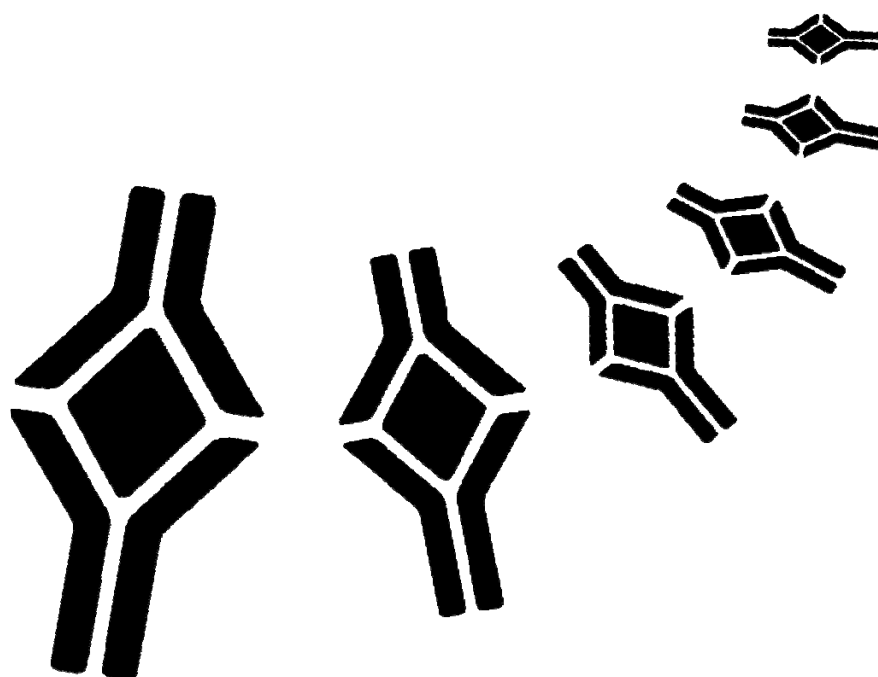


# BioVendor

Research  
and Diagnostic Products



## Human sVCAM-1 ELISA

Product Data Sheet

Cat. No.: RBMS232R

For Research Use Only

## CONTENTS

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|     |  |    |
|-----|--|----|
| 1.  | INTENDED USE                                 | 3  |
| 2.  | SUMMARY                                      | 3  |
| 3.  | PRINCIPLES OF THE TEST                       | 4  |
| 4.  | REAGENTS PROVIDED                            | 5  |
| 5.  | STORAGE INSTRUCTIONS – ELISA KIT             | 5  |
| 6.  | SPECIMEN COLLECTION AND STORAGE INSTRUCTIONS | 6  |
| 7.  | MATERIALS REQUIRED BUT NOT PROVIDED          | 6  |
| 8.  | PRECAUTIONS FOR USE                          | 7  |
| 9.  | PREPARATION OF REAGENTS                      | 8  |
| 10. | TEST PROTOCOL                                | 11 |
| 11. | CALCULATION OF RESULTS                       | 13 |
| 12. | LIMITATIONS                                  | 16 |
| 13. | PERFORMANCE CHARACTERISTICS                  | 16 |
| 14. | REFERENCES                                   | 20 |
| 15. | REAGENT PREPARATION SUMMARY                  | 22 |
| 16. | TEST PROTOCOL SUMMARY                        | 23 |

**»»** 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

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The human sVCAM-1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human sVCAM-1. **The human sVCAM-1 ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

## 2. SUMMARY

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The vascular cell adhesion molecule-1 (VCAM-1) or CD106 is a member of the immunoglobulin gene superfamily. The initial molecular cloning of VCAM-1 reported six extracellular Ig-like domains (6D VCAM-1).

This 6D VCAM-1 arises due to alternative splicing from a seven-domain VCAM-1 (7D VCAM-1). 7D VCAM-1 is the dominant form expressed by cultured human endothelial cells. Domains 1 through 3 are highly homologous to domains 4 through 6, suggesting that they arose by gene duplication. The cDNA of 7D VCAM-1 predicts a core protein of approximately 81 kDa with seven potential N-linked glycosylation sites. Upon complete glycosylation the mature protein has a molecular weight of approximately 102 kDa. This observation is in general agreement with immunoprecipitation studies that show a protein of approximately 110 kDa on cytokine-activated endothelium.

Murine and rat VCAM-1 have been cloned. In contrast to ICAM-1, VCAM-1 appears to have been highly conserved through evolution. Both rat and mouse VCAM-1 are highly homologous at the protein level to the human VCAM-1 (77% and 76%, respectively).

VCAM-1 supports the adhesion of lymphocytes, monocytes, natural killer cells, eosinophils, and basophils through its interaction with leukocyte very late antigen-4 (VLA-4). VCAM-1/VLA-4 interaction mediates firm adherence of circulating non-neutrophilic leukocytes to endothelium. VCAM-1 also participates in leukocyte adhesion outside of the vasculature, mediating precursor lymphocyte adhesion to bone marrow stromal cells and B cell binding to lymph node follicular dendritic cells.

VCAM-1 is not constitutively expressed on endothelium, but can be up-regulated in vitro in response to LPS, TNF- $\alpha$ , and IL-1, as well as to interferon- $\gamma$  and IL-4.

VCAM-1 is also present on tissue macrophages, dendritic cells, bone marrow fibroblasts, myoblasts and myotubes. A soluble form of VCAM-1 (sVCAM-1) has been described. Soluble VCAM-1 levels have been found in the serum of healthy individuals and increased levels of sVCAM-1 can be detected in several diseases:

Cancer: ovarian, gastric-intestinal, renal, bladder cancer, non-Hodgkin's lymphoma, breast cancer (cyst fluid);

Autoimmune diseases: multiple sclerosis (cerebrospinal fluid), systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis;

Infections: sepsis, meningitis, malaria;

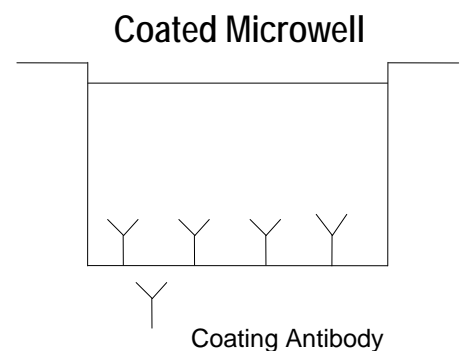
Inflammation: vasculitis, alcoholic cirrhosis, primary biliary cirrhosis, Wegener's

granulomatosis;

Others: impaired renal function, haemodialysis, hyperthyroidism, renal allograft.

### 3. PRINCIPLES OF THE TEST

An anti-human sVCAM-1 coating antibody is adsorbed onto microwells.



Human sVCAM-1 present in the sample or standard binds to antibodies adsorbed to the microwells. A conjugate mixture (biotin-conjugated anti-human sVCAM-1 antibody and Streptavidin-HRP) is added.

Figure 2

Biotin-conjugated anti-human sVCAM-1 antibody binds to human sVCAM-1 captured by the first antibody.

Streptavidin-HRP binds to the biotin-conjugated anti-human sVCAM-1 antibody.

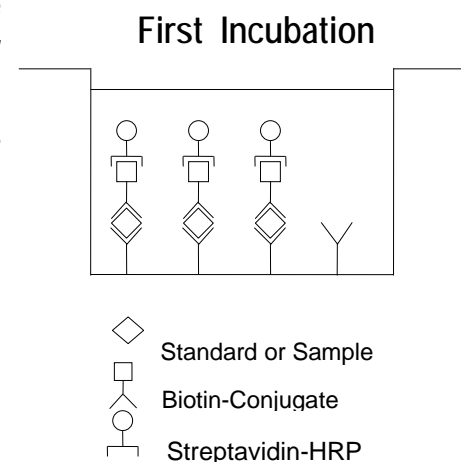
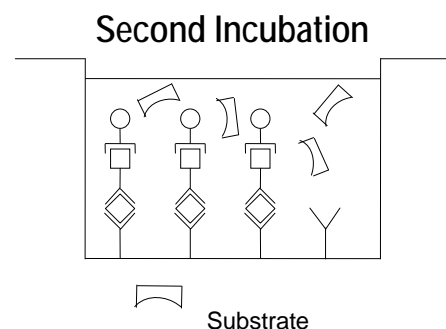


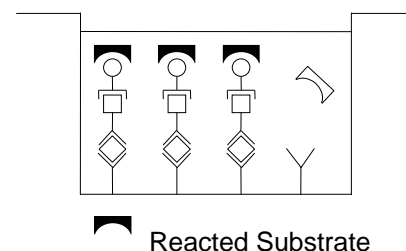
Figure 3

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 sVCAM-1 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 sVCAM-1 standard dilutions and human sVCAM-1 sample concentration determined.

Figure 4



#### 4. REAGENTS PROVIDED

- 1 aluminium pouch with a **Antibody Coated Microtiter Strips** with monoclonal antibody to human sVCAM-1
- 1 vial (100 µl) **Conjugate Mixture** containing **Biotin-Conjugate** anti-human sVCAM-1 monoclonal antibody mixed with **Streptavidin-HRP**
- 2 vials human sVCAM-1 **Standard** lyophilized, 200 ng/ml upon reconstitution
- 1 vial **Control high**, lyophilized
- 1 vial **Control low**, lyophilized
- 1 vial (5 ml) **Assay Buffer Concentrate 20x** (PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x** (PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml) **Blue-Dye**
- 1 vial (0.4 ml) **Green-Dye**
- 2 **Adhesive Films**

#### 5. STORAGE INSTRUCTIONS – ELISA KIT

Store kit reagents between 2° and 8°C except controls. Store lyophilized controls at -20°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C), controls to -20°C, respectively. 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

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Cell culture supernatant, serum, plasma (EDTA, citrate, heparin) and amniotic fluid 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 sVCAM-1. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5).

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

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- 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

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- 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 (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 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            | 25                                 | 475                  |
| 1 - 12           | 50                                 | 950                  |

### 9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

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

| Number of Strips | Assay Buffer Concentrate (20x) (ml) | Distilled Water (ml) |
|------------------|-------------------------------------|----------------------|
| 1 - 6            | 2.5                                 | 47.5                 |
| 1 - 12           | 5.0                                 | 95.0                 |

### 9.3 Conjugate Mixture

**Please note that the Conjugate Mixture should be used within 30 minutes after dilution.**

The **Conjugate Mixture** (Biotin-Conjugate mixed with Streptavidin-HRP)

must be diluted 1:100 with Assay Buffer (1x) just prior to use in a clean plastic test tube.

Conjugate Mixture may be prepared as needed according to the following table:

| Number of Strips | Conjugate Mixture (ml) | Assay Buffer (1x) (ml) |
|------------------|------------------------|------------------------|
| 1 - 6            | 0.03                   | 2.97                   |
| 1 - 12           | 0.06                   | 5.94                   |



#### 9.4 Human sVCAM-1 Standard

Reconstitute **human sVCAM-1 standard** by addition of distilled water.

Reconstitution volume is stated in the Quality Control Sheet. Swirl or mix gently to insure complete and homogeneous solubilization

(concentration of reconstituted standard = 200 ng/ml).

Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

Label 6 tubes, one for each standard point.

S1, S2, S3, S4, S5, S6

Then prepare 1:2 serial dilutions for the standard curve as follows:

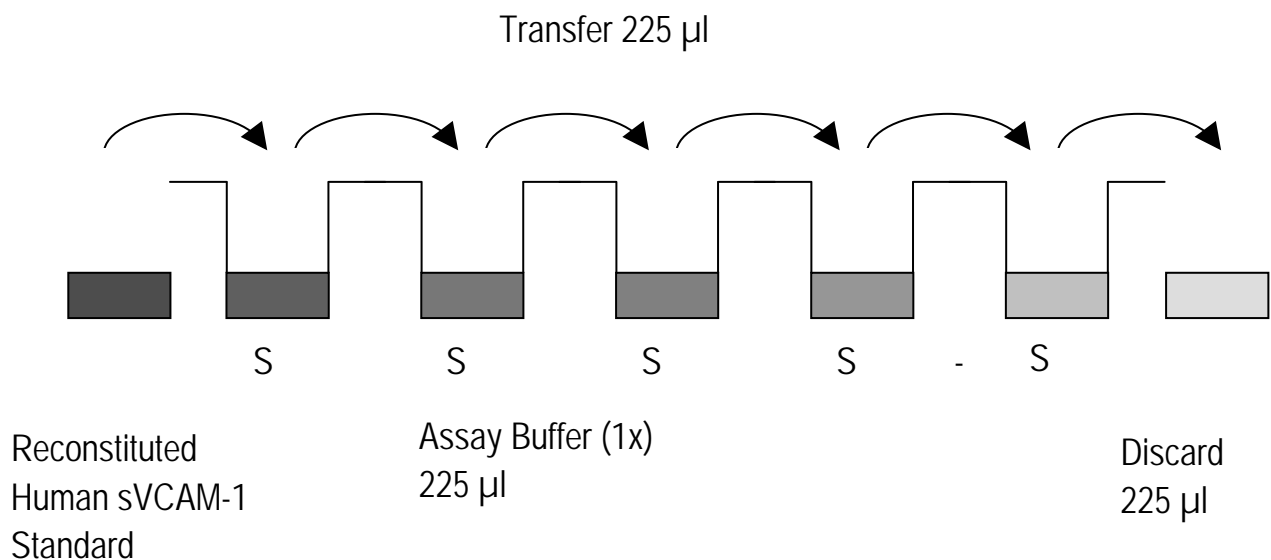
Pipette 225 µl of Assay Buffer (1x) into each tube.

Pipette 225 µl of reconstituted standard (concentration = 200 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 100 ng/ml).

Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer. Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 5).

Assay Buffer (1x) serves as blank.

Figure 5



## 9.5 Controls

Reconstitute by adding 150 µl distilled water to lyophilized **controls** (10-30 minutes). Swirl or mix gently to ensure complete and homogeneous solubilization. Further treat the controls like your samples in the assay. For control range please refer to certificate of analysis or vial label. Store reconstituted controls aliquoted at -20°C. Avoid repeated freeze and thaw cycles.

## 9.6 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 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 Assay Buffer (1x)  | 20 µl <b>Blue-Dye</b>  |
| 12 ml Assay Buffer (1x) | 48 µl <b>Blue-Dye</b>  |
| 50 ml Assay Buffer (1x) | 200 µl <b>Blue-Dye</b> |

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

|                        |                        |
|------------------------|------------------------|
| 3 ml Assay Buffer (1x) | 30 µl <b>Green-Dye</b> |
| 6 ml Assay Buffer (1x) | 60 µl <b>Green-Dye</b> |

## 10. TEST PROTOCOL

- a. Predilute your samples before starting with the test procedure. Dilute serum and plasma samples 1:50 with Assay Buffer (1x) according to the following scheme:  
10 µl sample + 490 µl Assay Buffer (1x)
- b. Prepare **Conjugate Mixture** (refer to Preparation of Reagents Conjugate Mixture 9.3.)
- c. 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 samples 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.
- d. 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.**
- e. Add 50 µl of diluted **Conjugate Mixture** to all wells, including the blank wells.
- f. Add 100 µl of prediluted **standards** (S1 - S6) (refer to Preparation of Reagents: Human SVCAM-1 Standard 9.4.) to 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 (100.0 ng/ml) | Standard 1 (100.0 ng/ml) | Sample 2 | Sample 2 |
| B | Standard 2 (50.0 ng/ml)  | Standard 2 (50.0 ng/ml)  | Sample 3 | Sample 3 |
| C | Standard 3 (25.0 ng/ml)  | Standard 3 (25.0 ng/ml)  | Sample 4 | Sample 4 |
| D | Standard 4 (12.5 ng/ml)  | Standard 4 (12.5 ng/ml)  | Sample 5 | Sample 5 |
| E | Standard 5 (6.3 ng/ml)   | Standard 5 (6.3 ng/ml)   | Sample 6 | Sample 6 |
| F | Standard 6 (3.1 ng/ml)   | Standard 6 (3.1 ng/ml)   | Sample 7 | Sample 7 |
| G | Blank                    | Blank                    | Sample 8 | Sample 8 |
| H | Sample 1                 | Sample 1                 | Sample 9 | Sample 9 |

- g. Add 100 µl of **Assay Buffer (1x)** in duplicate to the **blank wells**.
- h. Add 100 µl of each **prediluted sample** in duplicate to the **sample wells**.
- i. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 100 rpm.
- j. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point d. of the test protocol. Proceed immediately to the next step.
- k. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- l. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 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.
- m. 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.
- 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 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

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- 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 sVCAM-1 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 sVCAM-1 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 sVCAM-1 concentration.
- **If instructions in this protocol have been followed serum and plasma samples have been diluted 1:50 (10 µl sample + 490 µl Assay Buffer (1x)), the concentration read from the standard curve must be multiplied by the dilution factor (x 50).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human sVCAM-1 levels. Such samples require further external predilution according to expected human sVCAM-1 values with Assay Buffer (1x) in order to precisely quantitate the actual human sVCAM-1 level.**
- It is suggested that each testing facility establishes a control sample of known human sVCAM-1 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 6. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 6  
Representative standard curve for human sVCAM-1 ELISA. Human sVCAM-1 was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

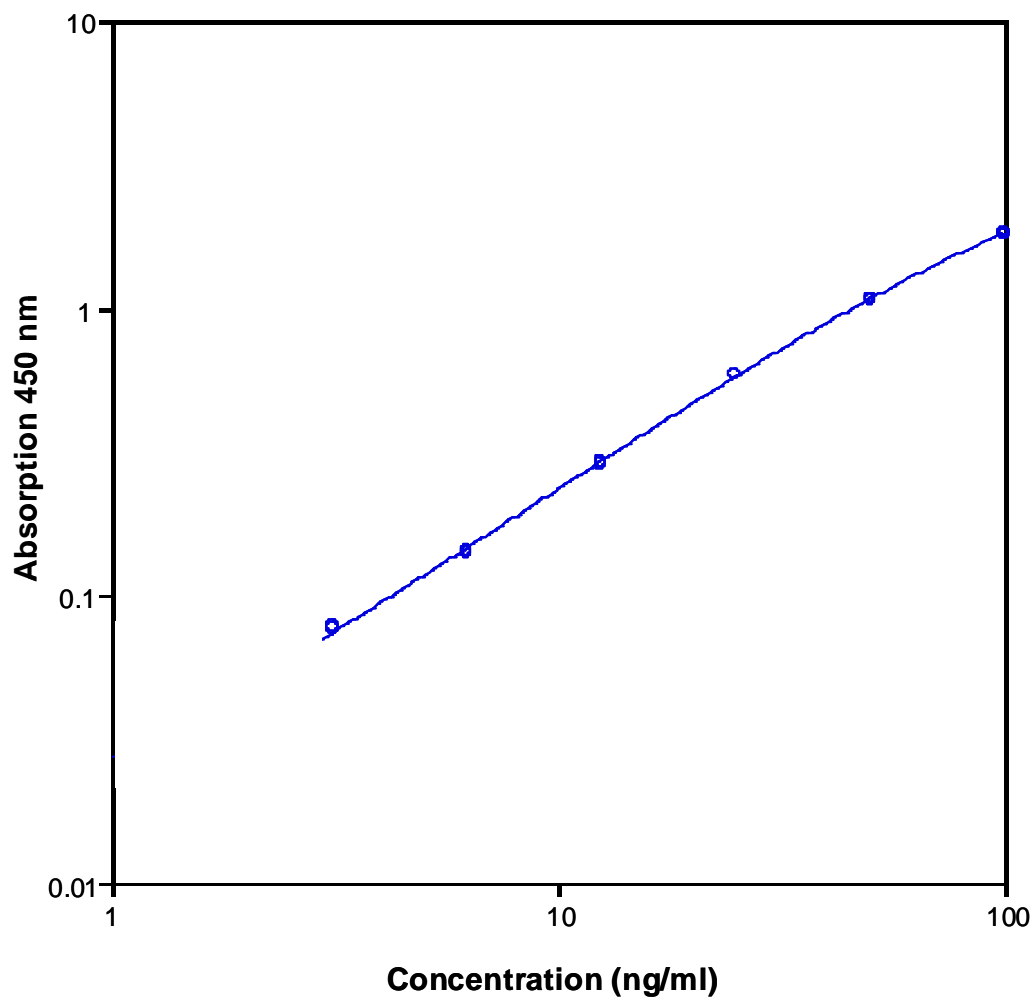


Table 2

Typical data using the human sVCAM-1 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

| Standard | Human sVCAM-1<br>Concentration (ng/ml) | O.D. at 450 nm | Mean O.D. at 450 nm | C.V. (%) |
|----------|--|----------------|---------------------|----------|
| 1        | 100.0                                  | 1.808<br>1.837 | 1.823               | 0.8      |
| 2        | 50.0                                   | 1.080<br>1.058 | 1.069               | 1.0      |
| 3        | 25.0                                   | 0.582<br>0.583 | 0.583               | 0.1      |
| 4        | 12.5                                   | 0.284<br>0.288 | 0.286               | 0.7      |
| 5        | 6.3                                    | 0.144<br>0.141 | 0.142               | 1.1      |
| 6        | 3.1                                    | 0.077<br>0.078 | 0.078               | 0.6      |
| Blank    | 0                                      | 0.032<br>0.028 | 0.030               | 6.7      |

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

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- 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

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### 13.1 Sensitivity

The limit of detection of human sVCAM-1 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 0.6 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 8 serum samples containing different concentrations of human sVCAM-1. 2 standard curves were run on each plate. Data below show the mean human sVCAM-1 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.1%.



Table 3

The mean human sVCAM-1 concentration and the coefficient of variation for each sample

| Sample | Experiment | Mean<br>sVCAM-1<br>Concentration (ng/ml) | Human<br>Coefficient of<br>Variation<br>(%) |
|--------|------------|--|---|
| 1      | 1          | 1550.5                                   | 1.9   |
|        | 2          | 1429.1                                   | 1.7   |
|        | 3          | 1397.5                                   | 0.3   |
| 2      | 1          | 689.2                                    | 1.4   |
|        | 2          | 698.3                                    | 2.6   |
|        | 3          | 660.6                                    | 3.0   |
| 3      | 1          | 781.8                                    | 3.2   |
|        | 2          | 808.7                                    | 1.5   |
|        | 3          | 732.5                                    | 1.3   |
| 4      | 1          | 543.2                                    | 6.1   |
|        | 2          | 575.5                                    | 2.4   |
|        | 3          | 500.3                                    | 2.7   |
| 5      | 1          | 395.4                                    | 3.4   |
|        | 2          | 461.1                                    | 7.4   |
|        | 3          | 430.4                                    | 8.3   |
| 6      | 1          | 435.3                                    | 1.4   |
|        | 2          | 458.4                                    | 3.3   |
|        | 3          | 431.5                                    | 2.5   |
| 7      | 1          | 384.7                                    | 6.0   |
|        | 2          | 412.1                                    | 2.0   |
|        | 3          | 368.1                                    | 0.6   |
| 8      | 1          | 460.0                                    | 3.2   |
|        | 2          | 498.6                                    | 2.8   |
|        | 3          | 458.8                                    | 4.5   |

### 13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human sVCAM-1. 2 standard curves were run on each plate. Data below show the mean human sVCAM-1 concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 5.2%.

Table 4

The mean human sVCAM-1 concentration and the coefficient of variation of each sample

| Sample | Mean Human sVCAM-1<br>Concentration<br>(ng/ml) | Coefficient of<br>Variation<br>(%) |
|--------|--|------------------------------------|
| 1      | 1459.0   | 5.5                                |
| 2      | 682.7  | 2.9                                |
| 3      | 774.3  | 5.0                                |
| 4      | 539.7  | 7.0                                |
| 5      | 428.9  | 7.7                                |
| 6      | 441.7  | 3.3                                |
| 7      | 388.3  | 5.7                                |
| 8      | 472.5  | 4.8                                |

### 13.3 Spiking Recovery

The spiking recovery was evaluated by spiking 7 levels of human sVCAM-1 into pooled normal serum samples. Recoveries were determined in 3 independent experiments with 6 replicates each.

The amount of endogenous human sVCAM-1 in unspiked serum was subtracted from the spike values.

The recovery ranged from 85% to 104% with an overall mean recovery of 89%.

### 13.4 Dilution Linearity

4 serum samples with different levels of human sVCAM-1 were analysed at serial 2 fold dilutions with 4 replicates each.

The recovery ranged from 87% to 120% with an overall recovery of 100% (see Table 5).

Table 5

| Sample | Dilution | Expected Human sVCAM-1 Concentration (ng/ml) | Observed Human sVCAM-1 Concentration (ng/ml) | Recovery of Expected Human sVCAM-1 Concentration (%) |
|--------|----------|--|--|--|
| 1      | 1:50     | --   | 1409.3                                       | --   |
|        | 1:100    | 704.6  | 639.5  | 91   |
|        | 1:200    | 352.3  | 306.0  | 87   |
|        | 1:400    | 176.2  | 166.2  | 94   |
| 2      | 1:50     | --   | 639.1  | --   |
|        | 1:100    | 319.5  | 308.3  | 97   |
|        | 1:200    | 159.8  | 174.2  | 109  |
|        | 1:400    | 79.9   | 86.9   | 109  |
| 3      | 1:50     | --   | 795.3  | --   |
|        | 1:100    | 397.7  | 368.2  | 93   |
|        | 1:200    | 198.8  | 189.5  | 95   |
|        | 1:400    | 99.4   | 94.5   | 95   |
| 4      | 1:50     | --   | 508.8  | --   |
|        | 1:100    | 254.4  | 244.8  | 96   |
|        | 1:200    | 127.2  | 152.7  | 120  |
|        | 1:400    | 63.6   | 70.1   | 110  |

### 13.5 Sample Stability

#### 13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human sVCAM-1 levels determined. There was no significant loss of human sVCAM-1 immunoreactivity detected by freezing and thawing.

#### 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 sVCAM-1 level determined after 24 h. There was no significant loss of human sVCAM-1 immunoreactivity detected during storage at -20°C, 2-8°C and RT.

A significant loss of human sVCAM-1 immunoreactivity (20%) was detected during storage at 37°C after 24 h.

### 13.6 Comparison of Serum and Plasma

From 2 individuals each, serum as well as EDTA, citrate, and heparin plasma was obtained at the same time and tested for human sVCAM-1. Concentrations were not significantly different and therefore all these blood preparations are suitable for use in the assay.

It is nevertheless highly recommended to assure the uniformity of blood preparations.

### 13.7 Specificity

The assay detects both natural and recombinant human sVCAM-1.

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human sVCAM-1 positive serum.

There was no crossreactivity detected, notably not with other members of the immunoglobulin gene superfamily.

### 13.8 Expected Values

Panels of 40 serum as well as EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human sVCAM-1.

The levels measured may vary with the sample collection used.

For detected human sVCAM-1 levels see Table 6.

Table 6

| Sample Matrix    | Number of Samples Evaluated | Range (ng/ml)  | Mean (ng/ml) | Standard Deviation (ng/ml) |
|------------------|-----------------------------|----------------|--------------|----------------------------|
| Serum            | 40                          | 400.6 – 1340.8 | 772.4        | 207.2                      |
| Plasma (EDTA)    | 40                          | 297.1 – 1563.3 | 704.7        | 304.1                      |
| Plasma (Citrate) | 40                          | 131.3 – 1222.7 | 763.5        | 224.4                      |
| Plasma (Heparin) | 40                          | 343.9 – 1254.2 | 670.2        | 235.5                      |

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## 15. REAGENT PREPARATION SUMMARY

### 15.1 Wash Buffer (1x)

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

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

### 15.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

| Number of Strips | Assay Buffer Concentrate (ml) | Distilled Water (ml) |
|------------------|-------------------------------|----------------------|
| 1 - 6            | 2.5                           | 47.5                 |
| 1 - 12           | 5.0                           | 95.0                 |

### 15.3 Conjugate Mixture

Make a 1:100 dilution of the **Conjugate Mixture** (Biotin-Conjugate mixed with Streptavidin-HRP) in Assay Buffer (1x) just prior to use:

| Number of Strips | Conjugate Mixture (ml) | Assay Buffer (1x) (ml) |
|------------------|------------------------|------------------------|
| 1 - 6            | 0.03                   | 2.97                   |
| 1 - 12           | 0.06                   | 5.94                   |

### 15.4 Human sVCAM-1 Standard

Reconstitute lyophilized **human sVCAM-1 standard** with distilled water. (Reconstitution volume is stated in the Quality Control Sheet.)

Prepare serial standard dilutions in Assay Buffer (1x).

### 15.5 Controls

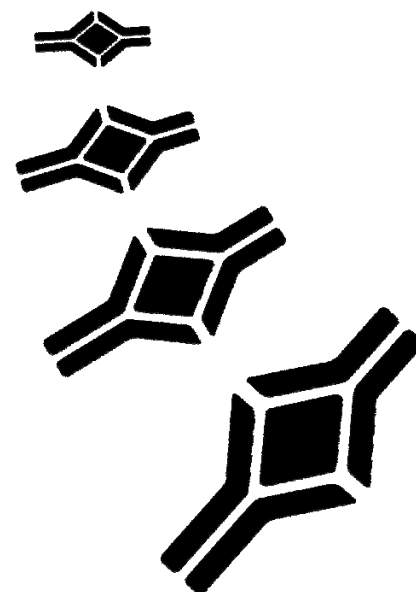
Add 150 µl distilled water to lyophilized **controls**.

## 16. TEST PROTOCOL SUMMARY

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1. Predilute serum and plasma samples with Assay Buffer (1x) 1:50.
2. Prepare Conjugate Mixture.
3. Determine the number of microwell strips required.
4. Wash microwell strips twice with Wash Buffer.
5. Add 50 µl Conjugate Mixture to all wells.
6. Add 100 µl of standard dilutions, in duplicate, to the standard wells.
7. Add 100 µl Assay Buffer (1x), in duplicate, to the blank wells.
8. Add 100 µl prediluted samples, in duplicate, to designated sample wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
10. Empty and wash microwell strips 3 times with Wash Buffer.
11. Add 100 µl of TMB Substrate Solution to all wells.
12. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
13. Add 100 µl Stop Solution to all wells.
14. Blank microwell reader and measure colour intensity at 450 nm.

**Note:** If instructions in this protocol have been followed serum and plasma samples have been diluted 1:50 (10 µl sample + 490 µl Assay Buffer (1x)), the concentration read from the standard curve must be multiplied by the dilution factor (x 50).



|   |   |   |   |  |
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