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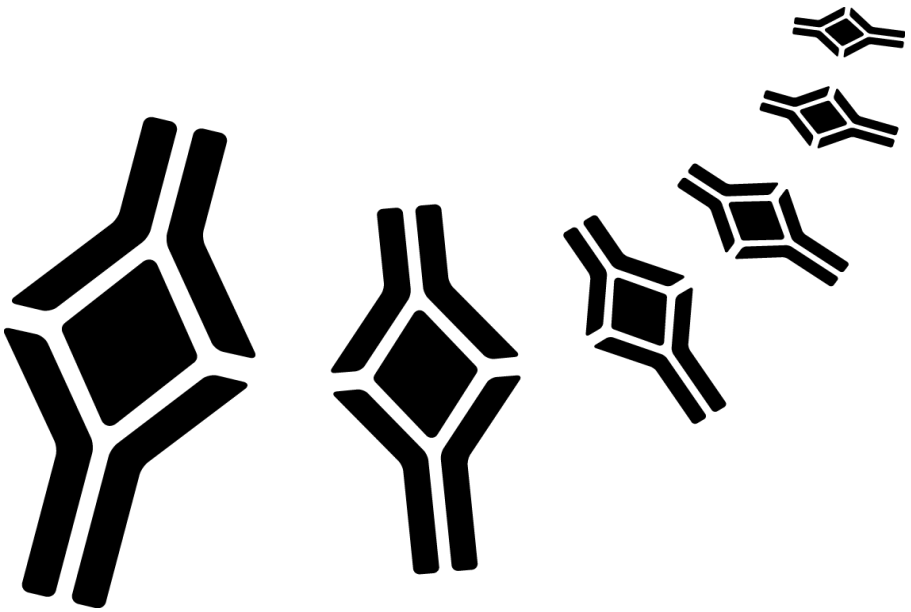


Monkey sPECAM-1 ELISA

Product Data Sheet

Cat. No.: RAF110R

For Research Use Only

CONTENTS

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 Monkey sPECAM-1 Coated ELISA Kit is an enzyme-linked immunosorbent assay for quantitative detection of soluble monkey platelet endothelial cell adhesion molecule-1.

2. SUMMARY

PECAM-1 (platelet endothelial cell adhesion molecule-1) also called CD31 and EndoCAM is a newly characterized adhesion molecule that belongs to the immunoglobulin superfamily. PECAM-1 is a transmembrane glycoprotein with a molecular weight of approximately 130 kDa, depending on the degree of glycosylation.

PECAM-1 is constitutively expressed on all vascular cells and has provided a useful immunohistochemical marker of blood vessels, particularly in the setting of angiogenesis. It has also been found on platelets, monocytes, neutrophils and CD8+ T-cells. Bone marrow stem cells and transformed cell lines of the myeloid and megakaryocytic lineage also express PECAM-1. Interestingly, PECAM-1 was also detected on solid tumor lines.

Recent studies suggest a role for PECAM-1 in the inflammatory process and leukocyte-endothelial interaction. The process of leukocyte emigration to the site of inflammation can be dissected into three successive stages:
(1) rolling, mediated by the selectins;
(2) tight adhesion mediated by ICAMs and their counter-receptors, the integrins; and
(3) transmigration of leukocytes through intercellular junctions of vascular endothelial cells which requires PECAM-1.
PECAM-1 appears to be able to interact both with itself (homophilic binding) and with other "non-PECAM-1" molecules (heterophilic binding).

PECAM-1 is an early and sensitive marker for tumor-induced angiogenesis. Several data have suggested that PECAM-1 may be involved in the process of angiogenesis in a developing vertebrate embryo as well as during metastases formation.

Besides the membrane-bound form of PECAM-1 a soluble form of the molecule exists, which is smaller than cell-associated PECAM-1, and contains the cytoplasmic tail. This form of soluble PECAM-1 is encoded by an alternatively spliced mRNA from which the exon containing the transmembrane domain has been removed. Soluble PECAM-1 can be detected in plasma.

3. PRINCIPLES OF TEST

An anti-monkey-PECAM-1 monoclonal coating antibody is adsorbed onto microwells.

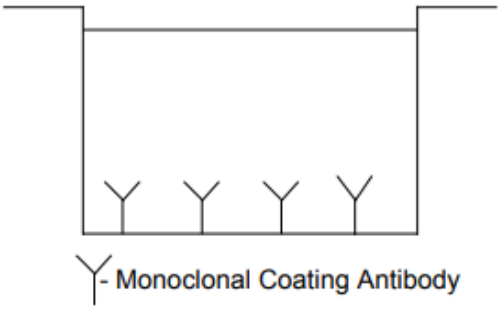


Figure 1 Coated microwell

Monkey PECAM-1 present in the sample or standard binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-monkey-PECAM-1 antibody is added and binds to monkey PECAM-1 captured by the first antibody.

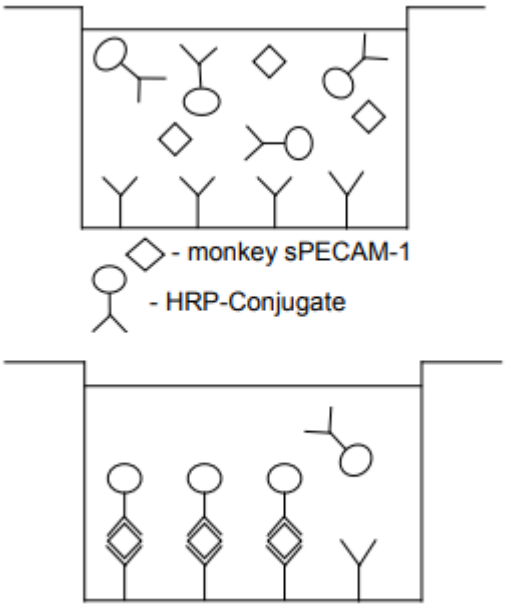


Figure 2 First incubation

Following incubation unbound enzyme conjugated anti-monkey-PECAM-1 is removed during a wash step and substrate solution reactive with HRP is added to the wells.

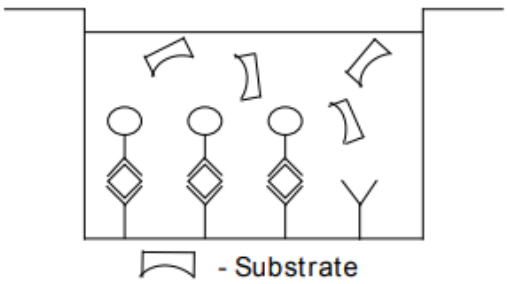


Figure 3 Second incubation

A colored product is formed in proportion to the amount of soluble monkey PECAM-1 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven monkey PECAM-1 standard dilutions and monkey PECAM-1 sample concentration determined.

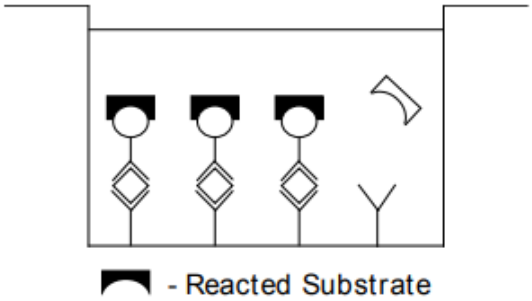


Figure 4 Stop reaction

4. REAGENTS PROVIDED

1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with Monoclonal Antibody (murine) to monkey PECAM-1
2 vials (5 µl) HRP-Conjugate anti-monkey-PECAM-1 monoclonal (murine) antibody
2 vials (60 U/ml) monkey PECAM-1 Standard
1 bottle (50 ml) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)
1 vial (5 ml) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)
1 vial (12 ml) Sample Diluent (buffered protein matrix)
1 vial (15 ml) Substrate Solution (tetramethyl-benzidine)
1 vial (15 ml) Stop Solution (1M Phosphoric acid)
2 adhesive Plate Covers

5. STORAGE INSTRUCTIONS

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C 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. SAMPLE COLLECTION

Cell culture supernatant and serum (baboon) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Pay attention to a possible *Hook Effect* due to high sample concentrations.

Samples containing a visible precipitate must be clar'fied 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 monkey sP-selectin. If samples are to be run within 24 hours, they may be stored at 2°C to 8°C.

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)
- Microplate 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 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 samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that 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 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 samples 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

1. Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
2. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

Wash Buffer (1x)

1. Pour entire contents (50 ml) of the Wash Buffer Concentrate (20x) into a clean 1000 ml graduated cylinder. Bring to final volume to 1000 ml with glass-distilled or deionized water.
2. Mix gently to avoid foaming.
3. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
4. 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

Assay Buffer (1x)

1. Pour the entire contents (5 ml) of the Assay Buffer Concentrate (20x) into a clean 100 ml graduated cylinder. Bring to final volume to 100 ml with distilled water. Mix gently to avoid foaming.
2. Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.
3. 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

Preparation of HRP-Conjugate

Note: The HRP-Conjugate should be used within 30 minutes after dilution.

1. Dilute the HRP-Conjugate 1:25 just prior to use by adding 120 µl Assay Buffer (1x) to the tube containing the HRP-Conjugate concentrate. Mix the contents of the tube well.
2. Make a further 1:100 dilution with Assay Buffer (1x) in a clean plastic tube.

3. The second dilution (1:100) of the HRP-Conjugate may be prepared as needed according to the following table:

Number of Strips	prediluted (1:25) HRP-Conjugate ml	Assay Buffer (1x) ml
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Monkey PECAM-1 standard

Standard dilutions can be prepared directly on the microwell plate or alternatively in tubes (see “External standard dilution“ on page 9).

External Standard Dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5 S6, S7.
2. Then prepare 1:2 serial dilutions for the standard curve as follows: Pipette 225 µl of Sample Diluent into each tube.
3. Pipette 225 µl of diluted standard (concentration = 60.0 U/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 30 U/ml).
   Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
4. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 5).

   Sample Diluent serves as blank.

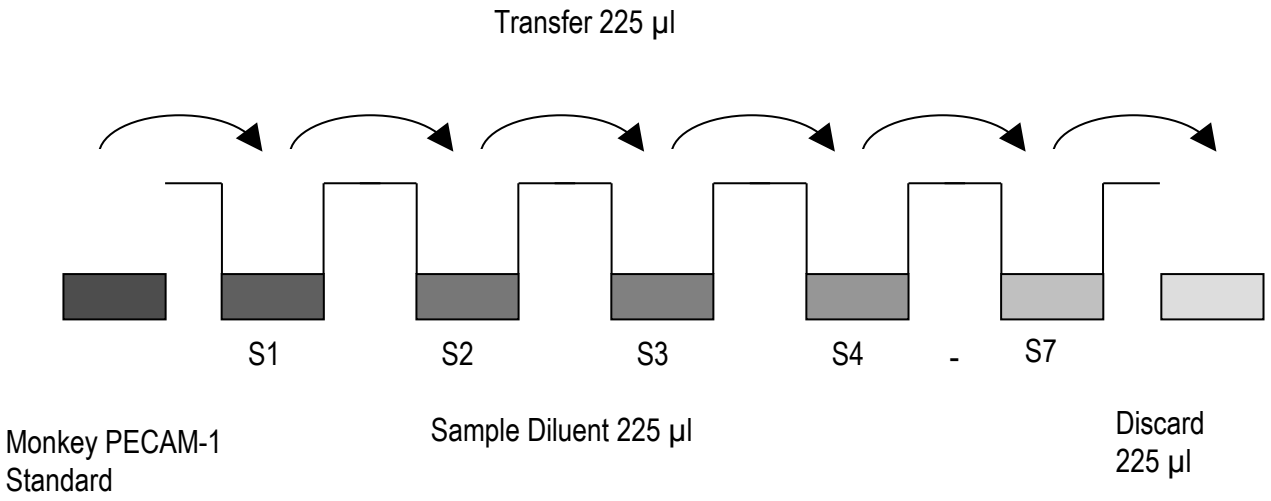


Figure 5 Dilute standards - tubes

10. TEST PROTOCOL

1. 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°C to 8°C sealed tightly.
2. 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, empty wells and 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.
3. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes – see "External standard dilution" on page 9):

   Add 100 µl of Sample Diluent in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of monkey PECAM-1 Standard, in duplicate, into well A1 and A2 (see Figure 5 and Figure 6). Mix the contents by repeated aspiration and ejection and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of monkey PECAM-1 standard dilutions ranging from 30.00 to 0.48 U/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

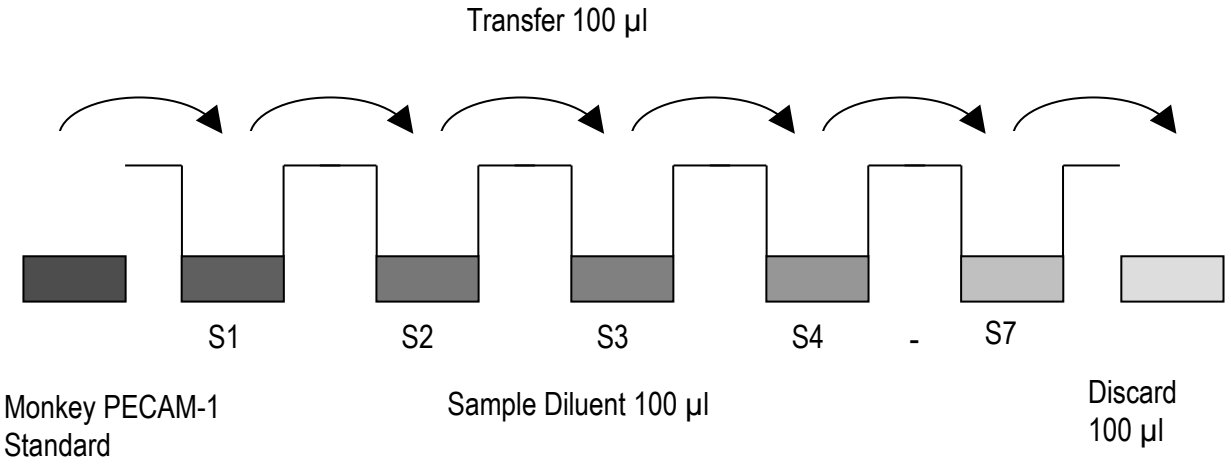


Figure 6 Dilute standards - microwell plate

In case of an external standard dilution (see “External standard dilution“ on page 9), pipette 100 µL of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1 Example of the arrangement of blanks, standards and samples in the microwell strips.

	1	2	3	4
A	Standard 1 30.00 U/ml	Standard 1 30.00 U/ml	Sample 1	Sample 1
B	Standard 2 15.00 U/ml	Standard 2 15.00 U/ml	Sample 2	Sample 2
C	Standard 3 7.50 U/ml	Standard 3 7.50 U/ml	Sample 3	Sample 3
D	Standard 4 3.75 U/ml	Standard 4 3.75 U/ml	Sample 4	Sample 4
E	Standard 5 1.90 U/ml	Standard 5 1.90 U/ml	Sample 5	Sample 5
F	Standard 6 0.95 U/ml	Standard 6 0.95 U/ml	Sample 6	Sample 6
G	Standard 7 0.48 U/ml	Standard 7 0.48 U/ml	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

4. Add 100 µl of Sample Diluent in duplicate to the blank wells.
5. Add 90 µl of Sample Diluent to the sample wells.
6. Add 10 µl of each Sample in duplicate to the sample wells.
7. Prepare HRP-Conjugate (see “Preparation of HRP-Conjugate“ on page 8).
8. Add 50 µL of diluted HRP-Conjugate to all wells, including the blank wells.
9. Cover with a Plate Cover and incubate at room temperature (18°C to 25°C) for 3 hours on a microplate shaker.
10. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point 2. of the test protocol. Proceed immediately to the next step.
11. Pipette 100 µl of TMB Substrate Solution to all wells, including the blank wells.
12. Incubate the microwell strips at room temperature (18°C to 25°C) for about 10 minutes. Avoid direct exposure to intense light. The color development on the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly recordable. Determination of the ideal time period for color 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 color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as an OD of 0.9 – 0.95 is reached.
13. 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°C to 8°C in the dark.

14. 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 monkey PECAM-1 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% of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the monkey PECAM-1 concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating monkey PECAM-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 monkey PECAM-1 concentration.
- If instructions in this protocol have been followed, samples have been diluted 1:10 and the concentration read from the standard curve must be multiplied by the dilution factor (x 10).
- Calculation of samples with a concentration exceeding standard 1 will result in incorrect, low monkey sP-selectin levels (*Hook Effect*). Such samples require further external predilution dilution according to monkey PECAM-1 values with Sample Diluent in order to precisely quantitate the actual monkey PECAM-1 level.
- It is suggested that each testing facility establishes a control sample of known monkey PECAM-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 representative standard curve is shown in Figure 7. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

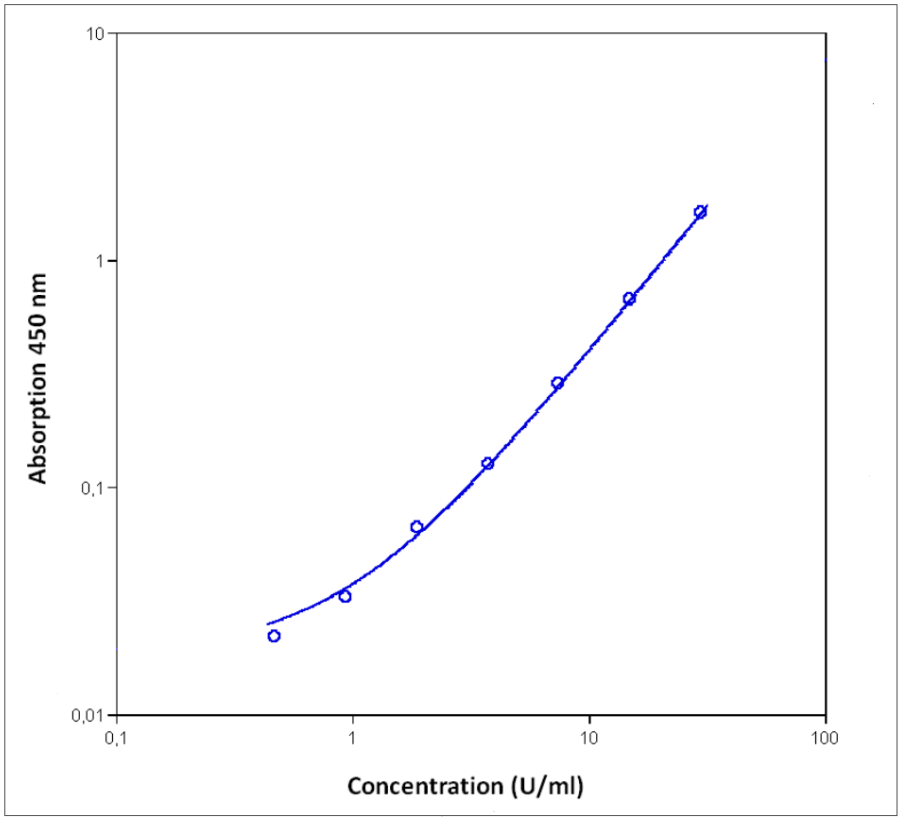


Figure 7 Representative standard curve for the monkey PECAM-1 ELISA. Monkey PECAM-1 was diluted in serial two-fold steps in Sample Diluent, symbols represent the mean of three parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

Table 1 Typical data using the monkey PECAM-1 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	monkey PECAM-1 Concentration U/ml	O.D. Mean	C.V. %
1	30.00	1.618	2.8
2	15.00	0.661	6.8
3	7.50	0.287	5.4
4	3.75	0.126	0.5
5	1.90	0.066	7.7
6	0.95	0.033	5.4
7	0.48	0.022	7.4
Blank	0.00	0.007	-

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 color 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. 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.

13. PERFORMANCE CHARACTERISTICS

13.1 Sensitivity

The limit of detection of monkey PECAM-1 defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 0.06 U/ml (mean of 6 independent assays).

13.2 Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in independent experiments. The overall Intra-assay coefficient of variation has been calculated to be 2 %

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in independent experiments by three technicians. The overall inter-assay coefficient of variation has been calculated to be <10%.

13.3 Spiking Recovery

Spiked samples were prepared by adding four different levels of monkey PECAM-1 to a monkey serum sample. The overall mean recovery was 110 %.

13.4 Dilution Linearity

Four serum samples with different levels of monkey PECAM-1 were assayed at four serial two-fold dilutions (1:10 - 1:80) with 4 replicates each. The overall mean recovery was 105 %.

13.5 Sample Stability

Freeze-Thaw stability

Aliquots of serum samples (unspiked or spiked with monkey PECAM-1) were stored at -20°C and thawed several times, and monkey PECAM-1 levels determined. There was no significant loss of monkey PECAM-1 concentration between 0 and 5 freeze-thaw cycles.

Storage Stability

Aliquots of a serum sample (unspiked or spiked with monkey PECAM-1) were stored at -20°C, 2°C to 8°C, room temperature (RT) and at 37°C, and the monkey PECAM-1 level determined after 24 hours. There was no significant loss of monkey PECAM-1 immunoreactivity during storage under above conditions.

14. REAGENT PREPARATION SUMMARY

14.1 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

14.2 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

14.3 HRP-Conjugate

Add 120 µl Assay Buffer (1x) to the tube containing HRP-Conjugate concentrate. Mix. Make further dilution according to table. Make a 1:100 dilution of HRP-Conjugate in Assay Buffer (1x):

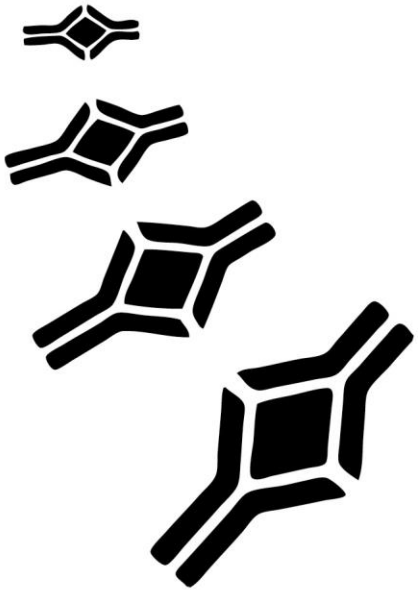
Number of Strips	HRP-Conjugate (prediluted 1:25) (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

15. TEST PROTOCOL SUMMARY

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to standard wells. Pipette 100 µl monkey PECAM-1 Standard into the first wells and create standard dilutions ranging from 30.00 to 0.48 U/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells. Alternatively external standard dilution in tubes: Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
5. Add 90 µl Sample Diluent to the sample wells.
6. Add 10 µl Sample, in duplicate, to designated wells.
7. Prepare HRP-Conjugate.
8. Add 50 µl of diluted HRP-Conjugate to all wells.
9. Cover microwell strips and incubate 3 hours at room temperature (18°C 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 including blank wells.
12. Incubate the microwell strips for about 10 minutes at room temperature (18°C to 25°C).
13. Add 100 µl Stop Solution to all wells including blank wells.
14. Blank microwell reader and measure color intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:10 and the concentration read from the standard curve must be multiplied by the dilution factor (x 10).

NOTES



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