

READ BEFORE OPENING

- Vials may contain small quantities of material, hence ensure that they are centrifuged prior to opening.
- This set of reagents is intended for use by persons experienced in the use of immunoassays. It is not suitable for use by inexperienced personnel.
- A sample protocol is included but please note that the protocol provided is a guideline. The type of substrate as well as all other reagents not included in the module set may influence test performance.

WORKING PROTOCOL FOR THE Cu/ZnSOD MODULE SET BMS222MST

1) Reagents provided (for 10 ELISA plates):

- 2.75 ml coating antibody (100 µg/ ml)
- 60 µl Cu/ZnSOD standard protein (400 ng/ ml)
- 55 µl HRP-conjugate

2) Buffers and further materials needed:

a) Phosphate buffered saline (PBS)

NaCl	8.00 g
KCl	0.20 g
Na ₂ HPO ₄ x 12 H ₂ O	2.85 g
KH ₂ PO ₄	0.20 g

Dissolve the salts in distilled water and adjust to 1 litre.

b) Assay Buffer:

Bovine Serum Albumin (BSA)	5 g
Tween 20	0.5 ml
PBS	adjust to 1 litre.

Dissolve ingredients in approx. 500 ml PBS, then adjust to 1 litre with PBS.

- c) Wash Buffer:
Add 0.5 ml Tween 20 to 1 litre of PBS and mix well.
- d) Microwell plate (Maxi sorb)
- e) *Substrate Solution: 1:2 mixture of H₂O₂ and Tetramethylbenzidine (KPL Gaithersburg, Maryland)*
- g) Stop Solution: 4N Sulfuric Acid (2 ml conc. (36N) Sulfuric Acid + 16 ml H₂O).

3) Storage condition:

Store the reagents of the module set at -20°C. Immediately after use reagents should be returned to -20°C storage. Avoid several freeze-thaw cycles. Aliquot reagents for use at different time points. Expiry of the reagents is stated on labels.

4) Preparation of reagents:

Please note: Centrifuge vials before opening to collect contents.

a) Preparation of the microwell Plate:

Coating:

The final antibody concentration is 2.5 µg/ ml; 100 µl of the coating solution are added to each well. Dilute the coating antibody as following for one microtiter plate:

$$\frac{10.725 \text{ ml PBS} + 275.0 \text{ } \mu\text{l coating antibody (100 } \mu\text{g/ ml)}}{11.0 \text{ ml coating solution}}$$

Immediately after coating, seal the plate with a plate cover and transfer to 2-8°C, allowing the binding process to take place over night.

Aspirate the contents of the wells and wash once with about 300µl of Wash Buffer according the Washing procedure described in the test protocol below.

Blocking:

Add 250 µl of Assay Buffer to each well and allow the binding reaction to take place for two hours at room temperature (alternatively the plate may be blocked over night at 2-8°C).

Wash the plate twice (see below) immediately before the samples are added to the wells. The blocked plates can be stored at 2-8°C up to one week.

Fixing:

If you want to store the coated plates for a longer period of time, just aspirate the blocking solution and proceed by adding 150 µl Fixing solution (PBS, 15% Sucrose) to each well. Incubate 1h at room temperature, aspirate and dry plates over night at 28°C. When

sealed with desiccant, the plates can be stored at 2-8 °C for at least 2 months.

b) Preparation of Standard:

The final concentration of the Cu/ZnSOD standard protein is 5 ng/ ml. Dilute the stock material as following for one standard curve:

6 µl	Standard Protein (400 ng/ ml)
474 µl	PBS
<hr/>	
480 µl	Standard Protein (5 ng/ ml)

c) Preparation of HRP-Conjugate:

The HRP-Conjugate must be diluted 1:1000 with PBS before use. Dilute the stock material as following for one microwell plate:

5.5 µl	HRP-Conjugate
5494.5 µl	PBS
<hr/>	
5500.0 µl	HRP-Conjugate

The reagents are now ready to be used in the test according to the test protocol below.

TEST PROTOCOL

- a. Predilute serum or plasma samples 1:20 with **PBS** according to the following dilution scheme:

10 μ l **Sample** + 190 μ l **PBS**

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

- b. Add 100 μ l of **PBS**, in duplicate, to the standard wells, leaving the first wells (5 ng/ ml) empty. Prepare standard dilutions by pipetting 200 μ l of **Cu/ZnSOD Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Transfer 100 μ l to wells B1 and B2 respectively. Mix the contents by repeated aspiration and ejection and transfer 100 μ l 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/ZnSOD standard dilutions ranging from 5 to 0.08 ng/ ml. Discard 100 μ l of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of Cu/ZnSOD standard dilutions:

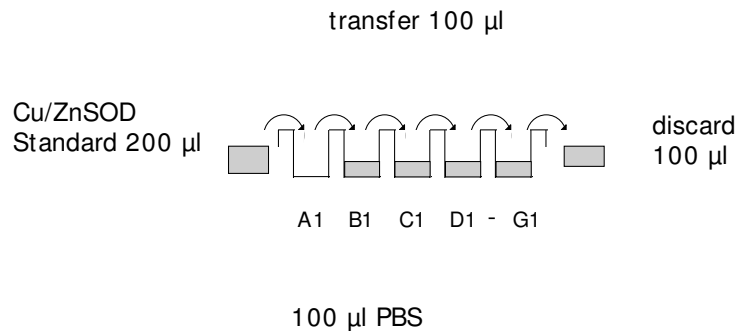


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (5 ng/ ml)	Standard 1 (5 ng/ ml)	Sample 1	Sample 1
B	Standard 2 (2.5 ng/ ml)	Standard 2 (2.5 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 (0.63ng/ ml)	Standard 4 (0.63ng/ ml)	Sample 4	Sample 4
E	Standard 5 (0.32ng/ ml)	Standard 5 (0.32ng/ 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

- c. Add 100 µl of **PBS**, in duplicate, to the blank wells.
- d. Add 90 µl of **PBS** to all wells designated for samples.
- e. Add 10 µl of each prediluted **Sample**, in duplicate, to the designated wells.
- f. Prepare HRP-Conjugate. (Refer to preparation of reagents)
- g. Add 50 µl of diluted **HRP-Conjugate** to all wells.
- h. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 1 hour, if available on a rotator set at 100 rpm.
- i. Prepare TMB Substrate Solution a few minutes prior to use.

- j. Remove Plate Cover and empty wells. Wash the microwell strips 3 times with approximately 400 μ l **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, 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.**

- k. Pipette 100 μ l of mixed **TMB Substrate Solution** to all wells, including the blank wells.

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

The colour development on the plate should be monitored and the substrate reaction stopped (see point m. of this protocol) before positive wells are no longer properly recordable.

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 620nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

- m. Stop the enzyme reaction by quickly pipetting 100 μ l 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 2 - 8°C in the dark.

- n. 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 Cu/ZnSOD standards.

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.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the Cu/ZnSOD concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating 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 Cu/ZnSOD concentration.
- **For samples which have been 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 (x 200).**

Note: Calculation of samples with an O.D. exceeding standard 1 may result in incorrect low Cu/ZnSOD levels. Such samples require further dilution with PBS in order to precisely quantitate the actual Cu/ZnSOD level.

- It is suggested that each testing facility establishes a control sample of known 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.

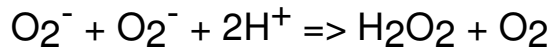
A basic understanding of immunoassay development and technical experience in ELISA performance are prerequisite for the successful use of this module set.

The protocol provided is just a guideline. The type of substrate as well as all other reagents not included in the module set may influence the test characteristics.

GENERAL INFORMATION

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)



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, chloroplasts 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 (15). The human Cu/Zn superoxide dismutase is a dimeric protein (3) 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 (13).

Cu/ZnSOD gene expression is induced by mediators of oxidative stress like sulfhydryl antioxidants (4, 12, 14), interleukin-1, tumor necrosis factor (7). 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 (8).

- monitoring of therapeutic treatments:

Cu/ZnSOD is a useful therapeutic tool in the treatment of chronic inflammation e.g. rheumatoid arthritis (2) or of the ischemic myocardium in the phase of reperfusion (6). 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 (13).

a) Patients with Down's Syndrome have significantly elevated serum and urine levels of Cu/ZnSOD (9).

b) Prenatal diagnosis of Down's Syndrome (10): 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 (5, 11).

Bibliography

- 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 interventions 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. (212-222), Elsevier/North-Holland, Amsterdam, New York.

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.

ORDERING INFORMATION

+ Europe

Bender MedSystems GmbH
Campus Vienna Biocenter 2
A-1030 Vienna, Austria, Europe
phone: +43 1 796 40 40 ext. 114
fax: +43 1 796 40 40 ext. 400

order@bendermedsystems.com

technical information:

techserv@bendermedsystems.com

+ USA

eBioscience, Inc.
10255 Science Center Drive
San Diego, CA 92121
phone: +1(888) 999 1371
fax: +1(858) 642 2046

contact@ebioscience.com

technical information:

tech@ebioscience.com

www.bendermedsystems.com

www.ebioscience.com