Quantikine® ELISA

Human VAP-1 Immunoassay

Catalog Number DVAP10

For the quantitative determination of human Vascular Adhesion Protein-1 (VAP-1) concentrations in cell culture supernates, serum, and plasma.

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

VAP-1 (vascular adhesion protein-1), also called AOC3 (amine oxidase, copper-containing 3) or SSAO (semicarbazide-sensitive amine oxidase), is one of a group of ectoenzymes that have both adhesive and enzymatic functions (1-3). VAP-1 is a copper amine oxidase with a topaquinone cofactor that catalyzes the oxidative deamination of small primary amines such as methylamine and aminoacetone (1-4). Its production of aldehydes and peroxide contribute to oxidative stress (5). VAP-1 is an 85-90 kDa type II integral membrane sialoglycoprotein that is found as a 170-180 kDa homodimer in the membrane (2). N-linked sialic acid-decorated oligosaccharides are present on the interacting surface of VAP-1 and promote both its adhesion and its enzymatic activity (6-8). A soluble, enzymatically active 180 kDa dimer produced by endothelial cells is the major source of normal SSAO activity in the circulation (8-11).

VAP-1 is expressed in tissues such as lungs, intestines, heart, brain, spleen, kidney, adrenals, pancreas, placenta, liver, and the developing thymus (1, 2, 5, 9). It is highly expressed on the surface of lung, intestinal and hepatic endothelia, smooth muscle, peripheral lymph node high endothelial venules, adipocytes, and blood vessel fibroblasts (2, 5, 7, 9-15). VAP-1 is stored intracellularly in vascular endothelia and translocates to the cell surface during inflammation (13). Lipid loading of adipocytes increases surface expression of VAP-1 and its release from the cell surface by matrix metalloproteinases is regulated by insulin and TNF-α (15, 16). VAP-1 is also associated with insulin-like activity, probably due to production of peroxide (11, 17). Increased circulating VAP-1 concentrations have been reported in diabetes, congestive heart failure, acute ischemic stroke, colorectal cancer, and inflammatory liver diseases (10, 11, 18-22).

VAP-1 binds multiple adhesion partners, including granulocyte Siglec-9 and lymphocyte Siglec-10; Siglec-10 is also a substrate for VAP-1 enzymatic activity (23, 24). VAP-1 contains an RGD motif for potential integrin adhesion (8). VAP-1 adhesion is important for interaction of lymphocytes, especially $CD8^+$ T cells, with inflamed endothelial tissues (2, 25). VAP-1 adhesion mediates slow rolling of inflammatory leukocytes on endothelium under flow, and facilitates leukocyte migration into the tissue (3, 26, 27). These activities are enhanced by VAP-1 enzymatic activity, but independent of other adhesion molecules such as selectins (3, 9, 23, 26, 27). Experimental deficiency of VAP-1 in mice results in a mild obesity, faster leukocyte rolling adhesion on vascular endothelium, and impaired leukocyte extravasation to lymphoid tissues and inflamed tissues including adipose tissue (3, 28, 29). VAP-1 is a potential therapeutic target for diabetes, oxidative stress, and inflammatory diseases (30).

The Quantikine Human VAP-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human VAP-1 in cell culture supernates, serum, and plasma. It contains *Sf* 21-expressed recombinant human VAP-1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human VAP-1 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 VAP-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VAP-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VAP-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VAP-1 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 VAP-1 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, further dilute the samples with 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 enzymes, binding proteins, and 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
VAP-1 Microplate	894167	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against VAP-1.	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.*
VAP-1 Conjugate	894168	21 mL of polyclonal antibody against VAP-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
VAP-1 Standard	894169	500 ng of recombinant human VAP-1 in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1-19	895467	11 mL of a buffer with preservatives.	
Calibrator Diluent RD6X	895152	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.	
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.
- 500 mL graduated cylinder.
- 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 VAP-1 Controls (optional; available from R&D Systems).

PRECAUTIONS

Calibrator Diluent RD6X 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. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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 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 heparin or EDTA 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.*

SAMPLE PREPARATION

Serum and plasma samples require at least a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD6X.

REAGENT PREPARATION

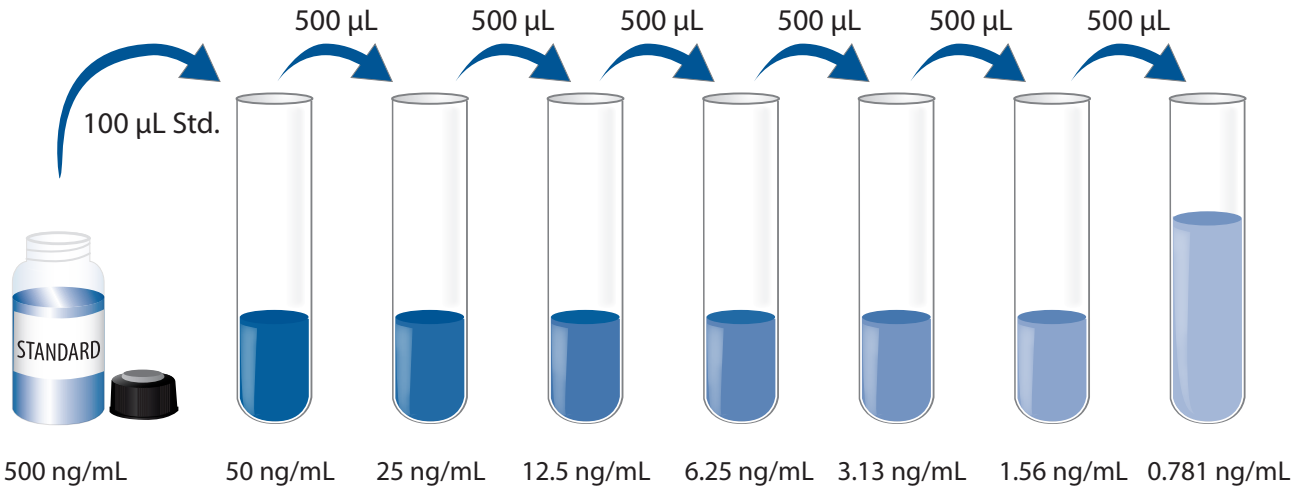
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into 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.

VAP-1 Standard - Reconstitute the VAP-1 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 500 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6X into the 50 ng/mL tube. Pipette 500 µL of Calibrator Diluent RD6X into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 50 ng/mL standard serves as the high standard. Calibrator Diluent RD6X 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 standards, samples, and controls be assayed in duplicate.

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 RD1-19 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.
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 VAP-1 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

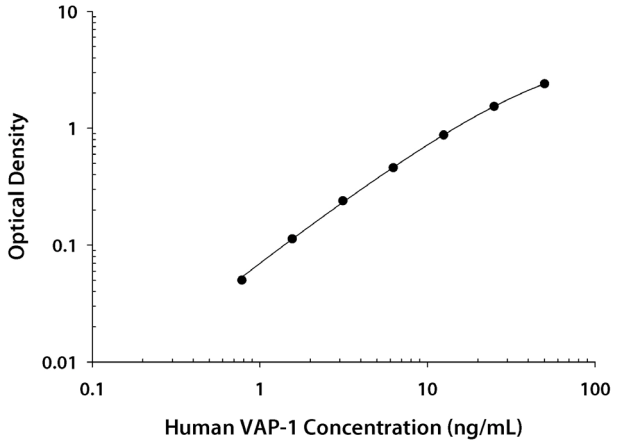
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 VAP-1 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

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.008 0.008	0.008	—
0.781	0.057 0.058	0.058	0.050
1.56	0.121 0.121	0.121	0.113
3.13	0.245 0.249	0.247	0.239
6.25	0.464 0.472	0.468	0.460
12.5	0.869 0.895	0.882	0.874
25	1.529 1.565	1.547	1.539
50	2.402 2.415	2.409	2.401

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	3.41	10.6	22.7	3.96	11.8	24.6
Standard deviation	0.071	0.163	0.535	0.180	0.567	1.16
CV (%)	2.1	1.5	2.4	4.5	4.8	4.7

RECOVERY

The recovery of VAP-1 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	104	95-114%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of VAP-1 were serially diluted with the 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)
1:2	Average % of Expected	98	106	108	105
	Range (%)	96-100	101-110	105-110	104-107
1:4	Average % of Expected	97	108	109	108
	Range (%)	92-101	103-111	104-113	104-114
1:8	Average % of Expected	96	108	109	109
	Range (%)	90-102	103-111	107-113	106-110
1:16	Average % of Expected	95	107	110	108
	Range (%)	89-100	105-112	106-114	105-112

*Samples were diluted prior to assay.

SENSITIVITY

Thirty-two assays were evaluated and the minimum detectable dose (MDD) of VAP-1 ranged from 0.007-0.077 ng/mL. The mean MDD was 0.024 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 *Sf* 21-expressed recombinant human VAP-1 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of VAP-1 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)	370	248-563	72.4
EDTA plasma (n=36)	364	235-531	72.8
Heparin plasma (n=36)	357	211-532	75.0

Cell Culture Supernates - Human peripheral blood leukocytes were cultured in DMEM supplemented with 5% bovine calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of natural VAP-1. All measured below the lowest standard, 0.781 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human VAP-1.

The factors listed below were prepared at 500 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant human VAP-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Siglec-9
Siglec-10
Siglec-11

Recombinant mouse:

VAP-1

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