PRODUCT INFORMATION AND MANUAL

human sICAM-3 Instant ELISA

BMS218INST

Enzyme-linked immunosorbent assay for quantitative

detection of human sICAM-3.

For research use only.

Not for diagnostic or therapeutic procedures.

128 Tests

human sICAM-3
BMS218INST

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1 Intended Use

The human sICAM-3 Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of soluble Intercellular Adhesion Molecule-3 in cell culture supernatants, human serum, plasma, urine, amniotic fluid, bile, or other body fluids. The human sICAM-3 Instant ELISA is **for research use only. Not for diagnostic or therapeutic procedures.**

2 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.

3 Principles of the Test

An anti-human sICAM-3 monoclonal coating antibody is adsorbed onto microwells. Human sICAM-3 present in the sample or standard binds to antibodies adsorbed to the microwells; an HRP-conjugated monoclonal anti-human sICAM-3 antibody binds to human sICAM-3 captured by the first antibody.
Following incubation unbound enzyme conjugated anti-human sICAM-3 is removed during a wash step and substrate solution reactive with HRP is added to the wells.

Figure 1

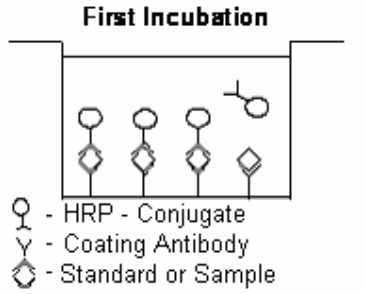
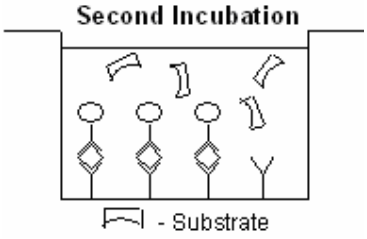
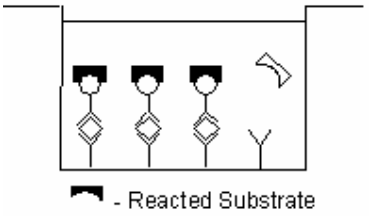


Figure 2



A coloured product is formed in proportion to the amount of human sICAM-3 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven human sICAM-3 standard dilutions and human sICAM-3 sample concentration determined.

Figure 3



4 Reagents Provided

1 aluminium pouch with **a Microwell Plate coated with Monoclonal Antibody** (murine) to human sICAM-3, **Sample Diluent** and **HRP-Conjugate** (anti-sICAM-3 monoclonal (murine) antibody), lyophilized

2 aluminium pouches with a **human sICAM-3 Standard curve (coloured)**

1 bottle (25 ml) **Wash Buffer Concentrate** 20x (phosphate-buffered saline with 1% Tween 20)

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

1 vial (12 ml) **Sample Diluent** (Use when an external predilution of the samples is needed)

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

2 adhesive **Plate Covers**

5 Storage Instructions

Store ELISA plate and Standard curves or whole kit at -20°C. The plate and the standard curves can also be removed, stored at -20°C, remaining kit reagents can be stored between 2° and 8°C. Expiry of the kit and reagents is stated on labels.
The 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, the reagent is not contaminated by the first handling.

6 Specimen Collection

Cell culture supernatants, human serum, plasma, urine, amniotic fluid, bile, or other body fluids are suitable for use in the assay. Remove the serum or plasma from the clot or red 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 grossly hemolyzed or lipemic specimens.

Samples must be stored frozen at -20°C to avoid loss of bioactive human sICAM-3. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13).
Avoid repeated freeze-thaw cycles. Prior to assay, frozen serum or plasma 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 linear 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 statements(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 substrate reagent.

- 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 and Samples

9.1 Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (25 ml) of the Wash Buffer Concentrate into a clean 500 ml graduated cylinder. Bring to final volume to 500 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 is stable for 30 days.

10 Test Protocol

- **Use plate immediately after removal from -20°C!**
- **Do not wait until pellets have completely dissolved before applying samples - the binding reaction in the standard strips starts immediately after addition of water!**
- **Do not try to dissolve pellets by pipetting up and down in the wells - some parts of the pellet could stick to the tip creating high variation of results**
- **Perform the washing step with at least 400 µl of washing buffer as stated in the manual or fill the wells completely - otherwise any pellet residues sticking to the rim of the well will not be removed and create high variation of results**
- **Allow the washing buffer to sit in the wells for a few seconds before aspiration**
- **Remove covers of the standard strips carefully in order that all the lyophilised pellets remain in the wells**

a. Determine the number of Microwell Strips required to test the desired number of samples plus Microwell Strips for blanks and standards (coloured). 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 -20°C sealed tightly. Place microwell strips containing the standard curve in position A1/A2 to H1/H2 (see Table 1).

b. Add 130 µl of distilled water to the sample wells.

c. Add distilled water to all standard and blank wells as indicated on the label of the standard strips (A1, A2 to H1, H2).

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 (50.00 ng/ml)	Standard 1 (50.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (25.00 ng/ml)	Standard 2 (25.00 ng/ml)	Sample 2	Sample 2
C	Standard 3 (12.50 ng/ml)	Standard 3 (12.50 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 (1.56 ng/ml)	Standard 6 (1.56 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

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

e. Cover with a **Plate Cover** and incubate at room temperature (18ºC to 25ºC) for 3 hours, if available on a microplate shaker at 100 rpm.

f. Remove **Plate Cover** and empty wells. Wash the microwell strips 3 times with approximately 400 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 no longer than 15 minutes. Do not allow wells to dry.

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

h. 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 i. 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 0.6 – 0.65.

i. 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 2 - 8°C in the dark.

j. 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 human sICAM-3 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.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human sICAM-3 concentration on the abscissa. Draw a best fit curve through the points of the graph.

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

- ***Samples have been diluted 1:5, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 5).**

- It is suggested that each testing facility establishes a control sample of known human 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 representative standard curve is shown in Figure 4. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

* N.B: There is a common dilution factor for samples due to the conjugate which must then be included in the calculation. The samples contribute 100 µl to the final volume per well. These 100 µl are composed of 80 µl of sample diluent plus 20 µl of the sample. This is a 1:5 dilution.
The remaining 50 µl to give 150 µl are due to the addition of 50 µl conjugate to all wells.
80 µl sample diluent and 50 µl conjugate results in 130 µl reconstitution volume, addition of 20 µl sample (80 µl + 20 µl = 1:5 dilution)

Figure 4
Representative standard curve for human sICAM-3 Instant ELISA. Human sICAM-3 was diluted in serial 2-fold steps in Sample Diluent, each symbol represents the mean of 3 parallel titrations.
Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

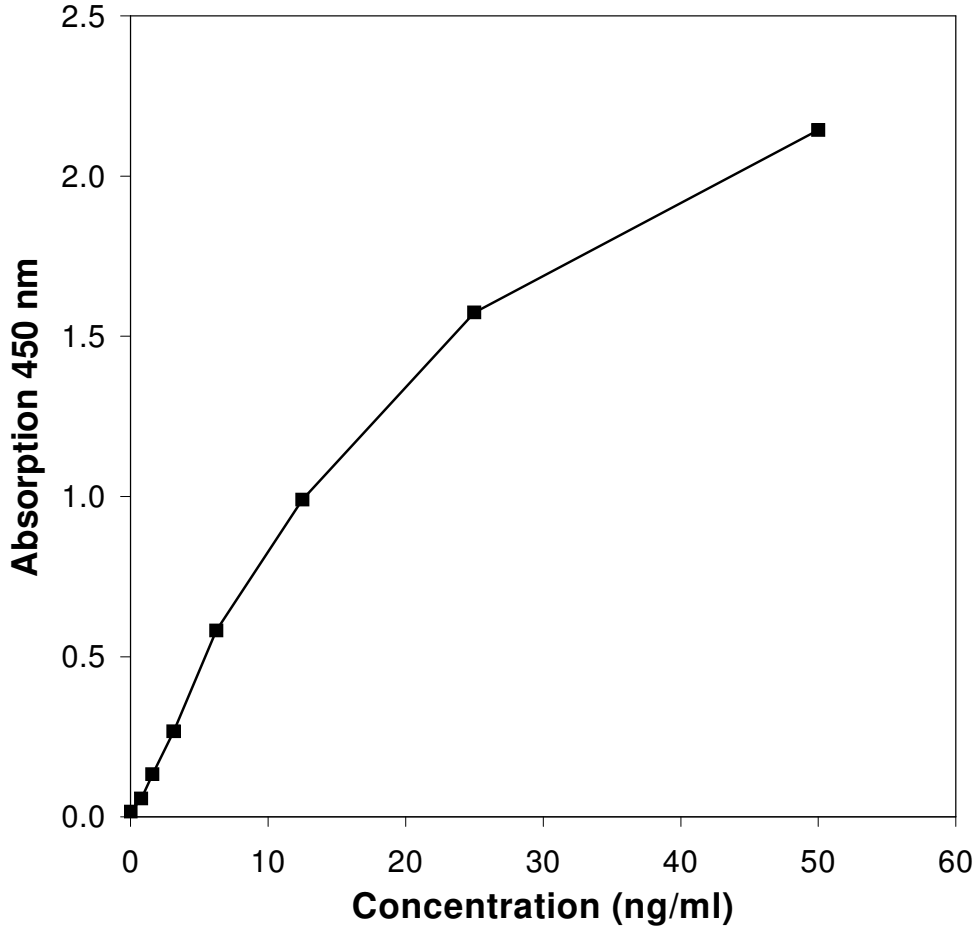


Table 2
Typical data using the human sICAM-3 INSTANT ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	human sICAM-3 Concentration (ng/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	50.00	2.188	2.144	2.9
	50.00	2.099		
2	25.00	1.552	1.574	2.0
	25.00	1.596		
3	12.50	0.973	0.990	2.4
	12.50	1.006		
4	6.25	0.619	0.581	9.2
	6.25	0.543		
5	3.13	0.284	0.267	9.0
	3.13	0.250		
6	1.56	0.135	0.133	2.1
	1.56	0.131		
7	0.78	0.056	0.057	2.5
	0.78	0.058		
Blank	0	0.015	0.016	
	0	0.017		

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 sICAM-3 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 0.38 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 6 serum samples containing different concentrations of human sICAM-3. 2 standard curves were run on each plate. Data below show the mean human sICAM-3 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.9%.

Table 3

The Mean human sICAM-3 concentration and the coefficient of variation for each sample.

Positive Sample	Experiment	human sICAM-3 Concentration (ng/ml)	Coefficient of Variation (%)
1	1	38	7.1
	2	41	5.4
	3	37	6.2
2	1	39	11.8
	2	41	2.8
	3	43	1.7
3	1	67	11.4
	2	62	5.9
	3	59	0.8
4	1	41	17.6
	2	47	3.5
	3	42	5.2
5	1	53	2.6
	2	52	2.5
	3	52	2.8
6	1	49	12.6
	2	51	2.8
	3	51	3.4

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments by 3 technicians. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of human sICAM-3. 2 standard curves were run on each plate. Data below (see Table 4) show the mean human sICAM-3 concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall coefficient of variation was 4.5%.

Table 4
The mean human sICAM-3 concentration and the coefficient of variation calculated on 18 determinations of each sample.

Sample	human sICAM-3 Concentration (ng/ml)	Coefficient of Variation (%)
1	39	5.5
2	42	4.5
3	63	6.7
4	44	7.9
5	53	0.4
6	51	1.9

13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human sICAM-3 into human serum. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments. The overall mean recovery has been determined to be 65%.

13.4 Dilution Parallelism

4 serum samples with different levels of human sICAM-3 were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 75% to 105% with an overall mean recovery of 85%.

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed 5 times, and the human sICAM-3 levels determined. There was no significant loss of human sICAM-3 immunoreactivity between 0 and 5 freeze-thaw cycles.

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 sICAM-3 level determined after 24, 48 and 96 h. There was no significant loss of human sICAM-3 immunoreactivity during storage under above conditions.

13.6 Comparison of Serum and Plasma

From 3 individuals, serum as well as EDTA, citrate and heparin plasma obtained at the same time point were evaluated. Human sICAM-3 levels were not significantly different and therefore all these blood preparations are suitable for human sICAM-3 determinations.

13.7 Specificity

To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed, notably there was no interference with human sICAM-1.

13.8 Expected Values

A panel of 20 randomly selected sera from apparently healthy blood donors (male and female) was tested for human sICAM-3. The detected human sICAM-3 levels ranged between 28.7 and 72.5 ng/ml with a mean level of 50 ng/ml and a standard deviation of 13.8 ng/ml. Normal human sICAM-3 levels may vary depending on the serum collective used.

14 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.

For literature update refer to **www.bendermedsystems.com/44.html**

15 Ordering Information

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Cat.No. BMS218INST human sICAM-3 INSTANT ELISA

16 Reagent Preparation Summary

16.1 Wash Buffer

Add **Wash Buffer Concentrate** 20 x (25 ml) to 475 ml distilled water

17 Test Protocol Summary

- Place standard strips in position A1/A2 to H1/H2.
- Add 130 µl **distilled water** to sample wells.
- Add **distilled water**, in duplicate, to all standard and blank wells as indicated on the label of the standard strips.
- Add 20 µl **Sample** to designated wells.
- Cover microwell strips and incubate 3 hours at room temperature (18° to 25°C) on a microplate shaker at 100 rpm.
- Empty and wash microwell strips 3 times with 400 µl **Wash Buffer**.
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells.
- Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
- Add 100 µl **Stop Solution** to all wells including blank wells.
- Blank microwell reader and measure colour intensity at 450 nm.

Note: Samples have been diluted 1:5, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 5).