Quantikine® ELISA

Human CD31/PECAM-1 Immunoassay

Catalog Number DCD310

For the quantitative determination of human CD31 concentrations in cell culture supernates, serum, plasma, saliva, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is a 130 kDa heavily glycosylated transmembrane protein belonging to the immunoglobulin (Ig) superfamily of cell adhesion molecules. CD31 is highly expressed on endothelial cells and at a lower level on platelets, granulocytes, macrophages, dendritic cells, T and B cells, and natural killer (NK) cells. It is involved in cell adhesion and is required for transepithelial migration of leukocytes (TEM) (1, 2). CD31 is composed of an extracellular domain (ECD) of 574 amino acids (aa) containing six Ig-like domains, a transmembrane domain, and a 118 aa cytoplasmic domain (3). The latter undergoes alternative splicing which generates multiple isoforms showing altered adhesive properties compared to full length CD31 (4). The human CD31 ECD shares 63% and 61% aa identity with mouse and rat CD31 respectively.

CD31 acts as a homophilic receptor through its extracellular domain and is also involved in binding via its cytoplasmic domain (5). This domain contains highly conserved ITIM motifs which, once tyrosine-phosphorylated, recruit and activate the signaling molecules Src and SHP-2 (6, 7). The resulting inhibition of TCR signaling increases the activation threshold of T cells, thus reinforcing peripheral tolerance and preventing development of autoimmunity (8). CD31 additionally regulates immune responses by acting as a key inhibitory receptor in dendritic cell development (9). Besides its role in TEM, CD31 appears to regulate T cell trafficking through a complex coordination of endothelial cell junctions and T cell extravasation (10). *In vitro*, a 110 kDa soluble form of CD31 is released following shedding of the extracellular domain during endothelial cell apoptosis (11). This ectodomain has also been described in the serum of patients suffering from myocardial infarction, acute ischaemic stroke, and multiple sclerosis, conditions that involve tissue damage and endothelial cell apoptosis (12, 13, 14).

The Quantikine Human CD31/PECAM-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human CD31 in cell culture supernates, serum, plasma, saliva, and urine. It contains CHO cell-expressed recombinant human CD31 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human CD31 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human CD31.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human CD31 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CD31 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human CD31 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of CD31 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human CD31 Microplate	894834	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human CD31.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human CD31 Standard	894836	2 vials of recombinant human CD31 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Calibrator Diluent RD6-9	895423	21 mL of a buffered animal serum with preservatives. *Use undiluted for serum/ plasma samples. Use diluted 1:5 for cell culture supernate/urine/saliva samples.*	Undiluted may be stored for up to 1 month at 2-8 °C.* Diluted, discard after use.
Human CD31 Conjugate	894835	21 mL of a polyclonal antibody specific for human CD31 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-76	895812	11 mL of a buffered protein base with and preservatives. *May contain a precipitate. Mix well before and during use.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human CD31 Controls (optional; R&D Systems, Catalog # QC206).

PRECAUTIONS

CD31 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

Calibrator Diluent RD6-9 contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *CD31 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

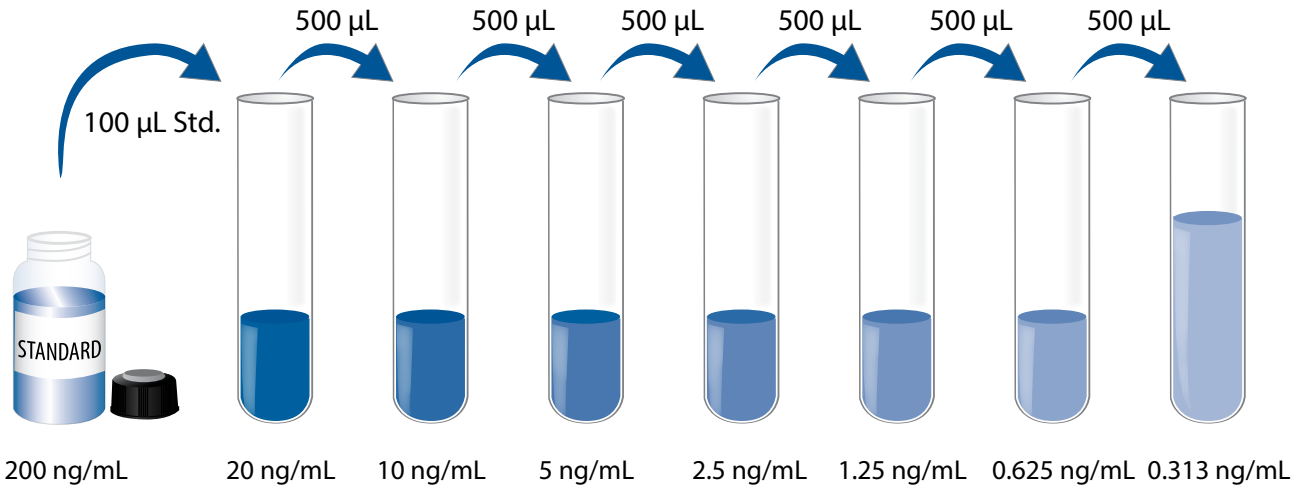
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD6-9 (diluted 1:5) - For cell culture supernate/saliva/urine samples only. Add 4 mL of Calibrator Diluent RD6-9 to 16 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD6-9 (diluted 1:5). **Discard after use.**

Human CD31 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human CD31 Standard with deionized or distilled water. This reconstitution produces a stock solution of 200 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6-9 (*for serum/plasma samples*) or Calibrator Diluent RD6-9 (diluted 1:5) (*for cell culture supernate/saliva/urine samples*) into the 20 ng/mL tube. Pipette 500 µL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *CD31 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-76 to each well.
4. Add 50 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human CD31 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

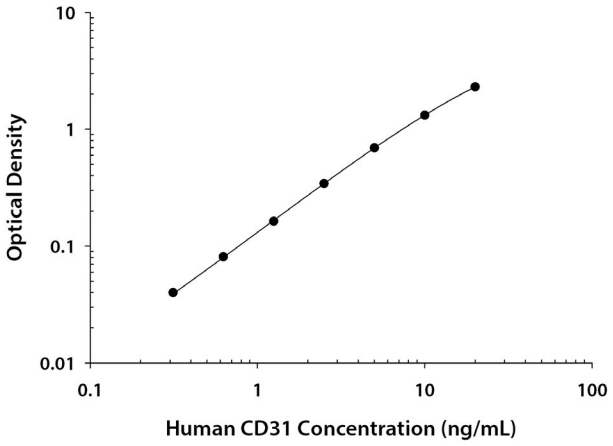
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human CD31 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

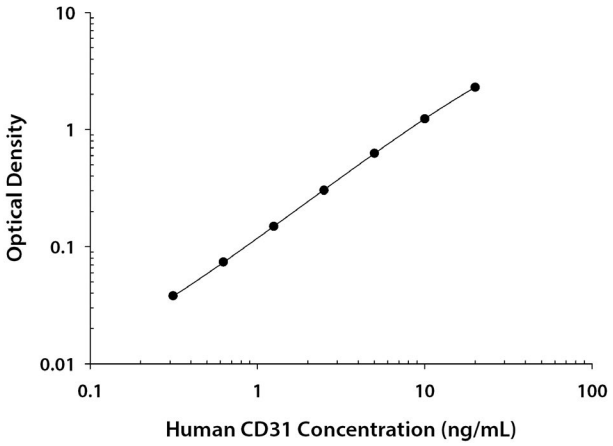
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/SALIVA/URINE ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.013 0.013	0.013	—
0.313	0.052 0.054	0.053	0.040
0.625	0.094 0.094	0.094	0.081
1.25	0.175 0.177	0.176	0.163
2.5	0.351 0.360	0.356	0.343
5	0.704 0.707	0.706	0.693
10	1.311 1.352	1.332	1.319
20	2.310 2.320	2.315	2.302

SERUM/PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.010 0.010	0.010	—
0.313	0.046 0.049	0.048	0.038
0.625	0.081 0.087	0.084	0.074
1.25	0.150 0.168	0.159	0.149
2.5	0.313 0.313	0.313	0.303
5	0.622 0.654	0.638	0.628
10	1.221 1.267	1.244	1.234
20	2.263 2.345	2.304	2.294

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE/SALIVA/URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	1.62	4.70	9.96	1.71	5.00	10.6
Standard deviation	0.065	0.117	0.372	0.104	0.252	0.411
CV (%)	4.0	2.5	3.7	6.1	5.0	3.9

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	2.01	5.86	12.3	1.90	5.71	12.0
Standard deviation	0.070	0.182	0.403	0.163	0.363	0.677
CV (%)	3.5	3.1	3.3	8.6	6.4	5.6

RECOVERY

The recovery of human CD31 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	96-112%
Serum* (n=4)	104	100-107%
EDTA plasma* (n=4)	100	97-106%
Heparin plasma* (n=4)	101	94-108%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human CD31 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)	Saliva (n=4)
1:2	Average % of Expected	100	106	106	106	96	93
	Range (%)	97-103	103-107	104-108	102-108	82-107	87-98
1:4	Average % of Expected	101	106	106	108	98	86
	Range (%)	99-104	99-109	102-112	100-113	85-108	83-89
1:8	Average % of Expected	101	107	106	108	101	———
	Range (%)	100-102	101-112	102-111	101-114	88-109	———
1:16	Average % of Expected	95	105	105	109	101	———
	Range (%)	89-98	93-114	97-114	99-119	———	———

SENSITIVITY

Forty-four assays were evaluated and the minimum detectable dose (MDD) of human CD31 ranged from 0.008-0.078 ng/mL. The mean MDD was 0.021 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified CHO cell-expressed recombinant human CD31 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human CD31 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=36)	13.1	8.72-18.4	2.37
EDTA plasma (n=36)	11.8	7.18-17.1	2.44
Heparin plasma (n=36)	11.3	7.89-17.1	2.57
Urine (n=10)	3.95	2.83-6.08	1.06

Sample Type	Mean of Detectable (ng/mL)	% Detecatable	Range (ng/mL)
Saliva (n=8)	1.12	75	ND-2.99

ND=Non-detectable

Cell Culture Supernates:

$CD8^+$ T cells were isolated from PBMCs, stimulated with anti-human CD3, anti-human CD28, and 20 ng/mL of recombinant human IL-2 for 5 days. Cells were then stimulated for 20 hours with 10 ng/mL PMA and 500 ng/mL ionomycin. An aliquot of the cell culture supernate was removed, assayed for human CD31, and measured 3.34 ng/mL.

THP-1 human acute monocytic leukemia cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 10 U/mL penicillin, and 10 µg/mL streptomycin sulfate until logarithmic growth phase was achieved. Cells were then cultured unstimulated or stimulated for 4 hours with 1 µg/mL LPS followed by 30 minutes of 5 mM ATP treatment. Aliquots of the cell culture supernates were removed and assayed for human CD31.

Condition	(ng/mL)
Unstimulated	1.50
Stimulated	3.58

SPECIFICITY

This assay recognizes natural and recombinant human CD31.

The factors listed below were prepared at 200 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range human CD31 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ALCAM
BCAM
Cadherin-8
Cadherin-11
CEACAM-1
CEACAM-5
CHL-1
Contactin-1
CNTN2
CNTN4
DC-SIGN R
Desmoglein-1
Desmoglein-2
DNAM-1
E-Cadherin
E-Calectin
Endocan
EpCAM
E-Selectin
FCRL1
FCRL2
FCRL3
FCRL4
FCRL5
FCRL6
Galectin-1
Galectin-2
Galectin-3
Galectin-4
Galectin-7
Galectin-8
ICAM-1
ICAM-2
ICAM-3
ICAM-5
Integrin αVβ3
JAM-1
JAM-2
JAM-3
LOX-1
L-Selectin
M-Cadherin
MCAM
N-Cadherin
NCAM-L1
NrCAM
P-Cadherin
P-Selectin
Siglec-2
Siglec-3
Siglec-5
Siglec-6
Siglec-7
Siglec-9
Siglec-10
TROP-2
VCAM-1
VE-Cadherin

Recombinant mouse:

ALCAM
CD31
CHL-1
ICAM-1
ICAM-2
ICAM-5
VCAM-1
E-Cadherin
Endocan
E-Selectin
Galectin-1
Galectin-3
Galectin-7
ICAM-5
JAM-1
JAM-2
JAM-3
LOX-1
L-Selectin
MAdCAM-1
P-Cadherin
P-Selectin
Siglec-2
Siglec-F
VE-Cadherin

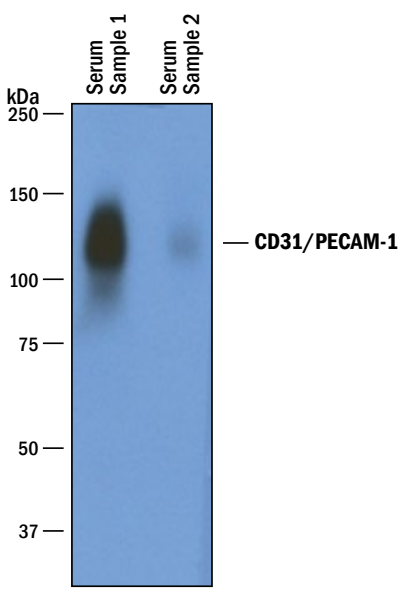
Recombinant rat:

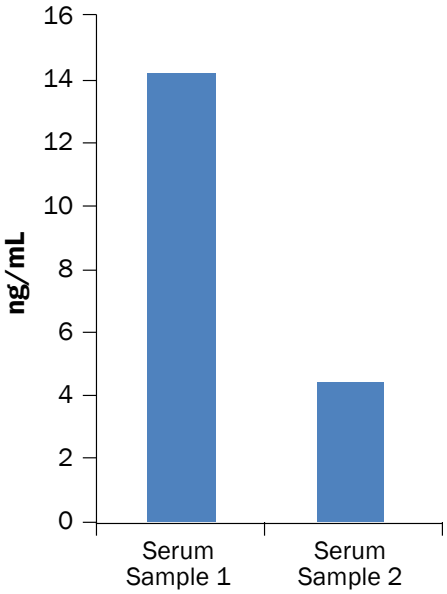
E-Selectin
ICAM-1
L-Selectin
MAG

Recombinant porcine:

PECAM-1

SPECIFICITY *CONTINUED*





Human serum samples were analyzed by Western Blot and Quantikine ELISA. Serum samples were resolved under reducing SDS-PAGE conditions, transferred to PVDF membrane, and immunoblotted with the detection antibody in this kit. The Western blot shows a direct correlation with the ELISA value for these samples.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.