

READ BEFORE OPENING

- Vials may contain small quantities of material, hence ensure that they are centrifuged prior to opening.
- This set of reagents is intended for use by persons experienced in the use of immunoassays. It is not suitable for use by inexperienced personnel.
- A sample protocol is included but please note that the protocol provided is a guideline. The type of substrate as well as all other reagents not included in the module set may influence test performance.

WORKING PROTOCOL FOR THE sVCAM-1 MODULE SET BMS232MST

1) Reagents provided (for 10 ELISA plates):

- 5.5 ml coating antibody (100 µg/ml)
- 0.5 µg sVCAM-1 standard protein (lyophilized)
- 70 µl Biotin-conjugate
- 45 µl Streptavidin-HRP

a) Phosphate buffered saline (PBS)

| | |
|--|--------|
| NaCl | 8.00 g |
| KCl | 0.20 g |
| Na ₂ HPO ₄ x 12 H ₂ O | 2.85 g |
| KH ₂ PO ₄ | 0.20 g |

Dissolve the salts in distilled water and adjust to 1 litre.

b) Assay Buffer:

| | |
|----------------------------|--------------------|
| Bovine Serum Albumin (BSA) | 5 g |
| Tween 20 | 0.5 ml |
| PBS | adjust to 1 litre. |

Dissolve ingredients in approx. 500ml PBS, then adjust to 1 litre with PBS.

- c) Wash Buffer:
Add 0.5 ml Tween 20 to 1 litre of PBS and mix well.
- d) Microwell plate (Maxi sorb)
- e) Substrate Solution: 1:2 mixture of H₂O₂ and Tetramethylbenzidine (KPL Gaithersburg, Maryland)
- f) Stop Solution: N Sulfuric Acid (2 ml conc. (36N) Sulfuric Acid + 16 ml H₂O).

3) Storage conditions:

Store the reagents of the module set at -20°C. Immediately after use reagents should be returned to -20°C storage. Avoid several freeze-thaw cycles. Aliquot reagents for use at different time points. Expiry of the reagents is stated on labels.

4) Preparation of reagents:

Please note: Centrifuge vials before opening to collect contents.

a) Preparation of the microwell Plate:

Coating:

The final antibody concentration is 5 µg/ml; 100 µl of the coating solution are added to each well. Dilute the coating antibody as following for one microtiter plate:

| | |
|----------|------------------------------|
| 10.45 ml | PBS |
| 550.0 µl | coating antibody (100 µg/ml) |
| <hr/> | |
| 11.0 ml | coating solution |

Immediately after coating, seal the plate with a plate cover and transfer to 2-8°C, allowing the binding process to take place over night.

Aspirate the contents of the wells and wash once with about 300 µl of Wash Buffer according the Washing procedure described in the test protocol below.

Blocking:

Add 250 µl of Assay Buffer to each well and allow the binding reaction to take place for two hours at room temperature (alternatively the plate may be blocked over night at 2-8°C).

Wash the plate twice (see below) immediately before the samples are added to the wells. The blocked plates can be stored at 2-8°C up to one week.

Fixing:

If you want to store the coated plates for a longer period of time, just aspirate the blocking solution and proceed by adding 150 µl Fixing solution (PBS, 15% Sucrose) to each well. Incubate 1 h at room temperature, aspirate and dry plates over night at 28°C. When

sealed with desiccant, the plates can be stored at 2-8 °C for at least 2 months.

b) Preparation of Standard:

The final concentration of the sVCAM-1 standard protein is 200 ng/ml. Reconstitute the lyophilized stock material by addition of distilled H₂O. Reconstitution volume is indicated on the label of the standard vial and proceed as following for one standard curve:

| | |
|--------|--|
| 23 µl | Standard Protein reconstituted (2 µg/ml) |
| 207 µl | Assay Buffer |
| <hr/> | |
| 230 µl | Standard Protein (200 ng/ml) |

Store the reconstituted Standard aliquoted at -20°C. Aliquoted Standard is stable for 3 months at -20°C

Label six tubes, one for each standard curve point.

100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.3 ng/ml, 3.2 ng/ml

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

Pipette 230 µl of Assay Buffer into each tube.

Pipette 230 µl of reconstituted standard into the first tube, labelled 100 ng/ml, and mix.

Pipette 230 µl of this dilution into the second tube, labelled 50 ng/ml, and mix.

Repeat serial dilutions three more times thus creating the points of the standard curve.

c) Preparation of Biotin-Conjugate:

The Biotin-Conjugate must be diluted 1:500 with Assay Buffer before use. Dilute the stock material as following for one microwell plate:

| | |
|---------|------------------|
| 6 µl | Biotin-Conjugate |
| 2994 µl | Assay Buffer |
| <hr/> | |
| 3000 µl | Biotin-Conjugate |

d) Preparation of Streptavidin-HRP

The stock solution must be diluted 1:1200 with Assay Buffer before use as following for one microwell plate:

$$\begin{array}{r} 2.5 \mu\text{l} \text{ Streptavidin-HRP} \\ 2997.5 \mu\text{l} \text{ Assay Buffer} \\ \hline 3000.0 \mu\text{l} \text{ Streptavidin-HRP} \end{array}$$

Make a 1:2 mixture of reagents c) + d).

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

TEST PROTOCOL

- a. Dilute serum or plasma samples 1:50 with Assay Buffer according to the following dilution scheme:
10 μ l Sample + 490 μ l Assay Buffer
- b. Prepare **Biotin-Conjugate/Streptavidin-HRP mixture** (refer to preparation of reagents).
- c. Add 50 μ l of diluted **Biotin-Conjugate/Streptavidin-HRP mixture** to all wells, including the blank wells.
- d. Add 100 μ l of prediluted **standards** to the designated wells according to Figure 1.

Figure 1

| | 1 | 2 | 3 | 4 |
|----------|----------------------------|----------------------------|----------|-----------|
| A | Standard 1 (100 ng/ml) | Standard 1 (100 ng/ml) | Sample 2 | Sample 2 |
| B | Standard 2 (50 ng/ml) | Standard 2 (50 ng/ml) | Sample 3 | Sample 3, |
| C | Standard 3 (25 ng/ml) | Standard 3 (25 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 (3.2 ng/ml) | Standard 6 (3.2 ng/ml) | Sample 7 | Sample 7 |
| G | Blank | Blank | Sample 8 | Sample 8 |
| H | Sample I | Sample I | Sample 9 | Sample 9 |

- e. Add 100 μ l of **Assay Buffer** in duplicate to the blank wells.
- f. Add 100 μ l of each 1:50 diluted **Sample**, in duplicate, to the designated wells.
- g. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, if available on a rotator set at 100 rpm.
- h. Prepare TMB Substrate Solution a few minutes prior to use.
- i. Remove Plate Cover and empty wells. Wash the microwell strips 3 times with approximately 400 μ l **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, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

- j. Pipette 100 µl of mixed **TMB Substrate Solution** to all wells, including the blank wells.
- k. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light.
The colour development on the plate should be monitored and the substrate reaction stopped (see point n. 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.
- l. Stop the enzyme reaction by pipetting 100 µl of **4N Sulfuric Acid** into each well, including the blank wells. It is important that the Sulfuric Acid is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Sulfuric Acid is added or within one hour if the microwell strips are stored at 2-8°C in the dark.
- m. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the sVCAM-1 standards.

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 sVCAM-1 concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating 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 sVCAM-1 concentration.
- **For samples which have been diluted according to the instructions given in this manual 1:50, the concentration read from the standard curve must be multiplied by the dilution factor (x 50).**

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low sVCAM-1 levels. Such samples require further dilution e.g. 1:100, 1:200 with Assay Buffer in order to precisely quantitate the actual sVCAM-1 level.

- It is suggested that each testing facility establishes a control sample of known sVCAM-1 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

A basic understanding of immunoassay development and technical experience in ELISA performance are prerequisite for the successful use of this module set.

The protocol provided is just a guideline. The type of substrate as well as all other reagents not included in the module set may influence the test characteristics.

GENERAL INFORMATION

Summary

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 (6,15). Domains 1 through 3 are highly homologous to domains 4 through 6, suggesting that they arose by gene duplication (6,15). The cDNA of 7D VCAM-1 predicts a core protein of approximately 81 kD with seven potential N-linked glycosylation sites. Upon complete glycosylation the mature protein has a molecular weight of approximately 102 kD. This observation is in general agreement with immunoprecipitation studies that show a protein of approximately 110 kD on cytokine-activated endothelium (5,25).

Murine and rat VCAM-1 have been cloned (14,31). 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) (14). 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 (27). VCAM-1 also participates in leukocyte adhesion outside of the vasculature, mediating precursor lymphocyte adhesion to bone marrow stromal cells (26) and B cell binding to lymph node follicular dendritic cells (10).

VCAM-1 is not constitutively expressed on endothelium, but can be up-regulated in vitro in response to LPS, TNF- α , and IL-1 (4,24), as well as to interferon- γ and IL-4 (21).

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 (7,20). Soluble VCAM-1 levels have been found in the serum of healthy

individuals (11) and increased levels of sVCAM-1 can be detected in several diseases (12):

Cancer: (2,9) ovarian, gastric-intestinal, renal, bladder cancer (12), non-Hodgkin's lymphoma (12), breast cancer (cyst fluid) (17);

Autoimmune diseases: multiple sclerosis (cerebrospinal fluid) (8), systemic sclerosis (13), systemic lupus erythematosus (23,28,29), rheumatoid arthritis (12,22,29);

Infections: sepsis (16), meningitis (16), malaria (16);

Inflammation: vasculitis (12), alcoholic cirrhosis (1), primary biliary cirrhosis (19), Wegener's granulomatosis (23);

Others: impaired renal function (11), haemodialysis (11), hyperthyroidism (30), renal allograft (3,12,18).

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Specificity

The assay recognizes both natural and recombinant human sVCAM-1. To define the specificity of this ELISA several polypeptides were tested for cross reactivity. There was no cross reactivity determined for any of the tested proteins, most notably there is no interference other members of the immunoglobulin superfamily.

Expected Values

A panel of 111 sera from apparently healthy blood donors (male and female) was tested for sVCAM-1. The detected sVCAM-1 levels ranged between 675 and 1693 ng/ml with a mean level of 1090 ng/ml and a standard deviation of ± 237 ng/ml.

ORDERING INFORMATION

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