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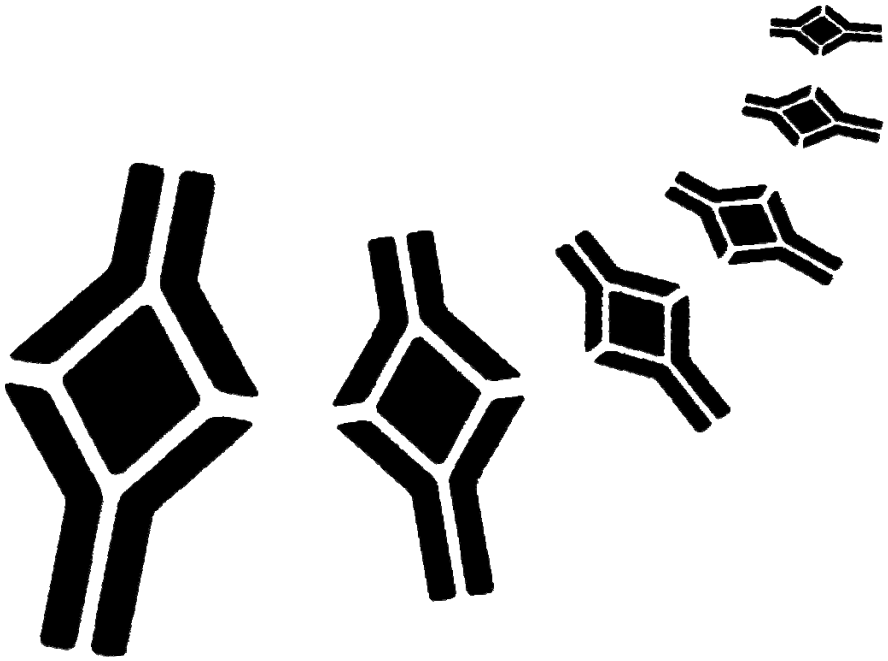

HUMAN CU/ZN SOD ELISA

Product Data Sheet

Cat. No.: RBMS222R

For Research Use Only

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» Use only the current version of Product Data Sheet enclosed with the kit!

1 INTENDED USE

The human Cu/ZnSOD ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Cu/ZnSOD. The human Cu/ZnSOD ELISA is for research use only. Not for diagnostic or therapeutic procedures.

2 SUMMARY

Superoxide Dismutases (SODs) (E.C.1.15.1.1.) 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)

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

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 (MnSOD) principally located in the matrix of mitochondria of all aerobes, copper/zinc (Cu/ZnSOD) mainly present in the cytoplasm of eukaryotic cells, iron (FeSOD), predominantly in the cytosol, chloropasts or mitochondria of prokaryotes as well as extracellular (ECSOD), 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/ZnSOD gene has been localized to chromosome 21q22.1.
Cu/ZnSOD 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/ZnSOD expression resulting in elevated levels of Cu/ZnSOD in human body fluids is of diagnostic value for measuring the activity of different diseases:

Nephropathies:
Cu/ZnSOD determination provides a tool for early diagnosis of nephropathies.

Monitoring of therapeutic treatments:
Cu/ZnSOD is a useful therapeutic tool in the treatment of chronic inflammation e.g. rheumatoid arthritis or of the ischemic myocardium in the phase of reperfusion. Due to the short half-life of SOD injected into the blood circulation, a rapid assay is necessary for monitoring SOD levels.

Trisomy 21 (Down's Syndrome):
In cases with Down's Syndrome an additional part of chromosome 21 is present in the genome of the patient as a structural chromosome aberration. The Cu/ZnSOD gene is localized on chromosome 21, closely associated with the gene complex responsible for the phenotype of Down's Syndrome. A gene-dosage effect for Cu/ZnSOD in Down's Syndrome providing a diagnostic marker for this syndrome has been described.

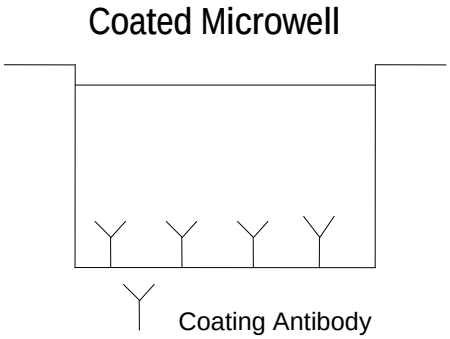
a) Patients with Down's Syndrome have significantly elevated serum and urine levels of Cu/ZnSOD.

b) Prenatal diagnosis of Down's Syndrome: Cu/ZnSOD levels are quantitated from erythrocytes of fetal umbilical vein blood and related to the number of cells, the content of haemoglobin and to the haematocrit. In case of Trisomy 21 the significantly elevated levels of Cu/ZnSOD are determined.

3 PRINCIPLES OF THE TEST

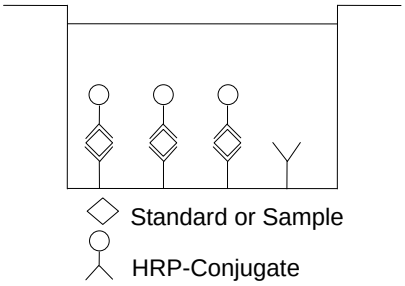
An anti-human Cu/ZnSOD coating antibody is adsorbed onto microwells.

Figure 1



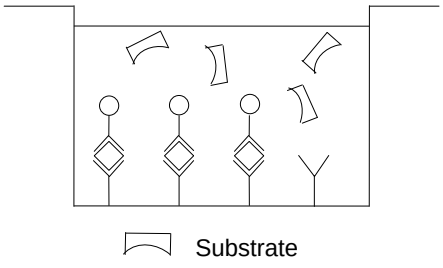
Human Cu/ZnSOD present in the sample or standard binds to antibodies adsorbed to the microwells. A HRP-conjugated anti-human Cu/ZnSOD antibody is added and binds to human Cu/ZnSOD captured by the first antibody.

Figure 2



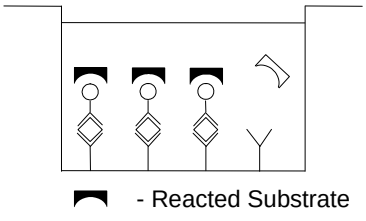
Following incubation unbound HRP-conjugated anti-human Cu/ZnSOD is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 3



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

Figure 4



4 REAGENTS PROVIDED

1 aluminium pouch with a **Antibody Coated Microtiter Strips** 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 **Standard**, 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**

5 STORAGE INSTRUCTIONS – ELISA KIT

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.

6 SPECIMEN COLLECTION AND STORAGE INSTRUCTIONS

Cell culture supernatant, serum, plasma (EDTA, citrate, heparin), amniotic fluid, urine and fetal umbilical vein blood were tested with this assay. Other biological samples 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.
Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use lipemic specimens. **Hemolyzed samples are not suitable for the assay.**
Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Cu/ZnSOD. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

7 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

8 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 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 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 specimens 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.

9 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.

9.1 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

9.2 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

9.3 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

9.4 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

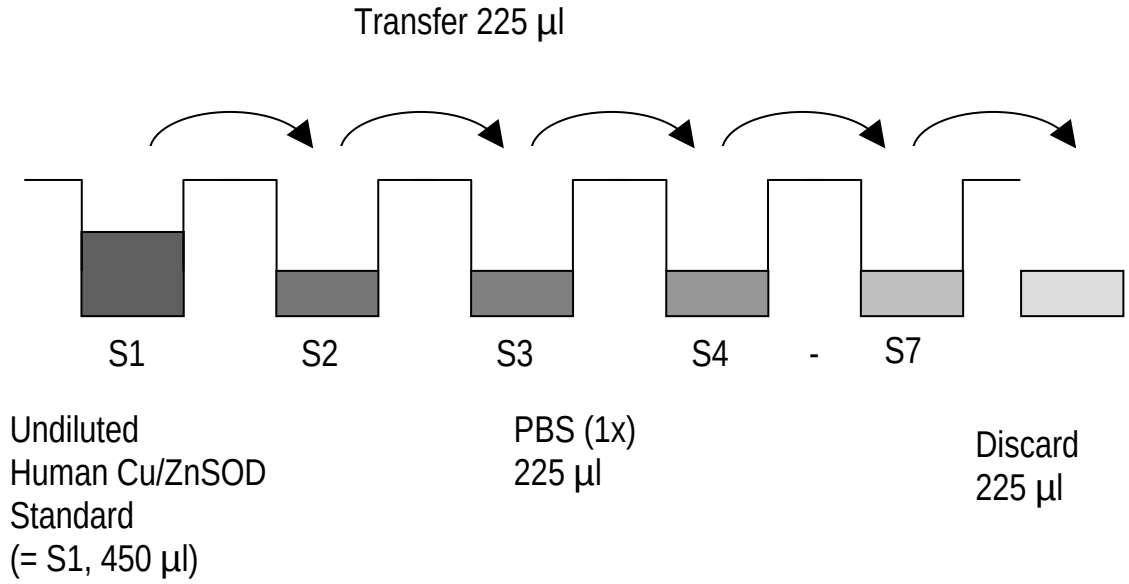
9.5 Human Cu/ZnSOD Standard

Standard dilutions can be prepared directly on the microwell plate (see 10.d) or alternatively in tubes (see 9.5.1).

9.5.1 External Standard Dilution

Label 7 tubes, one for each standard point.
S1, S2, S3, S4, S5, S6, S7
Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 µl of PBS (1x) into tubes S2 – S7.
Pipette 450 µl of undiluted standard (serves as the highest standard S1, concentration of standard 1= 5 ng/ml) into the first tube, labelled S1. Pipette 225 µl of this dilution into the second tube, labelled S2 (concentration of standard 2 = 2.5 ng/ml), and mix thoroughly before the next transfer. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 5).
PBS (1x) serves as blank.

Figure 5



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

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:

1. Diluent: Before standard and sample 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*

2. 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*

10 TEST PROTOCOL

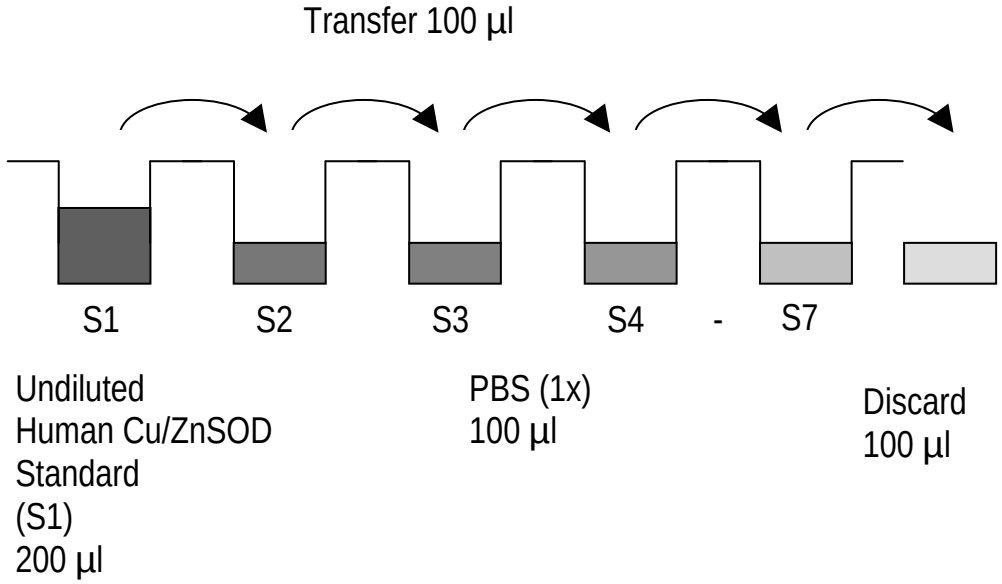
a. Predilute your samples before starting with the test procedure. Dilute serum and plasma samples 1:20 with PBS (1x) according to the following scheme: 10 µl sample + 190 µl PBS (1x) For fetal umbilical vein blood first adjust samples to $2x10^7$ erythrocytes/ml. (For sample preparation details please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

b. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample 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.

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 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. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry**. * Holzgreve W., P. Miny, and S. Tercanti. (1991). Prenatal inter-ventions for diagnosis and therapy in risk pregnancies. Diagnose Labor 41, 162-178. Porstmann T., R. Wietschke, G. Cobet, K. Lorenz, R. Grunow, S. Jahn, R. Bollmann, G. Stamminger, and R. von Baehr. (1990). Immunochemical quantification of Cu/Zn superoxide dismutase in prenatal diagnosis of Down's Syndrome. Hum. Genet. 85, 362-366. Porstmann T., R. Wietschke, G. Cobet, G. Stamminger, R. Bollmann, V. Rogalski, and P. Pas. (1991). Cu/Zn superoxide dismutase quantification from fetal erythrocytes - an efficient confirmatory test for Down's Syndrome after maternal serum screening and sonographic investigations. Prenat. Diagn. 11, 295-303.

d. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes - see 9.5.1): Add 100 µl of PBS (1x) in duplicate to **standard wells** B1/2- G1/2, leaving A1/A2 empty. Pipette 200 µl of undiluted **standard** (concentration = 5.00 ng/ml) in duplicate into well A1 and A2 (see Table 1). Transfer 100 µl to wells B1 and B2. Mix the contents of wells B1 and B2 by repeated aspiration and ejection, and transfer 100 µl to wells C1 and C2, respectively (see Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human Cu/ZnSOD standard dilutions ranging from 5.00 to 0.08 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 6



In case of an external standard dilution (see 9.5.1), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (5.00 ng/ml)	Standard 1 (5.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (2.50 ng/ml)	Standard 2 (2.50 ng/ml)	Sample 2	Sample 2
C	Standard 3 (1.25 ng/ml)	Standard 3 (1.25 ng/ml)	Sample 3	Sample 3
D	Standard 4 (0.63 ng/ml)	Standard 4 (0.63 ng/ml)	Sample 4	Sample 4
E	Standard 5 (0.31 ng/ml)	Standard 5 (0.31 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.16 ng/ml)	Standard 6 (0.16 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.08 ng/ml)	Standard 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 the sample wells.
g. Add 10 µl of each prediluted sample in duplicate to the sample wells.
h. Prepare HRP-Conjugate (see Preparation of HRP-Conjugate 9.4).
i. Add 50 µl of HRP-Conjugate to all wells.
j. 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.
k. Remove adhesive film and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
l. Pipette 100 µl of TMB Substrate Solution to all wells.
m. 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 standard 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 Standard 1 has reached an OD of 0.90 – 0.95.
n. 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.
o. Read absorbance of each microwell on a spectro-photometer 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 samples and the standards.

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

11 CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard 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 sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard 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 samples have been diluted 1:200 (1:20 external predilution, 1:10 dilution on the plate: 10 µl sample + 90 µl PBS (1x)), the concentration read from the standard curve must be multiplied by the dilution factor (x 200).
- Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Cu/ZnSOD levels. Such samples require further external predilution according to expected human Cu/ZnSOD values with PBS (1x) in order to precisely quantitate the actual human Cu/ZnSOD level.
- It is suggested that each testing facility establishes a control sample 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.
- A representative standard curve is shown in Figure 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7
Representative standard curve for human Cu/ZnSOD ELISA. Human Cu/ZnSOD was diluted in serial 2-fold steps in PBS (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

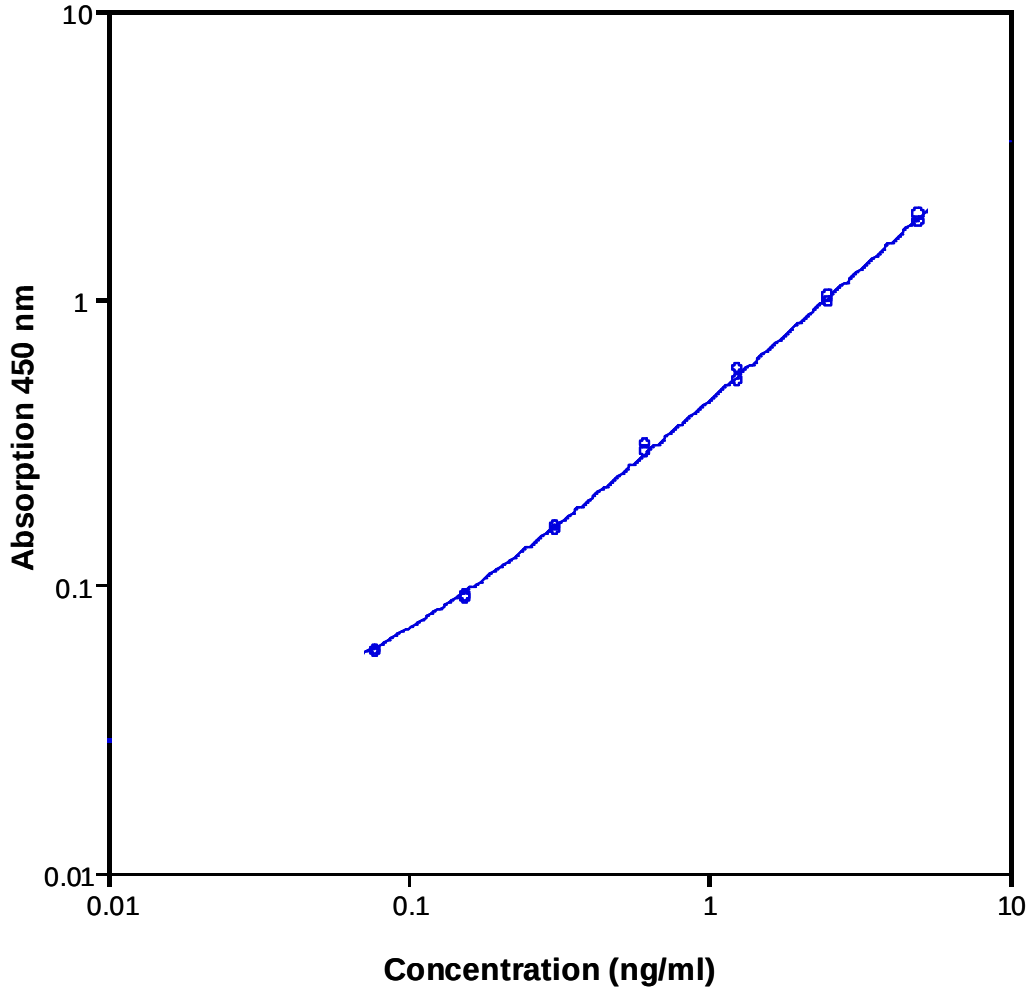


Table 2
Typical data using the human Cu/ZnSOD ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human Cu/ZnSOD Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	5.00	1.942 1.829	1.886	4.2
2	2.50	1.006 0.956	0.981	3.6
3	1.25	0.568 0.516	0.542	6.8
4	0.63	0.309 0.289	0.299	4.7
5	0.31	0.160 0.155	0.158	2.2
6	0.16	0.091 0.090	0.091	0.8
7	0.08	0.058 0.059	0.059	1.2
Blank	0	0.022 0.026	0.024	8.3

The OD values of the standard 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 colour intensity. Values measured are still valid.

12 LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen 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 solution, 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 antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples 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 sample.

13 PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

The limit of detection of human Cu/ZnSOD defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.04 ng/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human Cu/ZnSOD. 2 standard curves were run on each plate. Data below show the mean human Cu/ZnSOD concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.1%.

Table 3
The mean human Cu/ZnSOD concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human Cu/ZnSOD 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

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human Cu/ZnSOD. 2 standard curves were run on each plate. Data below show the mean human Cu/ZnSOD concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 5.8%.

Table 4
The mean human Cu/ZnSOD concentration and the coefficient of variation of each sample

Sample	Mean Human Cu/ZnSOD 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

13.3 Spiking Recovery

The spiking 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%.

13.4 Dilution Linearity

4 serum samples with different levels of human Cu/ZnSOD were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 80% to 107% with an overall recovery of 90% (see Table 5).
Table 5

Sample	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

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (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.

13.5.2 Storage Stability

Aliquots of serum samples (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.

13.6 Comparison of Serum and Plasma

From 22 individuals, 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.

13.7 Specificity

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

13.8 Expected Values

Panels of 40 serum as well as EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human Cu/ZnSOD. For detected human Cu/ZnSOD see Table 6. The levels measured may vary with the sample collection used. Measurement of human Cu/ZnSOD from erythrocytes of fetal umbilical vein blood ranged from 11.0 to 16.0 ng SOD/10^6 fetal erythrocytes for normals and > 20.0 ng SOD/10^6 fetal erythrocytes for fetuses with Down's Syndrome.

Table 6

Sample Matrix	Number of Samples Evaluated	Range (ng/ml)	% Detectable	Mean of Detectable (ng/ml)
Serum	40	nd *- 35.2	17.5	22.6
Plasma (EDTA)	40	nd *- 59.7	20	15.3
Plasma (Citrate)	40	nd *- 104.7	97.5	42.4
Plasma (Heparin)	40	nd *- 50.7	57.5	26.7

* n.d. = non-detectable, samples measured below the lowest standard point are considered to be non-detectable.

14 REFERENCES

1) Barra D., F. Martini, J. v. Bannister, M. E. Schinina, G. Rotilio, W. H. Bannister, and F. Bossa. (1980). The complete amino acid sequence of human Cu/Zn superoxide dismutase. FEBS Letters 120, 53-56.
2) Goebel K. M., and U. Storck. (1983). Effect of intra-articular orgotein versus a corticosteroid on rheumatoid arthritis of knees. Am. J. Med. 74, 124-128.
3) Hartz J. W., and H. F. Deutsch. (1972). Subunit structure of human superoxide dismutase. Biol. Chem. 247, 7043-7050.
4) Herouart D., M. Van-Montagu, and D. Inze. (1993). Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in Nicotiana. PNAS 90, 3108-3112.
5) Holzgreve W., P. Miny, and S. Tercanti. (1991). Prenatal inter-ventions for diagnosis and therapy in risk pregnancies. Diagnose Labor 41, 162-178.
6) Jolly S. R., W. S. Kane, M. B. Bailie, G. D. Abrams, and B. R. Lucchesi. (1984). Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. Circ. Res. 54, 277-285.
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.
9) Porstmann T., R. Wietschke, H. Schmechta, R. Grunow, B. Porstmann, R. Bleiber, M. Pergande, S. Stachat, and R. von Baehr. (1988). A rapid and sensitive enzyme immunoassay for Cu/Zn superoxide dismutase with polyclonal and monoclonal antibodies. Clin. Chim. Acta 171, 1-10.
10) Porstmann T., R. Wietschke, G. Cobet, K. Lorenz, R. Grunow, S. Jahn, R. Bollmann, G. Stamminger, and R. von Baehr. (1990). Immunochemical quantification of Cu/Zn superoxide dismutase in prenatal diagnosis of Down's Syndrome. Hum. Genet. 85, 362-366.
11) Porstmann T., R. Wietschke, G. Cobet, G. Stamminger, R. Bollmann, V. Rogalski, and P. Pas. (1991). Cu/Zn superoxide dismutase quantification from fetal erythrocytes - an efficient confirmatory test for Down's Syndrome after maternal serum screening and sonographic investigations. Prenat. Diagn. 11, 295-303.
12) Shull S., N. H. Heintz, M. Periasamy, M. Manohar, J. M. Janssen, J. P. Marsh, and B. T. Mossman. (1991). Differential regulation of antioxidant enzymes in response to oxidants. J. Biol. Chem. 266, 24398-24403.

13) Sinet P-M., J. Couturier, B. Dutrillaux, M. Poissonnier, O. Raoul, M-O. Rethove, D. Allard, J. Lejeune, and H. Jerome. (1976). Trisomie 21 et superoxyde dismutase-1 (IPO-A). Exp. Cell Res. 97, 47-55.
14) Suzuki H., A. Matsumori, Y. Matoba, B. S. Kyu, A. Tanaka, J. Fujita, and S. Sasayama. (1993). Enhanced expression of superoxide dismutase messenger RNA in viral myocarditis. J. Clin. Invest. 91, 2727-2733.
15) Walker J. E., A. D. Auffret, C. J. Brock, and H. M. Steinman. (1980). The significance of superoxide and superoxide dismutase: Chemical and biochemical aspects. Bannister J. V., and H. A. O. Hill eds. (212-222CE), Elsevier/North-Holland, Amsterdam, New York.

15 REAGENT PREPARATION SUMMARY

15.1 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

15.2 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

15.3 Phosphate Buffered Saline (PBS) (1x)

Add **PBS Concentrate** 20x (5 ml) to 95 ml distilled water.

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

15.4 HRP-Conjugate

Make a 1:5 predilution of the **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

16 TEST PROTOCOL SUMMARY

1. Predilute serum or plasma samples with PBS (1x) 1:20.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl PBS (1x), in duplicate, to all standard wells leaving the first wells empty. Pipette 200 µl standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
   Alternatively external standard dilution in tubes (see 9.5.1): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µl PBS (1x), in duplicate, to the blank wells.
6. Add 90 µl PBS (1x) to sample wells.
7. Add 10 µl prediluted sample in duplicate, to designated sample wells.
8. Prepare HRP-Conjugate.
9. Add 50 µl HRP-Conjugate to all wells.
10. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl of TMB Substrate Solution to all wells.
13. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
14. Add 100 µl Stop Solution to all wells.
15. Blank microwell reader and measure colour intensity at 450 nm.

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

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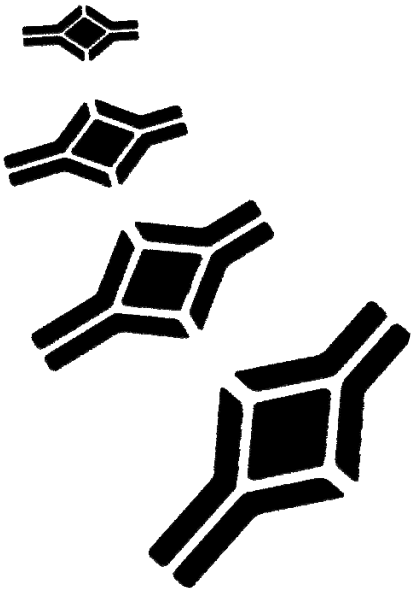

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