PRODUCT INFORMATION & MANUAL

Human sICAM-2 Platinum ELISA

BMS236

Enzyme-linked Immunosorbent Assay for quantitative detection of human sICAM-2.

For research use only.
Not for diagnostic or therapeutic procedures.

eBioscience®
An Affymetrix Company

Human sICAM-2 Platinum ELISA

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1 Intended Use

The human sICAM-2 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Intercellular Adhesion Molecule-2 (sICAM-2). The human sICAM-2 ELISA is for research use only. Not for diagnostic or therapeutic procedures.

2 Summary

Intercellular Adhesion Molecule-2 (ICAM-2) is a 60 kDa surface glycoprotein that promotes adhesion between immune cells and the vascular endothelium. ICAM-2 is a member of the Ig-superfamily with two immunoglobulin-like extracellular domains. ICAM-2 is much more closely related to the two N-terminal domains of ICAM-1 (34% identity) than either ICAM-1 or ICAM-2 is to other members of the Ig-superfamily, demonstrating the existence of a subfamily of Ig-like ligands that bind the same integrin receptor. ICAM-2 is broadly distributed on hematopoietic cells. On resting lymphocytes, the ICAM-2 expression is several-fold higher than that seen with ICAM-1, while monocytes express equivalent levels of ICAM-1 and ICAM-2. In contrast to ICAM-1, neutrophils stain negative for ICAM-2. Just little or no ICAM-2 staining can be demonstrated on other cell lines, the sole exception being (i) resting vascular endothelial cells, which possess high levels of ICAM-2, and (ii) small clusters of cells in lymphoid tissue germinal centres.

ICAM-2 expression is unaffected by a variety of inflammatory cytokines, while ICAM-1 is upregulated upon stimulation. This points toward the importance of ICAM-2 in the unstimulated resting state, before ICAM-1 expression is increased.

The integrin LFA-1 is the receptor for ICAM-2 and ICAM-1 as well. The functional characteristics of ICAM-2 as LFA-1 ligand can be summarized as follows:

ICAM-2 dominates over ICAM-1 on resting endothelial cells and seems to be involved in the recirculation of LFA-1 positive lymphocytes, e.g. facilitating T-memory cell recirculation. As resting T-cells express little or no ICAM-1, ICAM-2 may also be important in initial T-cell adhesion with antigen-presenting cells that bear LFA-1. Also the lysis of certain target

cells appears to occur in an ICAM-1 independent manner, possibly regulated by ICAM-2.

For literature update refer to **www.eBioscience.com**

3 Principles of the Test

An anti-human sICAM-2 coating antibody is adsorbed onto microwells.

Figure 1

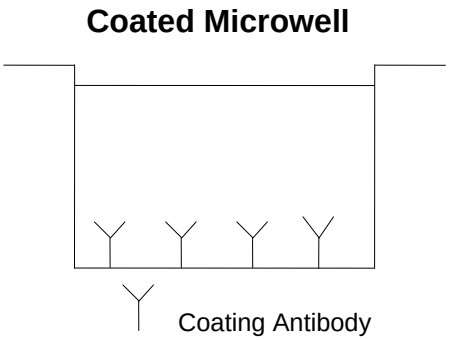


Figure 2

Human sICAM-2 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human sICAM-2 antibody is added and binds to human sICAM-2 captured by the first antibody.

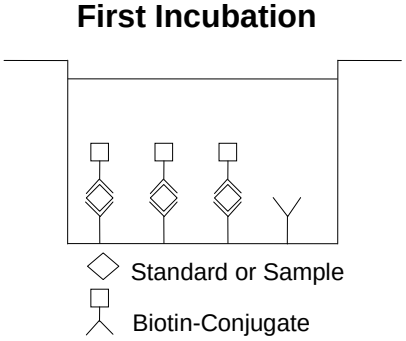


Figure 3

Following incubation unbound biotin-conjugated anti-human sICAM-2 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human sICAM-2 antibody.

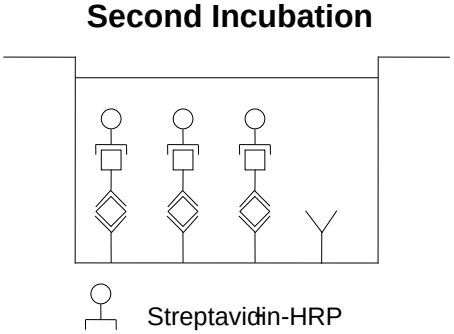
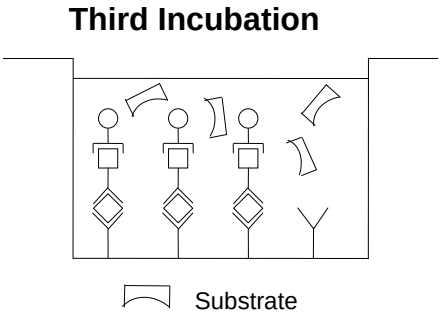


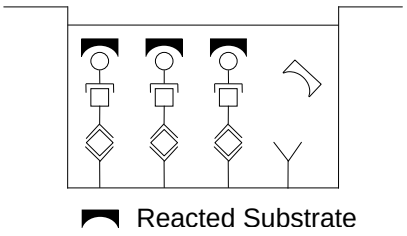
Figure 4

Following incubation unbound Streptavidin-HRP 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 human sICAM-2 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 6 human sICAM-2 standard dilutions and human sICAM-2 sample concentration determined.

Figure 5



4 Reagents Provided

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human sICAM-2

1 vial (0.4 ml) **Biotin-Conjugate** anti-human sICAM-2 monoclonal antibody

2 vials (5 µl) **Streptavidin-HRP-Concentrate**

2 vials human sICAM-2 **Standard** lyophilized, 24.00 U/ml upon reconstitution

1 bottle (23 ml) **Streptavidin-HRP Diluent**

1 bottle (25 ml) **Standard Buffer Diluent** (10x)

1 bottle (7.5 ml) **Biotin Conjugate Diluent**

1 bottle (10 ml) **Wash Buffer Concentrate** 200x

1 bottle (11 ml) **Substrate Solution** (tetramethyl-benzidine and buffered hydrogen peroxide)

1 vial (11 ml) **Stop Solution** (1N H_2SO_4)

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

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

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

4 **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, whole 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 grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human sICAM-2. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.3).
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 (10 ml) of the Wash Buffer Concentrate (200x) into a clean 2000 ml graduated cylinder. Bring to final volume of 2000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

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	2.5	497.5
1 - 12	5	955

9.2 Standard Buffer Diluent (1x)

Pour the entire contents (25 ml) of the concentrated **Standard Buffer Diluent** (10x) into a clean 250 ml graduated cylinder. Bring to final volume of 250 ml with distilled water. Mix gently to avoid foaming.

Standard Buffer Diluent (1x) may also be prepared as needed according to the following table:

Number of Strips	Standard Buffer Diluent (10x) (ml)	Distilled Water (ml)
1 - 6	5.0	45
1 - 12	10.0	90.0

9.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a dilution (1:27.5) of the concentrated **Biotin-Conjugate** solution with Biotin-Conjugate Diluent in a clean plastic tube as needed according to the following table:

Number of Strips	Concentrated Biotin-Conjugate (ml)	Biotin Conjugate Diluent (ml)
1 - 6	0.11	2.89
1 - 12	0.22	5.78

9.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Dilute the **Streptavidin-HRP** just prior to use by adding 500 µl Streptavidin-HRP Diluent to the tube containing the Streptavidin-HRP concentrate (5 µl). Mix the contents of the tube well.
Make a further dilution with Streptavidin-HRP Diluent in a clean plastic tube or reagent reservoir according to the following table:

Number of Strips	prediluted Streptavidin-HRP (ml)	Streptavidin-HRP Diluent (ml)
1 - 6	0.075	4.925
1 - 12	0.150	9.850

9.5 Human sICAM-2 Standard

Reconstitute **human sICAM-2 standard** by addition of Standard Buffer Diluent (1x). Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilisation (concentration of reconstituted standard = 24.00 U/ml). Allow the reconstituted standard to sit for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

9.5.1 External Standard Dilution

Label 5 tubes, one for each standard point.

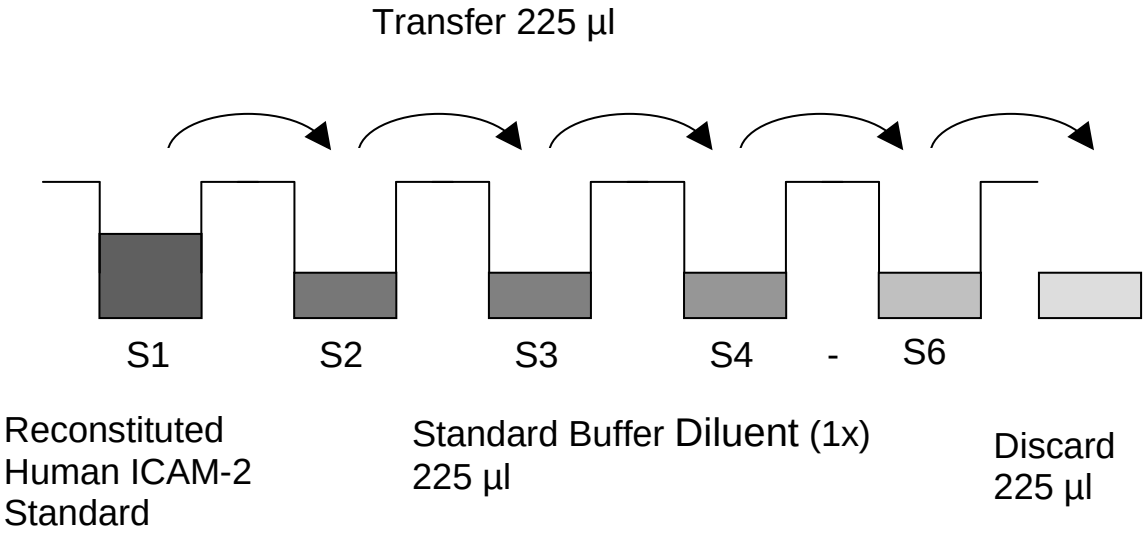
S2, S3, S4, S5, S6

Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 µl of Standard Buffer Diluent (1x) into tubes S2 – S6.

Pipette 225 µl of reconstituted standard (serves as the highest standard S1, concentration of standard 1 = 24.00 U/ml) into the first tube, labelled S2, and mix (concentration of standard 2 = 12.00 U/ml).
Pipette 225 µl of this dilution into the second tube, labelled S3, and mix thoroughly before the next transfer.
Repeat serial dilutions 3 more times thus creating the points of the standard curve (see Figure 6).

Standard Buffer Diluent (1x) serves as blank.

Figure 6



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

In order to help our customers to avoid any mistakes in pipetting the Platinum ELISAs, eBioscience offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

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, Red-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 Standard Buffer Diluent (1x)	20 µl ***Blue-Dye***
12 ml Standard Buffer Diluent (1x)	48 µl ***Blue-Dye***

2. Biotin-Conjugate: Before dilution of the concentrated Biotin-Conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Biotin Conjugate Diluent used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet: Preparation of Biotin-Conjugate.

3 ml Biotin Conjugate Diluent	30 µl ***Green-Dye***
6 ml Biotin Conjugate Diluent	60 µl ***Green-Dye***

3. Streptavidin-HRP: Before dilution of the Streptavidin-HRP Concentrate add the ***Red-Dye*** at a dilution of 1:250 (see table below) to Streptavidin-HRP Diluent used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet: Preparation of Streptavidin-HRP.

6 ml Streptavidin-HRP Diluent	24 µl ***Red-Dye***
12 Streptavidin-HRP Diluent	48 µl ***Red-Dye***

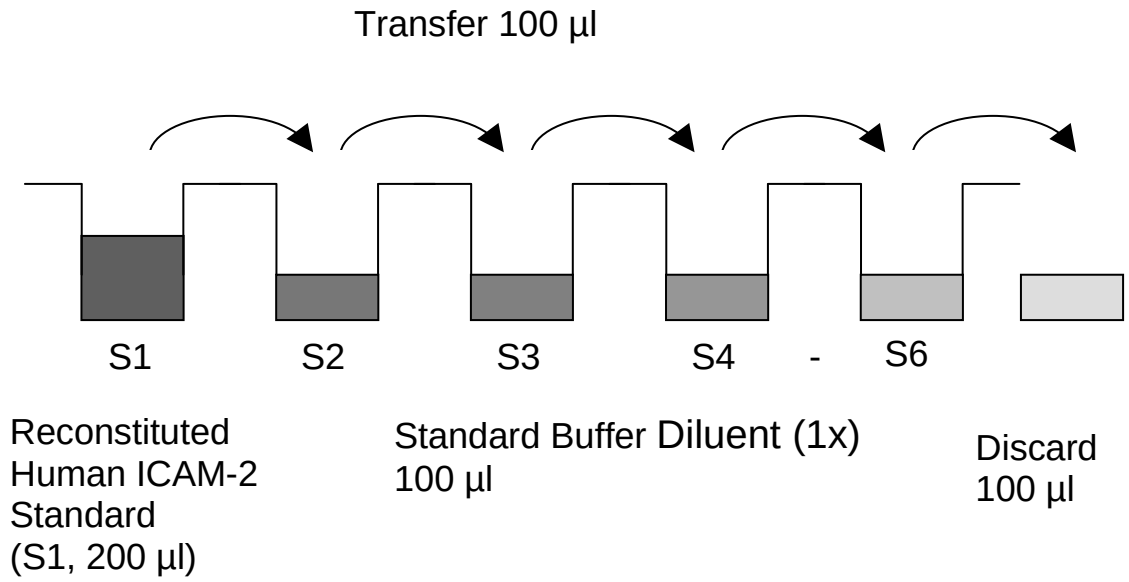
10 Test Protocol

a. Predilute your samples before starting with the test procedure. Dilute serum and plasma samples 1:20 with Standard Buffer Diluent (1x) according to the following scheme:
15 µl sample + 285 µl Standard Buffer Diluent (1x)

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. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.5.1.):
Add 100 µl of Standard Buffer Diluent (1x) in duplicate to **standard wells** B1/2- F1/2, leaving A1/A2 empty. Pipette 200 µl of prepared **standard** (see Preparation of Standard 9.5) in duplicate into well A1 and A2 (see Table 1) (concentration of standard 1, S1 = 24.00 U/ml), and transfer 100 µl to wells B1 and B2, respectively. 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 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 3 times, creating two rows of human sICAM-2 standard dilutions ranging from 24.00 to 0.75 U/ml. Discard 100 µl of the contents from the last microwells (F1, F2) used.

Figure 7



In case of an **external standard dilution** (see 9.5.1), pipette 100 µl of these standard dilutions (S1 – S6) 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 (24.00 U/ml)	Standard 1 (24.00 U/ml)	Sample 2	Sample 2
B	Standard 2 (12.00 U/ml)	Standard 2 (12.00 U/ml)	Sample 3	Sample 3
C	Standard 3 (6.00 U/ml)	Standard 3 (6.00 U/ml)	Sample 4	Sample 4
D	Standard 4 (3.00 U/ml)	Standard 4 (3.00 U/ml)	Sample 5	Sample 5
E	Standard 5 (1.50 U/ml)	Standard 5 (1.50 U/ml)	Sample 6	Sample 6
F	Standard 6 (0.75 U/ml)	Standard 6 (0.75 U/ml)	Sample 7	Sample 7
G	Blank	Blank	Sample 8	Sample 8
H	Sample 1	Sample 1	Sample 9	Sample 9

d. Add 100 µl of **Standard Buffer Diluent (1x)** in duplicate to the **blank wells**.

e. Add 100 µl of each prediluted **sample** in duplicate to the **sample wells**.

f. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).

g. Add 50 µl of prepared **Biotin-Conjugate** to all wells.

h. 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 400 rpm.

i. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).

j. Remove adhesive film 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. 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**.

k. Add 100 µl of prepared **Streptavidin-HRP** to all wells, including the blank wells.

l. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 30 minutes, if available on a microplate shaker set at 400 rpm.

m. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point j. of the test protocol. Proceed immediately to the next step.

n. Pipette 100 µl of **TMB Substrate Solution** to all wells.

o. Incubate the microwell strips at room temperature (18° to 25°C) for about 15 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 0.9 – 0.95.

p. 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.

q. 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 sICAM-2 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 sICAM-2 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-2 concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:20 (15 µl sample + 285 µl Standard Buffer Diluent (1x)), the concentration read from the standard curve must be multiplied by the dilution factor (x 20).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human sICAM-2 levels. Such samples require further external predilution according to expected human sICAM-2 values with Standard Buffer Diluent (1x) in order to precisely quantitate the actual human sICAM-2 level.**
- It is suggested that each testing facility establishes a control sample of known human sICAM-2 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 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 8

Representative standard curve for human sICAM-2 ELISA. Human sICAM-2 was diluted in serial 2-fold steps in Standard Buffer Diluent (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

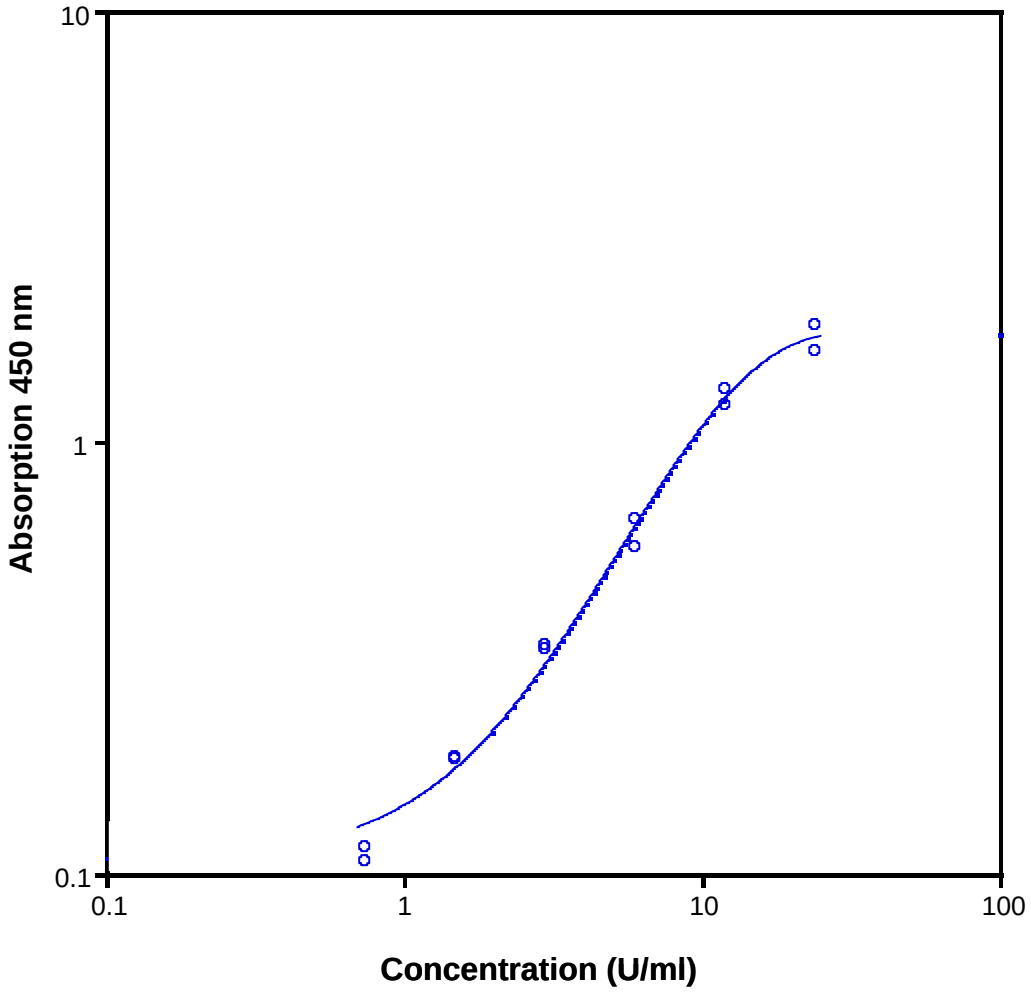


Table 2
Typical data using the human sICAM-2 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human sICAM-2 Concentration (U/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	24.00	1.627 1.869	1.748	6.9
2	12.00	1.220 1.328	1.274	4.3
3	6.00	0.569 0.665	0.617	7.8
4	3.00	0.331 0.339	0.335	1.2
5	1.50	0.183 0.186	0.184	0.7
6	0.75	0.115 0.107	0.111	3.6
Blank	0.00	0.032 0.032	0.032	0.0

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-2 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 0.30 U/ml (mean of 20 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 10 independent experiments. Each assay was carried out with 6 replicates of 2 serum samples containing different concentrations of human sICAM-2.
2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 6.7%.

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 3 replicates of 2 serum samples containing different concentrations of human sICAM-2. 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 7.0%.

13.3 Sample Stability

13.3.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human sICAM-2 levels determined. There was no significant loss of human sICAM-2 immunoreactivity detected by freezing and thawing.

13.3.2 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-2 level determined after 24 h. There was no significant loss of human sICAM-2 immunoreactivity detected during storage under above conditions.

13.4 Specificity

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into ICAM-2 positive serum. There was no crossreactivity detected, notably not with sICAM-1 and sICAM-3.

13.5 Expected Values

A panel of 80 sera samples from randomly selected apparently healthy donors (males and females) was tested for human sICAM-2.
The detected human sICAM-2 levels ranged between 44 and 405 U/ml with a mean level of 220 U/ml and a standard deviation of 74 U/ml.

14 Ordering Information

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15 Reagent Preparation Summary

15.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 200x (10 ml) to 2000 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	497.5
1 - 12	5	955

15.2 Standard Buffer Diluent (1x)

Add **Standard Buffer Diluent (10x)** (25 ml) to 225 ml distilled water.

Number of Strips	Standard Buffer Diluent (10x) (ml)	Distilled Water (ml)
1 - 6	5.0	45
1 - 12	10.0	90.0

15.3 Biotin-Conjugate

Make a 1:27.5 dilution of concentrated **Biotin-Conjugate** in Biotin-Conjugate Diluent:

Number of Strips	Concentrated Biotin-Conjugate (ml)	Biotin-Conjugate Diluent (ml)
1 - 6	0.11	2.89
1 - 12	0.22	5.78

15.4 Streptavidin-HRP

Dilute 5µl **Streptavidin-HRP** Concentrate in 500µl Streptavidin-HRP Diluent. Make a further dilution according to the following table :

Number of Strips	prediluted Streptavidin-HRP (µl)	Streptavidin-HRP Diluent (ml)
1 - 6	75	5
1 - 12	150	10

15.5 Human sICAM-2 Standard

Reconstitute lyophilized **human sICAM-2 standard** with Standard Buffer Diluent (1x). (Reconstitution volume is stated on the label of the standard vial.)

16 Test Protocol Summary

1. Predilute sample with Standard Buffer Diluent (1x)1:20.
2. Determine the number of microwell strips required.
3. Standard dilution on the microwell plate: Add 100 µl Standard Buffer Diluent (1x), in duplicate, to all standard wells leaving the first wells empty. Pipette 200 µl prepared 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.
4. Add 100 µl Standard Buffer Diluent (1x) in duplicate, to the blank wells.
5. Add 100 µl sample in duplicate, to designated sample wells.
6. Prepare Biotin-Conjugate.
7. Add 50 µl prepared Biotin-Conjugate to all wells.
8. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
9. Prepare Streptavidin-HRP.
10. Empty and wash microwell strips 3 times with Wash Buffer.
11. Add 100 µl prepared Streptavidin-HRP to all wells.
12. Cover microwell strips and incubate 30 minutes at room temperature (18° to 25°C).
13. Empty and wash microwell strips 3 times with Wash Buffer.
14. Add 100 µl of TMB Substrate Solution to all wells.
15. Incubate the microwell strips for about 15 minutes at room temperature (18° to 25°C).
16. Add 100 µl Stop Solution to all wells.
17. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:20 (15 µl sample + 285 µl Standard Buffer Diluent (1x)), the concentration read from the standard curve must be multiplied by the dilution factor (x 20).