Quantikine® ELISA

Human VEGF R1/Flt-1 Immunoassay

Catalog Number DVR100C
SVR100C
PDVR100C

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

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

Vascular endothelial growth factor receptor-1 (VEGF R1, also known as Flt-1) is a receptor tyrosine kinase (RTK) specific for the angiogenic factors VEGF (VEGF-A), PlGF, and VEGF-B (1-5). VEGF R1 is expressed as a membrane bound protein that triggers intracellular signaling as well as soluble forms (sVEGF R1) that can sequester ligands or dimerize with full-length receptor to prevent signal transduction (6, 7). Although VEGF R1 null mutations are lethal (8, 9), deletions of the kinase domain are not (10), suggesting that the soluble form, or at least the extracellular domain, is all that is necessary for normal vascular development. Experimental and clinical administrations of sVEGF R1 have been employed successfully in the prevention of neovasculogenesis and tumor growth (11-22). The serum/plasma level of sVEGF R1 may vary by pathology (23-27) and may have prognostic value (28). It has been suggested that sVEGF R1 levels should be presented alongside VEGF levels in experimental and pathological conditions (29). High levels of sVEGF R1 reportedly occur in the plasma during pregnancy and in patients with essential hypertension (25, 47, 48, 51-55). Significantly lower levels have been observed in the plasma of patients with cardiovascular disease and in smokers (24, 26).

Full length VEGF R1 is an approximately 180 kDa glycoprotein featuring seven extracellular immunoglobulin (Ig)-like domains, a transmembrane segment, and an intracellular tyrosine kinase domain containing a kinase insert sequence (1, 30-33). The truncated sVEGF R1 consists of only the first six extracellular Ig-like domains (6, 7). Alternative splicing generates additional soluble isoforms with varying substitutions. This kit detects both isoforms 2 and 3 equivalently. Isoform 2 has a substitution of amino acids 657-687 which includes the N-terminal portion of Ig-like domain 7. Isoform 3 has a substitution of aa 706-733 which includes a more C-terminal portion of Ig-like domain 7.

Ligand binding takes place within the first three N-terminal Ig-like domains (34), while the fourth Ig-like domain is responsible for receptor dimerization, a prerequisite for activation through trans-phosphorylation (35, 36). In addition to homodimers, VEGF R1 can form active heterodimers with VEGF R2 (37). The soluble form of VEGF R1 forms inactive heterodimers with VEGF R2 (7). VEGF, VEGF R1, and VEGF R2 represent a regulatory system essential for both normal and pathological angiogenesis. Although VEGF R1 is expressed in significant levels on monocyte/macrophage lineages (38, 39), expression of both VEGF receptors is primarily restricted to endothelial cells (40-42). VEGF, FGF basic, PECAM-1, cell-cell contact, and hypoxia are all reported to increase VEGF receptor expression (43-46). Peripheral blood monocyte expression of VEGF R1 is upregulated in response to monocyte activation (38, 39).

VEGF R1 has a greater affinity for VEGF than VEGF R2, yet VEGF R2 is phosphorylated approximately 10-fold more efficiently upon ligand binding. VEGF stimulates chemotaxis and proliferation of VEGF R2-transfected, but not VEGF R1-transfected human umbilical vein and porcine aortic endothelial cells (49, 50). Knockout mice missing either receptor usually die by day 10. Absence of VEGF R2 results in an undeveloped vasculature and few mature endothelial cells, while absence of VEGF R1 results in a preponderance of endothelial cells coalesced into disorganized tubules (8, 9). Mice lacking only the tyrosine kinase domain of VEGF R1, however, survive with near normal vasculature (10). These observations support the conclusion that VEGF R1 functions to limit VEGF/VEGF R2 mediated angiogenesis with the intact receptor acting as a decoy and the soluble form creating inert receptors by dimerization with VEGF R2 or sequestering free ligand. There is evidence, however, that the tyrosine kinase domain of VEGF R1 does play an angiogenic role. VEGF R1/R2 heterodimers can transduce signals (37). VEGF-induced nitric oxide (NO) release appears to be mediated by VEGF R1, and this NO release in turn acts as a molecular switch, inhibiting VEGF R2 mediated proliferation and affecting endothelial cell re-differentiation into capillary-like structures (50). Additionally, VEGF and PlGF mediated migration of monocytes/macrophages and production of tissue factor appears to be mediated by VEGF R1 (38, 39).

The Quantikine® Human VEGF R1/Flt-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human VEGF R1 in cell culture supernates, cell lysates, serum, plasma, and saliva. It contains NS0-expressed recombinant human VEGF R1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human 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 natural human VEGF R1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human VEGF R1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF R1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human 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 and color develops in proportion to the amount of VEGF R1 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 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 #	CATALOG # DVR100C	CATALOG # SVR100C	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human VEGF R1 Microplate	898352	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human VEGF R1.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human VEGF R1 Standard	898354	2 vials	12 vials	Recombinant human VEGF R1 in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Human VEGF R1 Conjugate	898353	1 vial	6 vials	21 mL/vial of polyclonal antibody specific for human VEGF R1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	1 vial	6 vials	11 mL/vial of a buffered protein solution with preservatives.	
Calibrator Diluent RD5P	895151	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DVR100C contains sufficient materials to run an ELISA on one 96 well plate.
SVR100C (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDVR100C). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

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 VEGF R1 Controls (optional; R&D Systems®, Catalog # QC239).

If using cell lysate samples, the following is also required:

- **Lysis Buffer 11** - 50 mM Tris (pH 7.4), 300 mM NaCl, 10% (w/v) glycerol, 3 mM EDTA, 1 mM MgCl2, 20 mM b-glycerophosphate, 25 mM NaF, 1% Triton X-100, 25 µg/mL Leupeptin, 25 µg/mL Pepstatin, and 3 µg/mL Aprotinin.

PRECAUTIONS

VEGF R1 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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

Some components in this kit contain a preservative 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.

Cell Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

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.*
Grossly hemolyzed samples are not suitable 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, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell lysate samples require a 10-fold dilution due to a matrix effect. A suggested 10-fold dilution is 30 µL of sample + 270 µL of Calibrator Diluent RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of VEGF R1 are found in saliva. Wear a face mask and gloves 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.

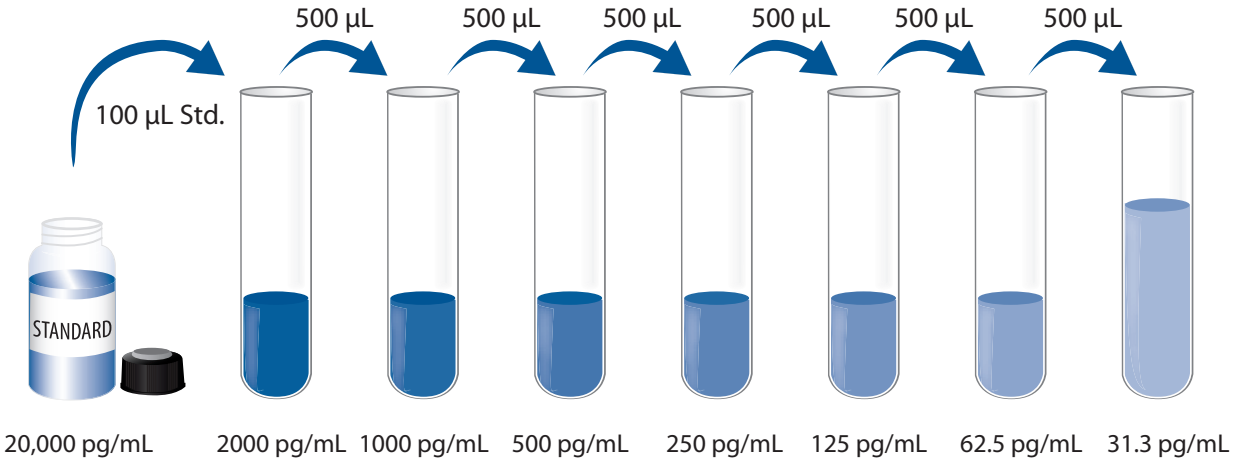
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:5) - Add 10 mL of Calibrator Diluent RD5P to 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5P (diluted 1:5).

Human VEGF R1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human VEGF R1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Note: *Standard must be used within 30 minutes.*

Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:5) into the 2000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) 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, samples, and controls be assayed in duplicate.

Note: *High concentrations of VEGF R1 are found in saliva. Wear a face mask and gloves 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 RD1W to each well.
4. Add 100 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 rpm ± 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 Human 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 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

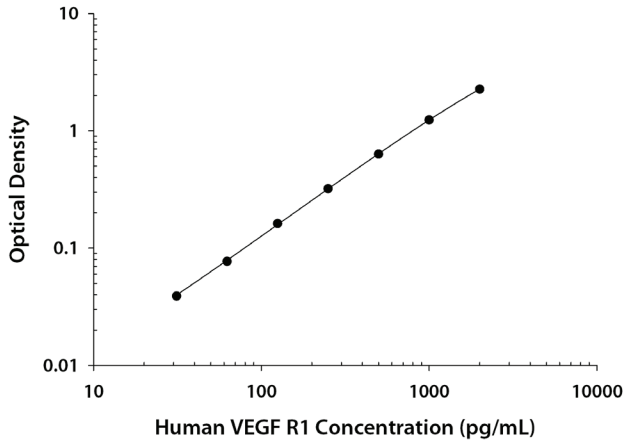
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 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	0.030 0.030	0.030	—
31.3	0.067 0.070	0.069	0.039
62.5	0.106 0.107	0.107	0.077
125	0.185 0.198	0.192	0.162
250	0.347 0.355	0.351	0.321
500	0.664 0.664	0.664	0.634
1000	1.240 1.295	1.268	1.238
2000	2.286 2.310	2.298	2.268

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	207	610	1207	207	616	1205
Standard deviation	5.16	14.9	35.6	13.9	38.9	74.0
CV (%)	2.5	2.4	2.9	6.7	6.3	6.1

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	99	87-110%
Lysis buffer* (n=4)	94	86-108%
Serum (n=4)	104	93-114%
EDTA plasma (n=4)	92	84-98%
Heparin plasma (n=4)	89	81-96%
Saliva (n=4)	109	96-124%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of human VEGF R1 ranged from 0.834-8.46 pg/mL. The mean MDD was 4.17 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 highly purified NS0-expressed recombinant human VEGF R1 produced at R&D Systems.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human VEGF R1 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Lysis buffer* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva (n=4)
1:2	Average % of Expected	100	103	98	99	100	101
	Range (%)	97-104	94-112	96-102	97-104	93-105	92-109
1:4	Average % of Expected	102	105	102	105	104	105
	Range (%)	97-109	94-111	100-104	103-107	98-110	94-117
1:8	Average % of Expected	101	104	107	107	106	106
	Range (%)	96-109	92-115	101-110	104-110	102-112	99-115
1:16	Average % of Expected	100	109	114	112	114	112
	Range (%)	94-103	92-121	112-116	106-116	111-117	108-118

*Samples were diluted prior to assay.

SAMPLE VALUES

Serum/Plasma/Saliva - Samples from apparently healthy volunteers were evaluated for the presence of human VEGF R1 in this assay. No medical histories were available for the donors used in this study. See references 51-55 for information regarding VEGF R1 levels during pregnancy and related conditions.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=30)	385	294-478	49.4
EDTA plasma (n=30)	292	208-391	43.1
Heparin plasma (n=30)	279	216-377	40.4
Saliva (n=10)	944	346-1827	502

Cell Culture Supernates - HUVEC human umbilical vein endothelial cells were cultured in EGM-2 media until confluent. Cells were untreated or placed under hypoxic conditions for 48 hours. An aliquot of the cell culture supernate was removed, assayed for human VEGF R1, and measured 13,050 pg/mL and 44,214 pg/mL, respectively.

Cell Lysates - HUVEC human umbilical vein endothelial cells placed under hypoxic conditions were solubilized in Lysis Buffer 11 and put on ice for 15 minutes. Tubes were centrifuged at 14,000 x g for 5 minutes to remove insoluble material. The remaining whole cell extract was removed, aliquoted into a clean test tube, and stored at ≤ 20 °C. Whole cell extract protein concentration was quantified using a total protein assay. 5 µg of the cell lysate was removed, assayed for human VEGF R1, and measured 1071 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human 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 50 ng/mL in a mid-range human VEGF R1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

β-ECGF
EGF
EG-VEGF
FGF acidic
FGF basic
FGF-3
FGF-4
FGF-5
FGF-6
FGF-8a
FGF-8e
FGF-8f
FGF-9
FGF-10
FGF-11
FGF-12
FGF-16
FGF-17
FGF-18
FGF-19
FGF-20
FGF-21
FGF-22
FGF-23
Flt-3
Flt-3 Ligand
G-CSF
GM-CSF
HB-EGF
HGF
IGF-I
IGF-II
KDR
KGF/FGF-7
M-CSF
MSP
MSPα
MSPα/β
MSPβ
β-NGF
NRG1
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PDGF-C
PDGF-D
PlGF
PlGF-2
PlGF-3
PlGF-4
$VEGF_{111}$
$VEGF_{121}$
$VEGF_{145}$
$VEGF_{162}$
$VEGF_{165}$
$VEGF_{165b}$
$VEGF_{189}$
$VEGF_{206}$
VEGF/PlGF
$VEGF\text{-}B_{167}$
VEGF-C
VEGF-D
VEGF R3

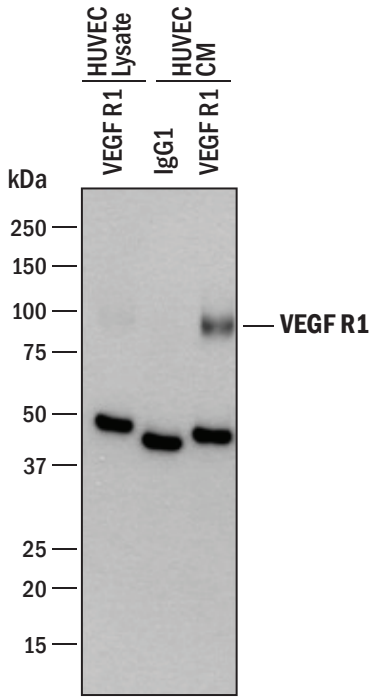
Recombinant mouse:

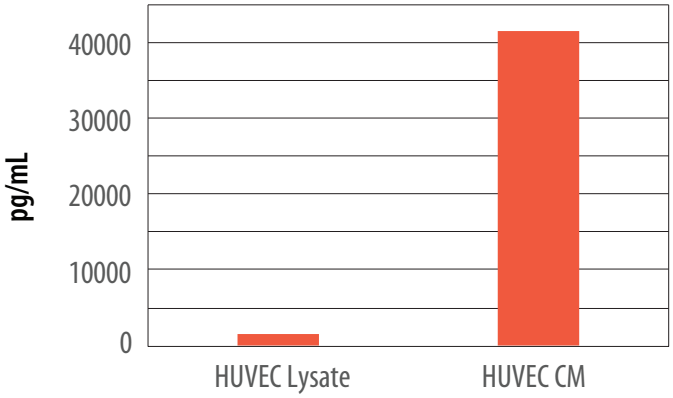
FGF-8c
VEGF R1

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF

SPECIFICITY *CONTINUED*





Lysate and conditioned media (CM) samples from HUVEC cells cultured in hypoxic conditions (1% O_2, 5% CO_2) were analyzed by Immunoprecipitation/Western Blot and Quantikine ELISA. For immunoprecipitation, samples were diluted in Sample Diluent Concentrate 2 (1X) (R&D Systems®, Catalog # DYC002), and incubated with 2 µg of the capture antibody provided in this kit plus 40 µL of Protein G Sepharose beads overnight. Immunoprecipitated samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with goat anti-human VEGF R1 (R&D Systems®, Catalog # AF321). The band intensity from the Immunoprecipitation/Western Blot correlates with Quantikine ELISA sample value.

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