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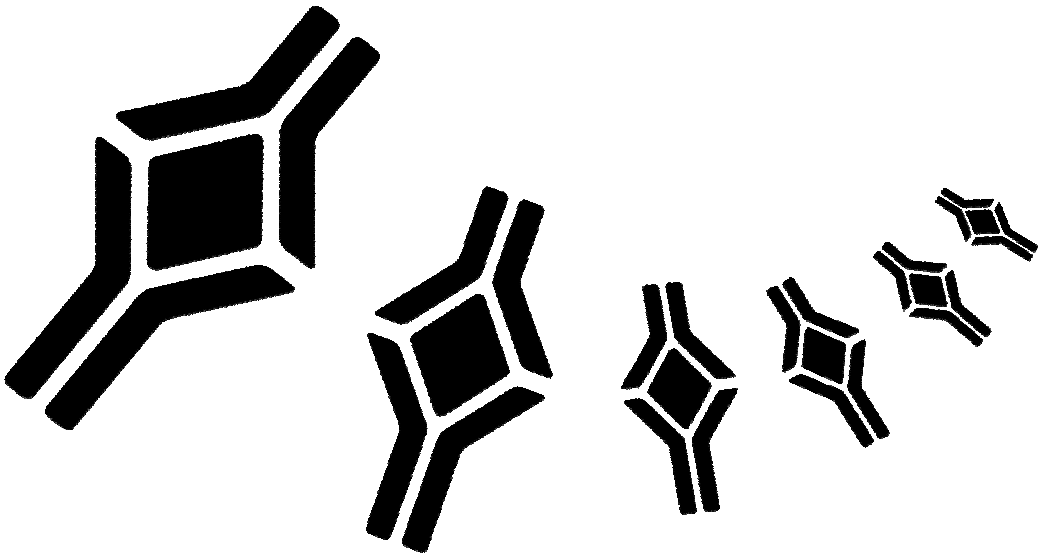

HUMAN sICAM-3 ELISA

Product Data Sheet

Cat. No.: RBMS218R

For Research Use Only

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» **This kit is manufactured by:**
BioVendor – Laboratorní medicína, a.s.

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

1 INTENDED USE

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

2 SUMMARY

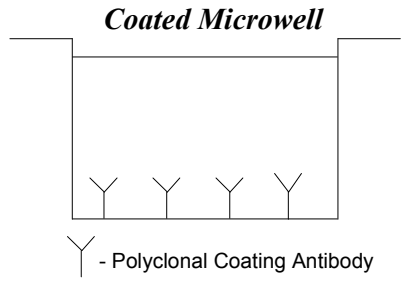
Intercellular Adhesion Molecule-3 (ICAM-3) is a member of the immunoglobulin supergene family (4) and functions as a ligand for the Lymphocyte Function-Associated Antigen-1 (LFA-1). Three counter-receptors have been described for LFA-1, intercellular adhesion molecule 1 (ICAM-1), ICAM-2 and ICAM-3 (3, 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).

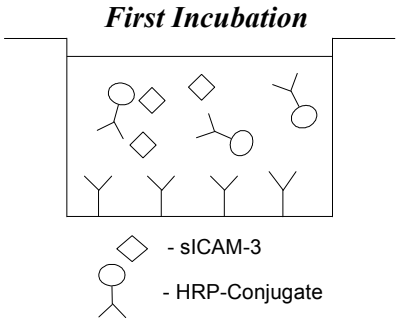
Furthermore, ICAM-3 expression has been shown for dendritic epidermal Langerhans cells, whereas it is absent on other dendritic cells from different lymphoid organs. Thus potential function of ICAM-3 at the initiation phase of LC-leukocyte interactions taking place during **skin localized immune reactions** 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

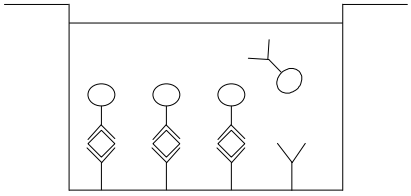
An anti-sICAM-3 monoclonal coating antibody is adsorbed onto microwells.



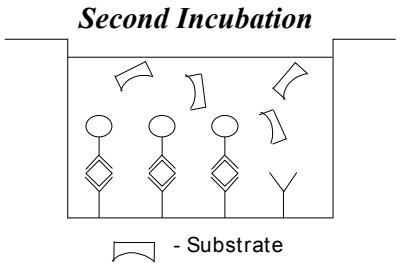
sICAM-3 present in the sample or standard binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-sICAM-3 antibody is added and binds to sICAM-3 captured by the first antibody.

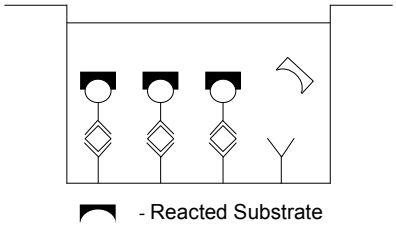


Following incubation unbound enzyme conjugated anti-sICAM-3 is removed during a wash step and substrate solution reactive with HRP is added to the wells.



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





4 REAGENTS PROVIDED

1 aluminium pouch with a **Antibody Coated Microtiter Strips** with Monoclonal Antibody (murine) to human sICAM-3

1 vial (6 ml) **HRP-Conjugate** anti-sICAM-3 monoclonal (murine) antibody, ready to use

2 vials (0.5 ml) 50 ng/ml **sICAM-3 Standard**

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (phosphate-buffered saline with 1% Tween 20)

1 bottle (12 ml) **Sample Diluent** (buffered protein matrix)

1 vial (15 ml) **Substrate Solution**

1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)

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

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

2 adhesive **Plate Covers**

Reagent Labels

5 STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use reagents should be returned to cold storage (2° to 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, EDTA, or heparinized plasma, urine, amniotic fluid, or other body fluids are suitable for use in the assay. Remove the serum or plasma from the clot or red cells, respectively, 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.

Pay attention to a possible **"Hook Effect"** due to high sample concentrations (see 11.).

Samples must be stored frozen at -20°C to avoid loss of bioactive sICAM-3. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles. Prior to assay, frozen sera or plasma should be brought to room temperature slowly and mixed gently and properly diluted with Sample Diluent within the microwells (**1 : 5** see page 14-15).

For sample stability refer to 13., page 26.

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 the conjugate and substrate reagents.

- Exposure to acids will inactivate 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 if 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

The reagents should be prepared before starting with the test procedure.

A. Wash Buffer

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

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 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. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

B. 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 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 Diluent	20 µl ***Blue-Dye***
12 ml Diluent	48 µl ***Blue-Dye***

2. HRP-Conjugate: Add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the HRP-Conjugate.

3 ml HRP-Conjugate	30 µl ***Green-Dye***
6 ml HRP-Conjugate	60 µl ***Green-Dye***

a. Mix all reagents thoroughly without foaming before use.

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 coated with Monoclonal Antibody** (murine) to human sICAM-3 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**.

d. 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:

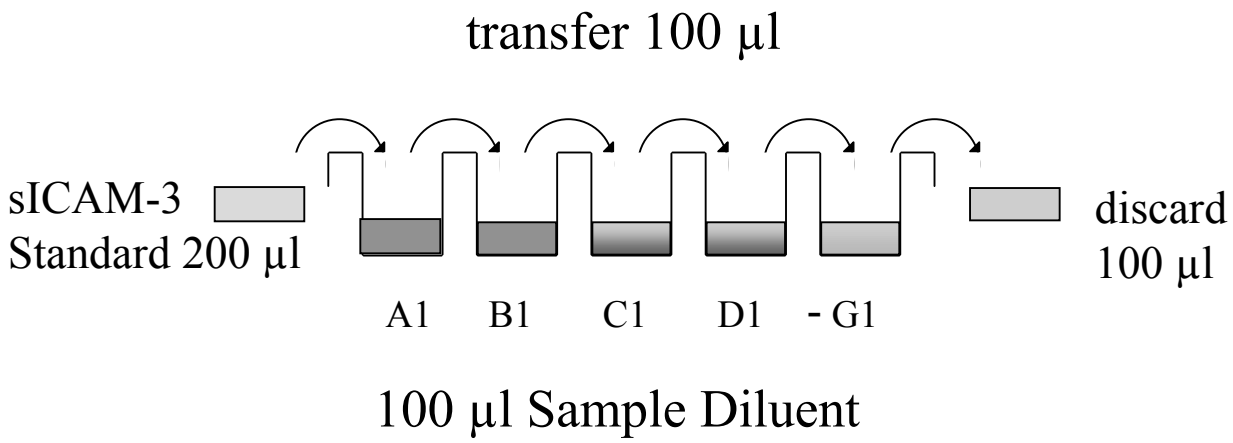


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	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

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

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

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

h. Add 50 µl of **HRP-Conjugate**, ready to use, to all wells.

i. 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.

j. Remove **Plate Cover** and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

k. Pipette 100 µl of **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 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.

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 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 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.

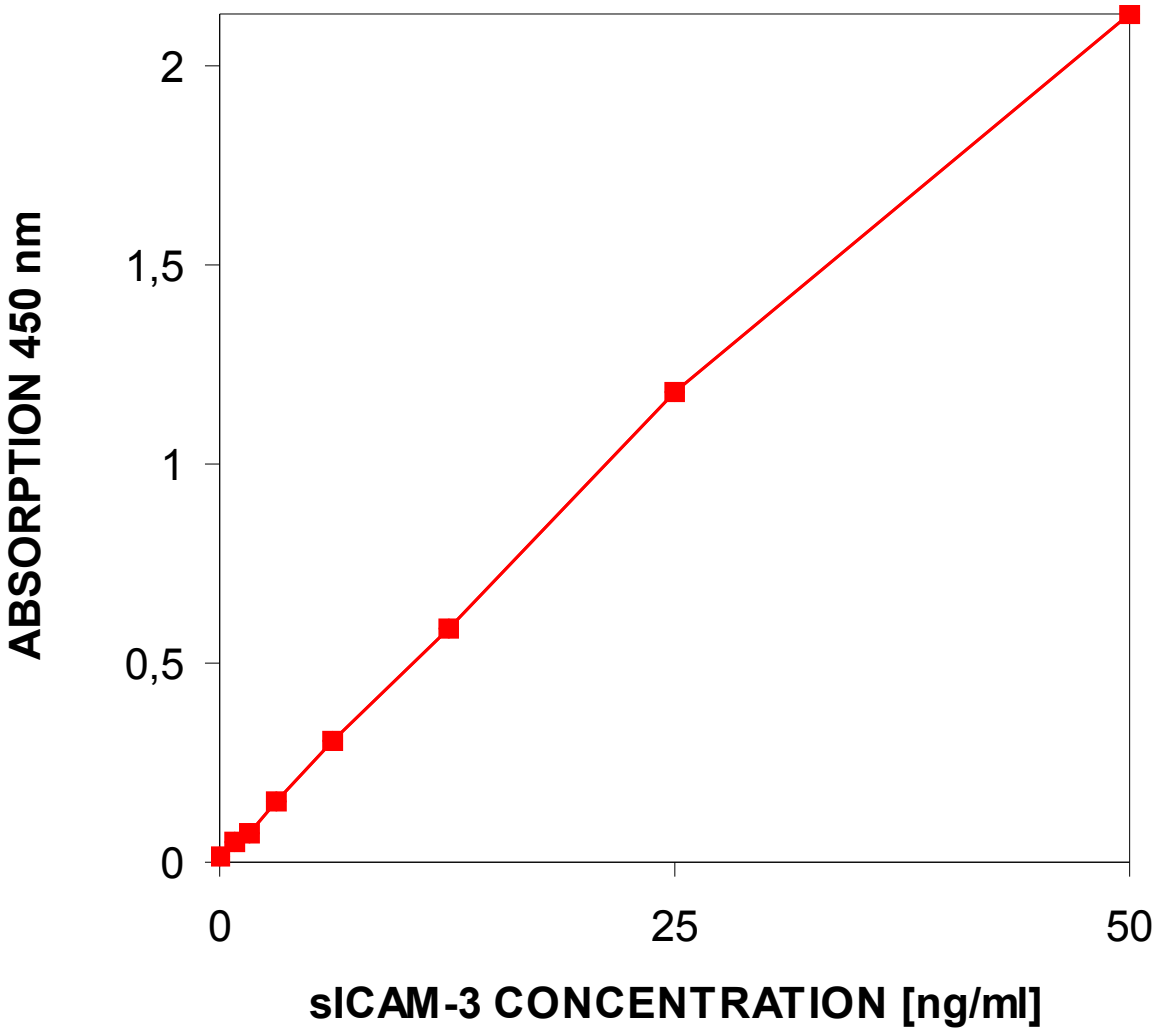
- **For samples which have been diluted according to the instructions given in this manual 1 : 5 the concentration read from the standard curve must be multiplied by the dilution factor (x 5).**

  Note: Calculation of samples with an O.D. exceeding S1 will result in incorrect low sICAM-3 levels (HOOK-Effect). Such samples require further external dilution 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 representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for sICAM-3 ELISA. Recombinant soluble ICAM-3 was diluted in serial two-fold steps in Sample Diluent; symbols represent the mean of three 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.



Typical data using the sICAM-3 ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	sICAM-3 Concentration (ng/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	50	2.098	2.132	2.3
	50	2.166		
2	25	1.226	1.182	5.3
	25	1.138		
3	12.5	0.607	0.586	5.2
	12.5	0.564		
4	6.25	0.318	0.305	6.2
	6.25	0.291		
5	3.13	0.151	0.149	1.2
	3.13	0.147		
6	1.6	0.072	0.074	2.9
	1.6	0.075		
7	0.78	0.050	0.049	2.9
	0.78	0.048		
Blank	0	0.020	0.016	
	0	0.011		

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 detergent before use.

- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, 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 significally increased the number of patients with human anti-mouse IgG antibody (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 analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample Diluent.

A. Sensitivity

The limit of detection for sICAM-3, defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 0.38 ng/ml (mean of 10 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sICAM-3. Two standard curves were run on each plate. Data below show the mean sICAM-3 concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 2.5%.

Positive Sample	Experiment	sICAM-3 Concentration (ng/ml)	Coefficient of Variation (%)
1	1	95.3	4.2
	2	98.2	2.2
	3	90.8	10.4
2	1	74.4	0.7
	2	74.1	0.6
	3	71.3	2.7
3	1	69.9	2.6
	2	69.1	2.4
	3	68.7	2.1
4	1	54.7	2.8
	2	56.9	4.3
	3	56.6	2.0
5	1	46.2	2.5
	2	45.2	2.2
	3	45.1	1.3
6	1	36.6	0.8
	2	37.5	4.3
	3	36.7	3.1
7	1	26.4	0.6
	2	27.3	1.5
	3	28.2	2.4
8	1	59.9	1.7
	2	59.5	1.1
	3	58.7	1.1

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sICAM-3. Two standard curves were run on each plate. Data below show the mean sICAM-3 concentration and the coefficient of variation calculated on 30 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 2.0 %.

Sample	sICAM-3 Concentration (ng/ml)	Coefficient of Variation (%)
1	94.8	4.0
2	73.3	2.3
3	69.3	0.9
4	56.1	2.2
5	45.5	1.4
6	36.9	1.4
7	27.3	3.1
8	59.3	1.0

C. Recovery Studies

Spiked samples were prepared by adding four different levels of recombinant sICAM-3 into human serum. As shown below, recoveries were determined in three independent experiments ranging from 98 % to 112 % with an overall mean recovery of 106 %.

sICAM-3 Spike (ng/ml)	Experiment	Recovery (%) sICAM-3
40	1	105
	2	110
	3	103
20	1	98
	2	111
	3	103
10	1	103
	2	108
	3	102
5	1	110
	2	112
	3	108

D. Dilution Linearity

Four serum samples with different levels of sICAM-3 were assayed at three serial two-fold dilutions (1:5 - 1:20) covering the working range of the standard curve. In the table below the percent recovery of expected values is listed. Recoveries ranged from 87 % to 104 % with an overall mean recovery of 96 %.

Sample	Dilution	sICAM-3Concentration (ng/ml) Expected Value	Observed Value	% Recovery of Exp. Value
1	1:5	--	59.6	--
	1:10	29.8	30.3	102
	1:20	14.9	15.5	104
2	1:5	--	60.6	--
	1:10	30.3	29.4	97
	1:20	15.2	14.1	93
3	1:5	--	78.8	--
	1:10	39.4	37.5	95
	1:20	19.7	18.1	92
4	1:5	--	102.7	--
	1:10	51.3	48.1	94
	1:20	25.7	22.3	87

E. Expected Values

A panel of 20 sera from apparently healthy blood donors (male and female) was tested for sICAM-3. The detected 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 sICAM-3 levels may vary depending on the serum collective used.

F. Sample Freeze-Thaw Stability

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

G. Sample Storage Stability

Aliquots of a serum sample (unspiked or spiked with sICAM-3) were stored at -20°C, 2-8°C, room temperature and at 37°C and the sICAM-3 level determined after 24, 48 and 96 hours. There was no significant loss of sICAM-3 immunoreactivity during storage under above conditions.

H. Comparison of Serum and Plasma

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

I. 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.

14 REFERENCES

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15 REAGENT PREPARATION SUMMARY

A. Wash Buffer Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

- Wash Microwell Strips twice with **Wash buffer**
- Add 100 µl **Sample Diluent**, in duplicate, to standard wells except the first wells (50 ng/ml)
- Pipette 200 µl **sICAM-3 Standard** into the first standard wells and create standard dilutions ranging from 50 to 0.78 ng/ml by transferring 100 µl from well to well; Discard 100 µl from the last wells.
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 80 µl **Sample Diluent** to sample wells
- Add 20 µl **Sample** to designated wells
- Add 50 µl **HRP-Conjugate,** ready to use, to all wells
- Cover microwell strips and incubate 2 hour at room temperature (18° to 25°C)
- Empty and wash microwell strips 3 times with **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: 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 (x5). Calculation of samples with an O.D. exceeding S1 will result in incorrect low sICAM-3 levels (HOOK-Effect). Such samples require further external dilution with Sample Diluent in order to precisely quantitate the actual sICAM-3 level.

NOTES

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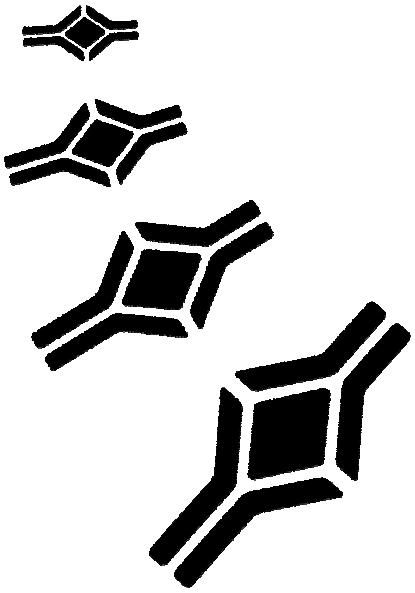

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