Quantikine® ELISA

Mouse VEGF R1/Flt-1 Immunoassay

Catalog Number MVR100

For the quantitative determination of mouse soluble Vascular Endothelial Growth Factor Receptor 1 (VEGF R1) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
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INTRODUCTION

The vascular endothelial growth factor (VEGF) family comprises five members including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) (1, 2). These proteins exist as homodimers. In addition, naturally occurring heterodimers of PlGF/VEGF and VEGF/VEGF-B have been reported (3-5). The biological activities of these ligands are mediated by three receptor tyrosine kinases (RTKs): VEGF R1 (Flt-1), VEGF R2 (KDR) and VEGF R3 (Flt-4) (1, 6). These three receptors are characterized by the presence of seven immunoglobulin-like domains (Ig) in their extracellular regions and constitute a subfamily within the RTK superfamily.

Mouse VEGF R1 is a 180 kDa type I transmembrane glycoprotein. It is synthesized as a 1533 amino acid (aa) precursor with a 22 aa signal sequence, a 737 aa extracellular region containing seven Ig-like domains, a 22 aa transmembrane domain and a 752 aa cytoplasmic region containing the split kinase domain (7-9). By alternative splicing, a 633 aa truncated soluble mouse VEGF R1 containing only the first six Ig-like domains and 31 unique aa residues at the C-terminus is also produced (9-11). Mouse VEGF R1 shares approximately 92% and 81% amino acid sequence identity with rat and human VEGF R1, respectively (7, 12, 13). The first three amino-terminal Ig-like domains of VEGF R1 are important for ligand binding, while the fourth Ig-like domain is required for ligand-induced receptor dimerization (8, 14). VEGF R1 binds VEGF-A, -B and PlGF. The ligand binding affinities are similar between the soluble VEGF R1 and the transmembrane receptor (1, 14-17).

VEGF R1 is expressed primarily on endothelial cells (8, 18). Among non-endothelial cells, VEGF R1 expression is detected on monocytes/macrophages (19, 20), uterine smooth muscle cells (21), endometrial epithelium (22), testicular Sertoli and Leydig cells (23), synctiotrophoblast cells (24), cytotrophoblast cells (25), hepatic stellate/Ito cells (26), renal mesangial cells (27) and mesothelium (19). During pregnancy in the mouse, a stage-dependent expression of soluble VEGF R1 and transmembrane VEGF R1 has been reported, with soluble VEGF R1 expression in the trophoblasts increasing from mid to late gestation as transmembrane VEGF R1 expression decreases (10). Soluble VEGF R1 has been detected in various biological fluids including serum/plasma, amniotic fluid and cell-conditioned media (10, 24).

The VEGF family of ligands and receptors are key mediators of angiogenesis. VEGF R2 binds VEGF-A, -C and -D, and transduces signals for blood vessel growth. VEGF R3 binds VEGF-C and -D and mediates lymphangiogenesis (1, 6). The exact role of VEGF R1 in angiogenesis is not clear. Although VEGF R1 binds VEGF with higher affinity than VEGF R2, the kinase activity of VEGF R1 is much lower than that of VEGF R2 (8). While VEGF R1 null mutations are embryonic lethals, tyrosine kinase domain mutants of VEGF R1 develop normal blood vessels. Based on these observations, it has been suggested that the kinase domain-mediated signals are not critical for angiogenesis, and that VEGF R1 likely functions as a decoy receptor to limit VEGF/VEGF R2 mediated angiogenesis (2, 8). Some cellular responses that are mediated directly by the kinase domain of VEGF R1 have been reported, including monocyte chemotaxis and the increased expression of urokinase type plasminogen activator and plasminogen activator inhibitor 1 on endothelial cells (16, 28, 29). VEGF R1 and VEGF R2 can form heterodimers in addition to homodimers (1). The activating ligands for, and the signals transduced by, the heterodimeric receptors are not well understood. Soluble VEGF R1 is a potent inhibitor of VEGF activity and may play an important regulatory role during pregnancy and in vasculogenesis and angiogenesis (8, 10, 18, 22, 30).

The Quantikine Mouse VEGF R1/Flt-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse VEGF R1 in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant mouse VEGF R1 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural mouse VEGF R1 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 mouse VEGF R1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse VEGF R1 has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse VEGF R1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse VEGF R1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse soluble VEGF R1 bound in the initial step. The sample values are then read off the standard curve.

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 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.
- It is recommended that samples be pipetted within 10 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

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
Mouse VEGF R1 Microplate	892222	96 well polystyrene microplate (12 strips of 8 wells) coated with polyclonal antibody specific for mouse VEGF R1.	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.*
Mouse VEGF R1 Standard	892224	2 vials (16 ng/vial) of recombinant mouse VEGF R1 in a buffered protein base with preservatives; lyophilized.	Aliquot and store for up to 1 month at ≤ -20 °C* **in a manual defrost freezer.** Avoid repeated freeze-thaw cycles.
Mouse VEGF R1 Control	892225	2 vials of recombinant mouse VEGF R1 in a buffered protein base with preservatives; lyophilized. The concentration range of mouse VEGF R1 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.	Use a new Control for each assay. Discard after use.
Mouse VEGF R1 Conjugate	892223	12 mL of a polyclonal antibody against mouse VEGF R1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-21	895215	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	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 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	895174	23 mL of diluted hydrochloric 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.
- **Polypropylene** test tubes for dilution of standards and samples.

PRECAUTIONS

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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 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 as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles..

Note: *Heparin and Citrate plasma have not been validated for use in this assay.*
Grossly hemolyzed or lipemic samples may not be suitable for use in this assay.

SAMPLE PREPARATION

Use polypropylene tubes.

Serum and plasma samples require a 2-fold dilution prior to assay. A suggested 2-fold dilution is 75 µL of sample + 75 µL of Calibrator Diluent RD5-3.

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

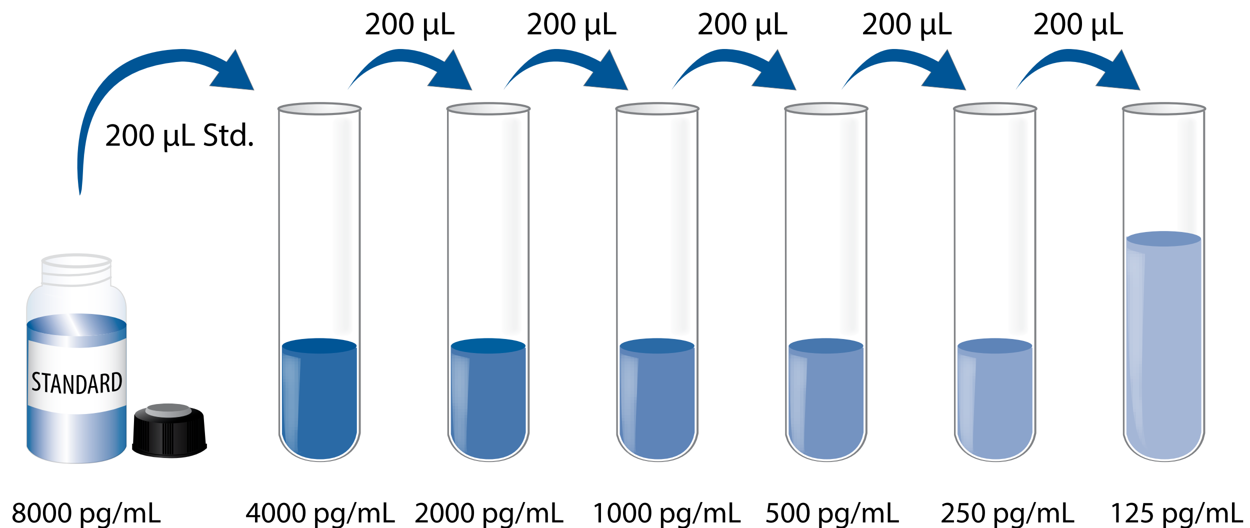
Mouse VEGF R1 Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

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. 100 µL of the resultant mixture is required per well.

Mouse VEGF R1 Standard - Reconstitute the Mouse VEGF R1 Standard with 2.0 mL of Calibrator Diluent RD5-3. Do not substitute other diluents. This reconstitution produces a stock solution of 8000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted mouse VEGF R1 Standard serves as the high standard (8000 pg/mL). Calibrator Diluent RD5-3 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

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

1. Prepare all reagents, standard dilutions, Control, 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 50 µL of Assay Diluent RD1-21 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 four times for a total of five 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 100 µL of Mouse VEGF R1 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 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 100 µL of Stop Solution to each well. 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

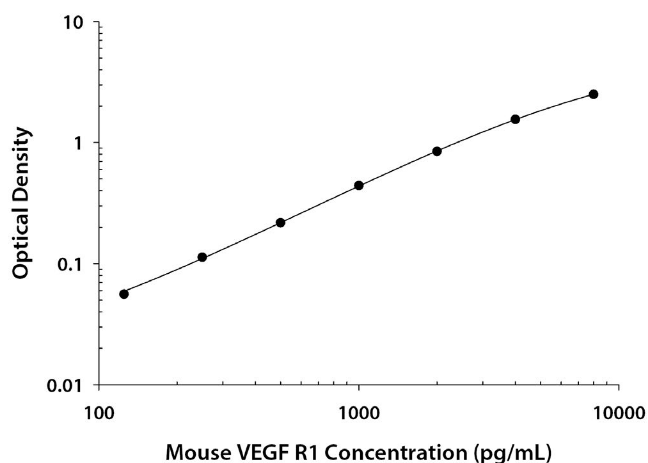
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse VEGF R1 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.



(pg/mL)	O.D.	Average	Corrected
0	0.066 0.067	0.066	—
125	0.122 0.122	0.122	0.056
250	0.178 0.180	0.179	0.113
500	0.280 0.288	0.284	0.218
1000	0.497 0.520	0.508	0.442
2000	0.907 0.918	0.912	0.846
4000	1.588 1.650	1.619	1.553
8000	2.545 2.589	2.567	2.501

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 (pg/mL)	265	1383	2174	250	1339	2139
Standard deviation	19	55	69	21	96	134
CV (%)	7.2	4.0	3.2	8.4	7.2	6.3

RECOVERY

The recovery of mouse VEGF R1 spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=6)	96	82-110%
Serum* (n=6)	106	96-119%
EDTA plasma* (n=6)	106	92-120%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse VEGF R1 in each matrix were diluted with Calibrator Diluent and then assayed.

		Cell culture supernates (n=6)	Serum* (n=6)	EDTA plasma* (n=6)
1:2	Average % of Expected	103	96	99
	Range (%)	98-112	93-100	87-110
1:4	Average % of Expected	102	94	97
	Range (%)	93-108	86-105	87-104
1:8	Average % of Expected	104	91	95
	Range (%)	94-111	81-98	89-104
1:16	Average % of Expected	105	88	96
	Range (%)	95-110	82-99	87-103

*Samples were diluted prior to assay.

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SENSITIVITY

Ten assays were evaluated and the minimum detectable dose (MDD) of mouse VEGF R1 ranged from 3.8-15.2 pg/mL. The mean MDD was 9.8 pg/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 NS0-expressed recombinant mouse VEGF R1/Fc Chimera produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of VEGF R1 in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)
Serum (n=18)	3634	1816-7062
EDTA plasma (n=20)	4635	2750-8026
Pregnant mouse serum (n=2)	28,405	22,740-34,070

Cell Culture Supernates:
Mouse splenocytes (1 x 10^6 cells/mL) were cultured for 4 days in RPMI supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol and 10 ng/mL of recombinant human IL-2. The cell culture supernate was removed, assayed for mouse VEGF R1, and measured 3720 pg/mL.

Two mouse lungs (1-2 mm pieces in 40 mL of medium) were cultured for 5 days in RPMI supplemented with 10% fetal calf serum and stimulated with 10 μg/mL of ConA. The cell culture supernate was removed, assayed for mouse VEGF R1, and measured 6807 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse VEGF R1.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 12.5 ng/mL in a mid-range mouse VEGF R1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:
PlGF-2
VEGF-B
VEGF R2

Recombinant human:
VEGF
VEGF/PlGF

Recombinant mouse VEGF and recombinant rat VEGF cross-react approximately 0.6% in this assay.

Recombinant human VEGF R1 cross-reacts approximately 0.8% in this assay.

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