PRODUCT INFORMATION

Cu/ZnSOD (Human) ELISA

Enzyme-linked immunosorbent assay for

detection of human Cu/ZnSOD.

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

REF ***IB49622***

Manufactured for:
Immuno-Biological Laboratories, Inc. (IBL-America)
8201 Central Ave NE, Suite P, Minneapolis, MN 55432
Toll Free: (888) 523-1246 Fax: (763) 780-2988
www.ibl-america.com / info@ibl-america.com

TABLE OF CONTENTS

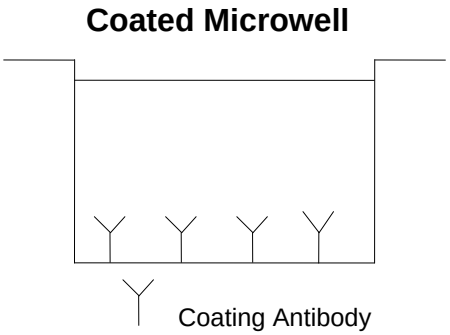
1. Intended Use

The human Cu/ZnSOD ELISA is an enzyme-linked immunosorbent assay for the detection of human Cu/ZnSOD. For research use only, not for use in diagnostic procedures.

2. Principle of the Test

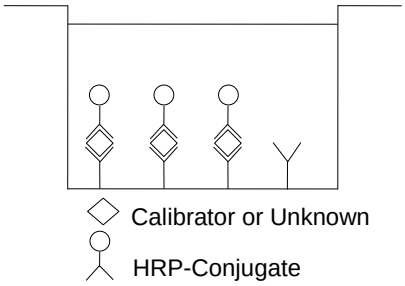
An anti-human Cu/ZnSOD coating antibody is adsorbed onto microwells.

Figure 1



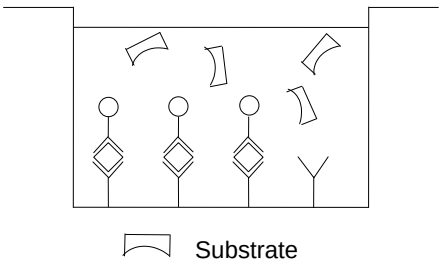
Human Cu/ZnSOD present in the unknown or calibrator binds to antibodies adsorbed to the microwells. A HRP-conjugated anti-human Cu/ZnSOD antibody is added and binds to human Cu/ZnSOD captured by the first antibody.

Figure 2



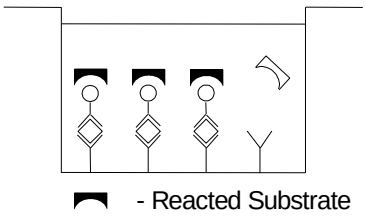
Following incubation unbound HRP-conjugated anti-human Cu/ZnSOD is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 3



A coloured product is formed in proportion to the amount of human Cu/ZnSOD present in the unknown or calibrator. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibrator curve is prepared from 7 human Cu/ZnSOD calibrator dilutions and human Cu/ZnSOD concentration determined.

Figure 4



3. Reagents Provided

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human Cu/ZnSOD

2 vials (20 µl) **HRP-Conjugate** anti-human Cu/ZnSOD monoclonal antibody

2 vials (500 µl) human Cu/ZnSOD **Calibrator**, 5 ng/ml

1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)

1 vial (5 ml) **Phosphate Buffered Saline Concentrate** (PBS) 20x

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml) **Blue-Dye**

1 vial (0.4 ml) **Green-Dye**

2 **Adhesive Films**

4. Storage Instructions

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels. Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

5. Collection and Handling of Unknowns

Cell culture supernatant, serum, plasma (EDTA, citrate, heparin), amniotic fluid, urine and fetal umbilical vein blood were tested with this assay. Other biologicals might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Pay attention to a possible **“Hook Effect”** due to high concentrations of unknown (see chapter 10).

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

Unknowns should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Cu/ZnSOD. If unknowns are to be run within 24 hours, they may be stored at 2° to 8°C.
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen unknown should be brought to room temperature slowly and mixed gently.

6. Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

7. 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. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- 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 unknowns are handled.
- Avoid contact of skin or mucous membranes with kit reagents or unknowns.
- Rubber or disposable latex gloves should be worn while handling kit reagents or unknowns.
- 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 unknowns which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose unknowns and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- 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.

8. 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.

8.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also 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

8.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also 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

8.3 Phosphate Buffered Saline (PBS) (1x)

Mix the contents of the bottle well. Add contents of **PBS concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the PBS (1x) is stable for 30 days.

PBS (1x) may also 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

8.4 HRP-Conjugate

Please note that the HRP-Conjugate should be used within 30 minutes after dilution.

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 or reagent reservoir 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

8.5 Human Cu/ZnSOD Calibrator

8.5.1 Calibrator Dilution

Label 7 tubes, one for each calibrator point.

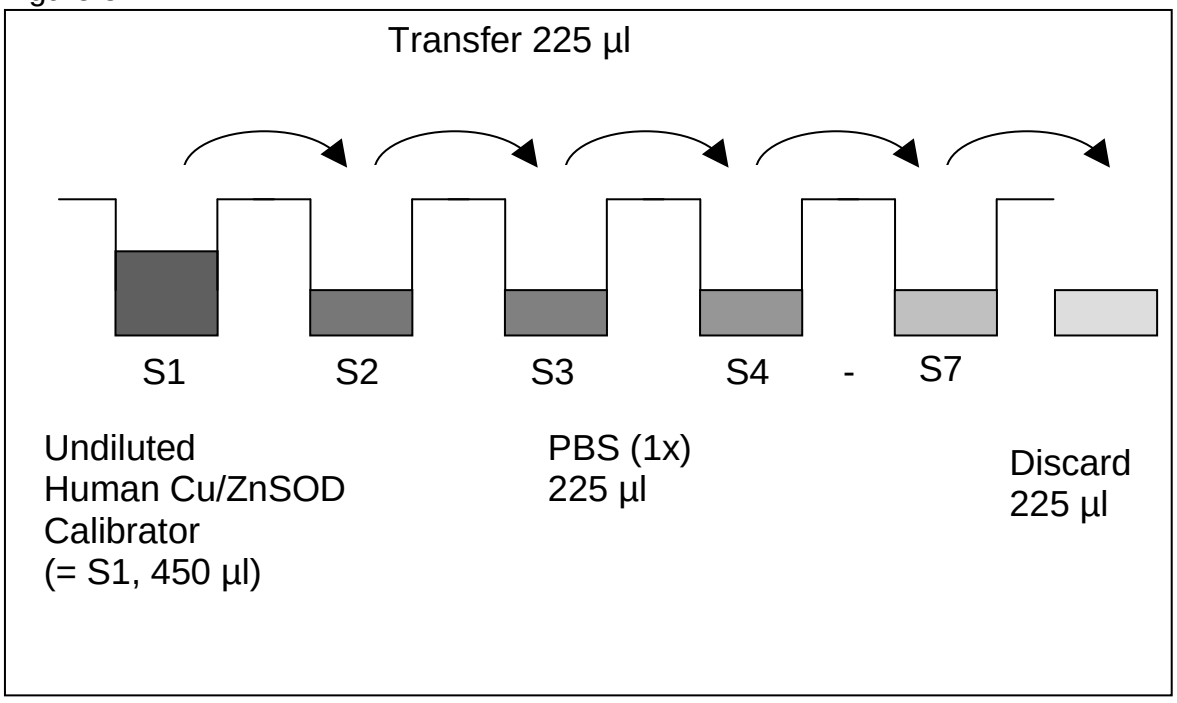
S1, S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the calibrator curve as follows:
Pipette 225 µl of PBS (1x) into tubes S2 – S7.

Pipette 450 µl of undiluted calibrator (serves as the highest calibrator S1, concentration of calibrator 1= 5 ng/ml) into the first tube, labelled S1.
Pipette 225 µl of this dilution into the second tube, labelled S2 (concentration of calibrator 2 = 2.5 ng/ml), and mix thoroughly before the next transfer.
Repeat serial dilutions 5 more times thus creating the points of the calibrator curve (see Figure 5).

PBS (1x) serves as blank.

Figure 5



8.6 Addition of Colour-giving Reagents: Blue-Dye, Green-Dye

In order to help our customers to avoid any mistakes in pipetting the IBL-America ELISAs, IBL-America offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye***) can be added to the reagents according to the following guidelines:

Diluent: Before calibrator and unknown dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml PBS (1x)	20 µl ***Blue-Dye***
12 ml PBS (1x)	48 µl ***Blue-Dye***
50 ml PBS (1x)	200 µl ***Blue-Dye***

HRP-Conjugate: Before dilution of the concentrated HRP-Conjugate add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet: Preparation of HRP-Conjugate.

3 ml Assay Buffer (1x)	30 µl ***Green-Dye***
6 ml Assay Buffer (1x)	60 µl ***Green-Dye***

9. Test Protocol

1. Predilute your unknowns before starting with the test procedure.
   Dilute serum and plasma unknowns 1:20 with PBS (1x) according to the following scheme:
   10 µl unknown + 190 µl PBS (1x)
   For fetal umbilical vein blood first adjust unknowns to $2x10^7$ erythrocytes/ml. Then proceed as above.

2. Determine the number of microwell strips required to test the desired number of unknowns plus appropriate number of wells needed for running blanks and calibrators. Each unknown, calibrator, blank and optional control should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

3. 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 surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. **Do not allow wells to dry**.

4. Pipette 100 µl of the prepared calibrator dilutions (see above, section 8.5.1) in the calibrator wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, calibrators and unknowns in the microwell strips:

	1	2	3	4
A	Calibrator 1 (5.00 ng/ml)	Calibrator 1 (5.00 ng/ml)	Unknown 1	Unknown 1
B	Calibrator 2 (2.50 ng/ml)	Calibrator 2 (2.50 ng/ml)	Unknown 2	Unknown 2
C	Calibrator 3 (1.25 ng/ml)	Calibrator 3 (1.25 ng/ml)	Unknown 3	Unknown 3
D	Calibrator 4 (0.63 ng/ml)	Calibrator 4 (0.63 ng/ml)	Unknown 4	Unknown 4
E	Calibrator 5 (0.31 ng/ml)	Calibrator 5 (0.31 ng/ml)	Unknown 5	Unknown 5
F	Calibrator 6 (0.16 ng/ml)	Calibrator 6 (0.16 ng/ml)	Unknown 6	Unknown 6
G	Calibrator 7 (0.08 ng/ml)	Calibrator 7 (0.08 ng/ml)	Unknown 7	Unknown 7
H	Blank	Blank	Unknown 8	Unknown 8

5. Add 100 µl of **PBS (1x)** in duplicate to the **blank wells**.

6. Add 90 µl of **PBS (1x)** to the wells designated for **unknowns**.

7. Add 10 µl of each **prediluted unknown** in duplicate to the appropriately assigned wells designated for unknowns.

8. Prepare **HRP-Conjugate** (see Preparation of HRP-Conjugate 8.4).

9. Add 50 µl of **HRP-Conjugate** to all wells.

10. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.

11. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point 3. of the test protocol. Proceed immediately to the next step.

12. Pipette 100 µl of **TMB Substrate Solution** to all wells.

13. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

    The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour 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 colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Calibrator 1 has reached an OD of 0.9 – 0.95.

14. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. 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 2 - 8°C in the dark.

15. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the unknowns and the 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.

10. Results

- Calculate the average absorbance values for each set of duplicate calibrators and unknowns. Duplicates should be within 20 per cent of the mean value.

- Create a calibration curve by plotting the mean absorbance for each calibrator concentration on the ordinate against the human Cu/ZnSOD 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 human Cu/ZnSOD for each unknown, 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 human Cu/ZnSOD concentration.

- **If instructions in this protocol have been followed unknowns have been diluted 1:200 (1:20 external predilution, 1:10 dilution on the plate: 10 µl unknown + 90 µl PBS (1x)), the concentration read from the calibration curve must be multiplied by the dilution factor (x 200).**

- **Calculation of unknowns with a concentration exceeding calibrator 1 may result in incorrect, low human Cu/ZnSOD levels (Hook Effect). Such unknowns require further external predilution according to expected human Cu/ZnSOD values with PBS (1x) in order to precisely determine the actual human Cu/ZnSOD level.**

- It is suggested that each testing facility establishes a control of known human Cu/ZnSOD concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

11. Limitations

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every run.

- Bacterial or fungal contamination of either screen unknowns 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 may result in inaccurate results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

- Human anti-mouse IgG antibodies (HAMA) may interfere with assays utilizing murine monoclonal antibodies leading to inaccurate results. Serum unknowns containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the unknown.

12. Performance Characteristics

12.1 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human Cu/ZnSOD into 2 normal pooled serum unknowns. 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%.

12.2 Dilution Parallelism

4 serums with different levels of human Cu/ZnSOD were analyzed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 80% to 107% with an overall recovery of 90% (see Table 2).

Table 2

Unknown	Dilution	Expected Human Cu/ZnSOD Concentration (ng/ml)	Observed Human Cu/ZnSOD Concentration (ng/ml)	Recovery of Expected Human Cu/ZnSOD Concentration (%)
1	1:200	--	216.3	--
	1:400	108.2	96.1	89
	1:800	54.1	42.7	79
	1:1600	27.1	24.0	89
2	1:200	--	123.8	--
	1:400	61.9	52.8	85
	1:800	30.9	30.1	97
	1:1600	15.5	14.2	92
3	1:200	--	146.1	--
	1:400	73.1	63.7	87
	1:800	36.5	29.2	80
	1:1600	18.3	19.6	107
4	1:200	--	53.0	--
	1:400	26.5	25.9	98
	1:800	13.3	11.1	83
	1:1600	6.6	6.5	98

12.3 Stability of Unknowns

12.3.1 Freeze-Thaw Stability

Aliquots of serum (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human Cu/ZnSOD levels determined. There was no significant loss of human Cu/ZnSOD immunoreactivity detected by freezing and thawing.

12.3.2 Storage Stability

Aliquots of serum (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human Cu/ZnSOD level determined after 24, 48 and 96 h. There was no significant loss of human Cu/ZnSOD immunoreactivity detected during storage under above conditions.

12.3.3 Comparison of Serum and Plasma

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

12.4 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.

13. Ordering Information

This kit is manufactured for Immuno-Biological Laboratories, Inc. (IBL-America). For ordering information, please contact:

Immuno-Biological Laboratories, Inc. (IBL-America)
8201 Central Ave NE, Suite P
Minneapolis, MN 55432
Toll Free: (888) 523-1246
Fax: (763) 780-2988
www.ibl-america.com
info@ibl-america.com