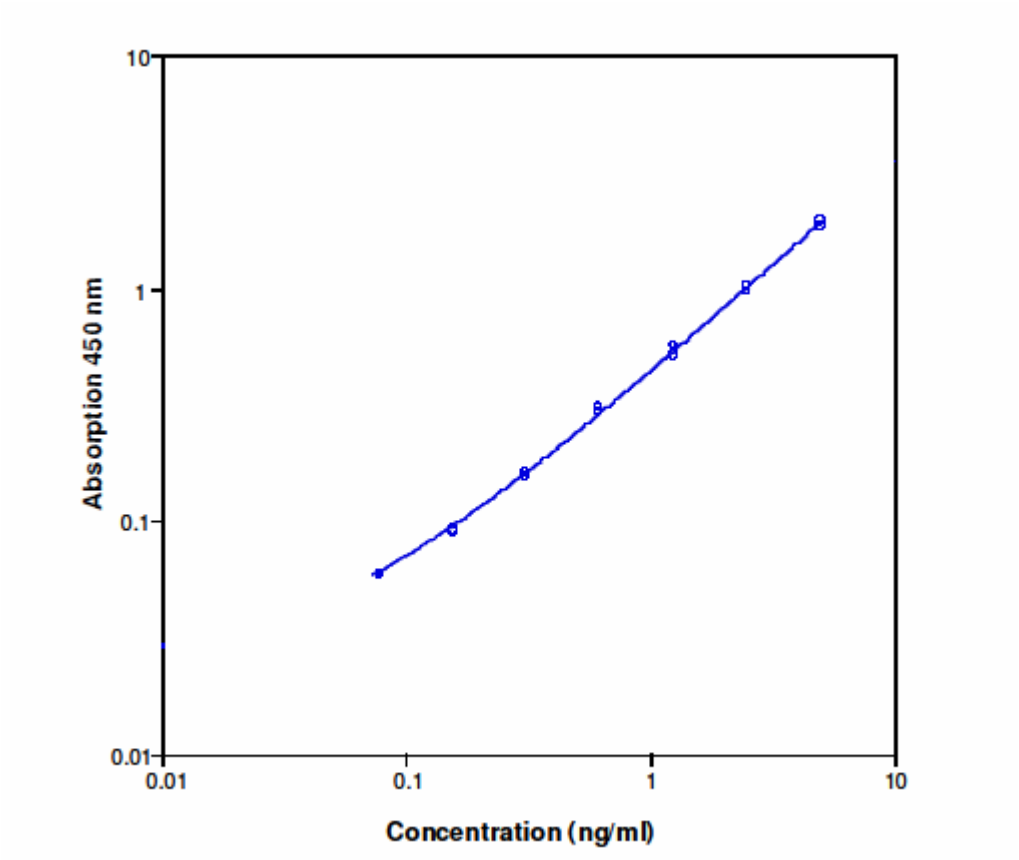
o. Read absorbance of each microwell on a microplate reader using 450 nm as the primary wavelength (optionally 620 nm as the reference wavelength; 610 nm to 650 nm is acceptable). Blank the microplate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the Cu/Zn SOD calibrators.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

CALCULATIONS

- Calculate the mean absorbance values for each set of duplicate calibrators and samples. Duplicates should be within 20 percent of the mean.
- Create a calibration curve by plotting the mean absorbance for each calibrator concentration on the ordinate against the Cu/Zn SOD concentration on the abscissa. Draw a best-fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating Cu/Zn SOD for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the calibration curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding Cu/Zn SOD concentration.
- **For samples which have been diluted according to the instructions given in this package insert (1:200), the concentration read from the calibration curve must be multiplied by the dilution factor (x 200).**
- **Note: Calculation of samples with a concentration exceeding calibrator 1 may result in incorrect, low Cu/Zn SOD levels (Hook Effect). Such samples require further dilution with PBS (1X) in order to precisely quantitate the actual Cu/Zn SOD level.**
- It is suggested that each testing facility establishes a control sample of known Cu/Zn SOD concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.
- A representative calibration curve is shown in Figure 3. This curve cannot be used to derive assay results. Every laboratory must prepare a calibration curve for each assay run.

Figure 3. Representative calibration curve for Human Cu/Zn SOD ELISA



Cu/Zn SOD was diluted in serial two-fold steps in PBS (1X). Do not use this calibration curve to derive assay results. A calibration curve must be run for each assay run.

Typical data using the Human Cu/Zn SOD ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Calibrator	Cu/Zn SOD Concentration (ng/mL)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	5.00	1.942	1.886	4.2
	5.00	1.829		
2	2.50	1.006	0.981	3.6
	2.50	0.956		
3	1.25	0.568	0.542	6.8
	1.25	0.516		
4	0.63	0.309	0.299	4.7
	0.63	0.289		
5	0.31	0.160	0.158	2.2
	0.31	0.155		
6	0.16	0.091	0.091	0.8
	0.16	0.090		
7	0.08	0.058	0.059	1.2
	0.08	0.059		
Blank	0.00	0.022	0.024	8.3
	0.00	0.026		

The OD values of the calibration curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

LIMITATIONS

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every assay run.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred. Reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely 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.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (HAMA). HAMA may interfere with assays utilizing mouse monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to mouse immunoglobulins can still be analyzed in such assays when mouse immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of Cu/Zn SOD defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 0.04 ng/mL (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of Cu/Zn SOD. Two calibration curves were run on each plate. Data below show the mean Cu/Zn SOD concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 5.1%.

Positive Sample	Experiment	Cu/Zn SOD Concentration (ng/mL)	Coefficient of Variation (%)
1	1	110.1	4.7
	2	99.2	6.1
	3	93.9	7.0
2	1	194.9	2.2
	2	185.1	1.0
	3	179.4	3.6
3	1	129.4	4.2
	2	123.7	6.1
	3	124.8	3.4
4	1	48.1	1.8
	2	45.6	10.7
	3	38.0	8.2
5	1	149.2	1.4
	2	145.5	4.7
	3	150.1	1.8
6	1	64.2	7.8
	2	53.3	7.8
	3	58.5	7.0
7	1	133.5	7.5
	2	122.5	9.5
	3	121.0	2.5
8	1	42.8	5.9
	2	40.1	4.6
	3	42.1	2.1

b. Inter-assay

Assay-to-assay reproducibility within one laboratory was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of Cu/Zn SOD. Two calibration curves were run on each plate. Data below show the mean Cu/Zn SOD concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 5.8%.

Sample	Cu/Zn SOD Concentration (ng/mL)	Coefficient of Variation (%)
1	101.1	8.2
2	186.5	4.2
3	126.0	2.4
4	43.9	11.9
5	148.3	1.6
6	58.7	9.3
7	125.7	5.4
8	41.6	3.4

C. Recovery Studies

The spike recovery was evaluated by spiking 4 levels of human Cu/ZnSOD into 2 normal pooled serum samples. The amount of endogenous human Cu/ZnSOD in unspiked serum was subtracted from the spike values. The recovery ranged from 89% to 108% with an overall mean recovery of 98%.

D. Dilution Parallelism

Four serum samples with different levels of Cu/Zn SOD were assayed at four serial two-fold dilutions (1:200-1:1,600) covering the working range of the calibration curve. In the table below the percent recovery of expected values is listed. Recoveries ranged from 80% to 107% with an overall mean recovery of 90%.

Sample	Dilution	Cu/Zn SOD Concentration (ng/mL) Expected Value	Observed Value	% Recovery of Exp. Value
1	1:200	--	216.3	--
	1:400	108.2	96.1	89
	1:800	54.1	42.7	79
	1:1,600	27.1	24.0	89
2	1:200	--	123.8	--
	1:400	61.9	52.8	85
	1:800	30.9	30.1	97
	1:1,600	15.5	14.2	92
3	1:200	--	146.1	--
	1:400	73.1	63.7	87
	1:800	36.5	29.2	80
	1:1,600	18.3	19.6	107
4	1:200	--	53.0	--
	1:400	26.5	25.9	98
	1:800	13.3	11.1	83
	1:1,600	6.6	6.5	98

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored frozen at -20°C and thawed five times, and Cu/Zn SOD levels determined. There was no significant loss of Cu/Zn SOD between 0 and 5 cycles of freezing and thawing.

b. Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 4°C, room temperature and at 37°C, and the Cu/Zn SOD level determined after 24, 48 and 96 hours. There was no significant loss of Cu/Zn SOD immunoreactivity during storage at above conditions.

F. Comparison of Serum and Plasma

From 22 individuals, serums as well as EDTA, citrate and heparin plasma obtained at the same time point were evaluated. All these blood preparations are suitable for Cu/Zn SOD determinations. It is nevertheless highly recommended to assure the uniformity of blood preparations used in one assay.

G. Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human Cu/ZnSOD positive serum. There was no cross reactivity detected.

REAGENT PREPARATION SUMMARY

A. Wash Buffer (1X) Add **Wash Buffer Concentrate (20X)** (50 mL) to 950 mL distilled water.

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

B. Assay Buffer (1X)

Add **Assay Buffer Concentrate (20X)** (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. PBS (1X)

Add **PBS Concentrate (20X)** (5 mL) to 95 mL distilled water.

Number of Strips	PBS (20X) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

D. HRP-Conjugate

Make a 1:5 predilution of **HRP-Conjugate** by adding 80 µL Assay Buffer (1X) to the tube containing the HRP-Conjugate concentrate. Make a further 1:100 dilution in Assay Buffer (1X):

Number of Strips	Prediluted (1:5) HRP-Conjugate (mL)	Assay Buffer (1X) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

ASSAY PROTOCOL SUMMARY

- Predilute serum or plasma samples with **PBS (1X)** 1:20.
- Wash **Microwell Strips** twice with **Wash Buffer**.
- Add 100 µL **PBS (1X)**, in duplicate, to calibrator wells except the first wells (5 ng/mL).
- Pipette 200 µL **Cu/Zn SOD Calibrator** into the first calibrator wells and create calibrator dilutions ranging from 5 to 0.08 ng/mL by transferring 100 µL from well to well; Discard 100 µL from the last wells.
- Add 100 µL **PBS (1X)**, in duplicate, to the blank wells.
- Add 90 µL **PBS (1X)** to the sample wells.
- Add 10 µL 1:20 prediluted **Sample**, in duplicate, to designated wells.
- Prepare **HRP-Conjugate**.
- Add 50 µL diluted **HRP-Conjugate** to all wells.
- Cover **Microwell Strips** and incubate 1 hour at RT (18° to 25°C).
- Empty and wash **Microwell Strips** 3 times with **Wash Buffer**.
- Add 100 µL of **TMB Substrate Solution** to all wells including blank wells.
- Incubate the **Microwell Strips** for about 10 minutes at RT (18° to 25°C).
- Add 100 µL **Stop Solution** to all wells including blank wells.
- Blank microplate reader and measure color intensity at 450 nm.

Note: For samples which have been diluted according to the instructions given in this package insert (1:200), the concentration read from the calibration curve must be multiplied by the dilution factor (x 200).

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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