Bender MedSystems

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 sICAM-3 MODULE SET BMS218MST

1) Reagents provided (for 10 ELISA plates):

10 ml coating antibody (100 µg/ml)
100 µl sICAM-3 standard protein (2.8 µg/ml)
20 µl HRP-conjugate
100 ml Sample Diluent

2) Buffers and further materials needed:

a) Phosphate buffered saline (PBS)

NaCl	8.00 g
KCl	0.20 g
$Na_2HPO_4 \times 12\ H_2O$	2.85 g
KH_2PO_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 H_2O_2 and Tetramethylbenzidine (KPL Gaithersburg,Maryland)

f) Stop Solution: 4N Sulfuric Acid (2 ml conc. (36N) Sulfuric Acid + 16 ml H_2O).

3) Storage condition:

Store the reagents of the module set at -20ºC. Immediately after use reagents should be returned to -20ºC 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 10 µg/ml; 100 µl of the coating solution are added to each well. Dilute the coating antibody as following for one microtiter plate:

9	ml	PBS
1	ml	coating antibody (100 µg/ml)
10	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 300 µl 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 under vacuum. 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 sICAM-3 standard protein is 50 ng/ ml. Dilute the stock material as following for one standard curve:

8.9	µl	Standard Protein (2.8 µg/ml)
491	µl	Assay Buffer
500	µl	Standard Protein (50 ng/ml)

c) Preparation of HRP-Conjugate:
The HRP-Conjugate must be diluted 1:3200 with Assay Buffer before use. Dilute the stock material as following for one microwell plate:

1.6	µl	HRP-Conjugate
4998.4	µl	Assay Buffer
5000.0	µl	HRP-Conjugate

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

TEST PROTOCOL

a. Add 100 µl of **Sample Diluent**, in duplicate, to the standard wells, leaving the first wells (50 ng/ml) empty. Prepare standard dilutions by pipetting 200 µl of **sICAM-3 Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Transfer 100 µl to well B1 and B2 respectively. Take care not to scratch the inner surface of the microwells. Mix the contents of well B1 and B2 and transfer 100 µl to well C1 and C2 respectively. Continue this procedure four times, creating two rows of sICAM-3 standard dilutions ranging from 50 to 0.78 ng/ ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of sICAM-3 standard dilutions:

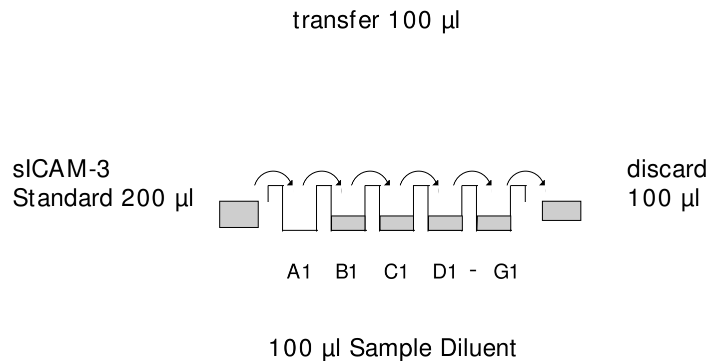

Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (50 ng/ml)	Standard 1 (50 ng/ml)	Sample 1	Sample 1
B	Standard 2 (25 ng/ml)	Standard 2 (25 ng/ml)	Sample 2	Sample 2
C	Standard 3 (12.5 ng/ml)	Standard 3 (12.5 ng/ml)	Sample 3	Sample 3
D	Standard 4 (6.25 ng/ml)	Standard 4 (6.25 ng/ml)	Sample 4	Sample 4
E	Standard 5 (3.13 ng/ml)	Standard 5 (3.13 ng/ml)	Sample 5	Sample 5
F	Standard 6 (1.6 ng/ml)	Standard6 (1.6 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.78 ng/ml)	Standard 7 (0.78 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

b. Add 100 µl of **Sample Diluent**, in duplicate, to the blank wells.

c. Add 80 µl **Sample Diluent** to all wells designated for samples.

d. Add 20 µl of each **Sample**, in duplicate, to the designated wells and mix the contents.

e. Prepare **HRP-Conjugate** (Refer to preparation of reagents).

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

g. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, if available on a rotator set at 100 rpm.

h. Prepare **TMB Substrate Solution** a few minutes prior to use.

i. Remove **Plate Cover** and empty wells. Wash the microwell strips 3 times with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

   After the last wash, 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.

j. Pipette 100 µl of mixed **TMB Substrate Solution** to all wells, including the blank wells.

k. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 to 20 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light. The point at which the substrate reaction is stopped is often determined by the ELISA reader being used. Many ELISA readers record absorbance only up to 2.0 O.D. **The O.D. values at the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly recordable.**

l. Stop the enzyme reaction by quickly pipetting 100 µl of **4N Sulfuric Acid** into each well, including the blank wells. It is important that the Sulfuric Acid is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Sulfuric Acid is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

m. 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 sICAM-3 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 sICAM-3 concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating sICAM-3 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 sICAM-3 concentration.

- **For samples which have been diluted according to the instructions given in this manual 1 : 5 the concentration read from the standard curve must be multiplied by the dilution factor (x 5).**

  Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low sICAM-3 levels. Such samples require further dilution of 1:10 - 1:20 with Sample Diluent in order to precisely quantitate the actual sICAM-3 level.

- It is suggested that each testing facility establishes a control sample of known sICAM-3 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

Intercellular Adhesion Molecule-3 (ICAM-3) is a member of the immunoglobulin supergene family (4) and functions as a ligand for the Lymphocyte Function-Associated Antigen-1 (LFA-1). Three counter-receptors have been described for LFA-1, intercellular adhesion molecule 1 (ICAM-1), ICAM-2 and ICAM-3 (3, 4, 10, 11, 12). LFA-1, an alpha-beta complex, is a member of the leukocyte integrin family (9) which mediate lymphocyte adhesion.

ICAM-3 is a heavily glycosylated protein of 124 kDa with a polypeptide core of 57 kDa (5, 13). The integral membrane protein with five immunoglobulin-like domains shares high homology to ICAM-1 and ICAM-2 in the extracellular region. In contrast to ICAM-1 and ICAM-2, ICAM-3 is absent on endothelia. ICAM-3 is expressed on resting lymphocytes, monocytes and neutrophils, representing the major LFA-1 ligand on these cells (4, 7). The finding that adhesion of resting T lymphocytes to LFA-1 occurs primarily via ICAM-3 combined with the fact that ICAM-3 is much better expressed than other LFA-1 ligands on monocytes and resting lymphocytes implies an important role for ICAM-3 in the initiation of immune responses (8).
ICAM-3 was found to be involved in the regulation of LFA-1/
ICAM-1 dependent leukocyte intercellular interactions. The initial interaction of ICAM-3 with LFA-1 might increase LFA-1-mediated cell binding to ICAM-1 (2).
Furthermore, ICAM-3 expression has been shown for dendritic epidermal Langerhans cells, whereas it is absent on other dendritic cells from different lymphoid organs. Thus potential function of ICAM-3 at the initiation phase of LC-leukocyte interactions taking place during **skin localized immune reactions** can be postulated (1).
Recent data suggest that ICAM-3 expression can be induced on endothelial cells in lymphoid neoplasms as shown for **Hodgkin's** and **non-Hodgkin's disease** (6).
ICAM-3 is a very interesting molecule involved in the initial immune response thus suggesting an important role as a disease marker for a number of different indications and pathological situations.

Bibliography

1) Acevedo A., M. A. del Pozo, A. G. Arroyo, P. Sanchez-Mateos, R. Gonzalez-Amaro, and F. Sanchez-Madrid. (1993). Distribution of ICAM-3-bearing cells in normal human tissues - expression of a novel counter-receptor for LFA-1 in epidermal Langerhans cells.
Am. J. Pathol. 143, 774-783.

2) Campanero M. R., M. A. del Pozo, A. G. Arroyo, P. Sanchez-Mateos, T. Hernandez-Caselles, A. Craig, R. Pulido, and F. Sanchez-Madrid. (1993). ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathway.
J. Cell Biol. 123, 1007-1016.

3) De Fougerolles A. R., S. A. Stacker, R. Schwarting, and T. A. Springer. (1991). Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1.
J. Exp. Med. 174, 253- 267.

4) De Fougerolles A. R., and T. A. Springer. (1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes.
J. Exp. Med. 175, 185-190.

5) De Fougerolles A. R., L. B. Klickstein, and T. A. Springer. (1993). Cloning and expression of intercellular adhesion molecule 3 reveals strong homology to other immunoglobulin family counter-receptors for lymphocyte function-associated antigen 1.
J. Exp. Med. 177, 1187-1192.

6) Doussis-Anagnostopoulou I., L. Kaklamanis, J. Cordell, M. Jones, H. Turley, K. Pulford, D. Simmons, D. Mason, and K. Gatter. (1993). ICAM-3 expression on endothelium in lymphoid malignancy.
Am. J. Pathol. 143, 1040-1043.

7) Fawcett J., C. L. L. Holness, L. A. Needham, H. Turley, K. C. Gatter, D. Y. Mason, and D. L. Simmons. (1992). Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes.
Nature 360, 481-484.

8) Hernandez-Caselles F., G. Rubio, M. R. Campanero, M. A. Delpozo, M. Muro, F. Sanchez-Madrid, and P. Aparicio. (1993). ICAM-3, the third LFA-1 counterreceptor is a co-stimulatory molecule for both resting and activated T-lymphocytes.
Eur. J. Immunol. 23, 2799-2806.

9) Marlin S. D., and T. A. Springer. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1).
Cell 51, 813-819.

10) Nortamo P., R. Salcedo, T. Timonen, M. Patarroyo, and C. G. Gahmberg. (1991). A monoclonal antibody to the human leukocyte adhesion molecule intercellular adhesion molecule-2: cellular distribution and molecular characterization of the antigen.
J. Immunol. 146, 2530-2535.

11) Rothlein R., M. L. Dustin, S. D. Marlin, and T. A. Springer. (1986). A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1.
J. Immunol. 137, 1270-1274.

12) Springer T. A. (1990). Adhesion receptors of the immune system. Nature 346, 425-434.

13) Vazeux R. P. A. Hoffman, J. K. Tomita, E. S. Dickinson, R. L. Jasman, T. S. John, and W. M. Gallatin. (1992). Cloning and characterization of a new intercellular adhesion molecule ICAM-R. Nature 360, 485-488.

Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a sICAM-3 positive serum. There was no detectable cross reactivity with any of the tested proteins, notably there was no interference with sICAM-1.

ORDERING INFORMATION

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