

Quantikine[®]

Human VEGF Immunoassay

Catalog Number DVE00

For the quantitative determination of human vascular endothelial growth factor (VEGF) concentrations in cell culture supernate, serum, and plasma.

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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INTRODUCTION

Vascular endothelial growth factor (VEGF) (1), also known as vascular permeability factor (VPF) (2) or vasculotropin (3), is a homodimeric 34 - 42 kDa, heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells. The amino acid sequence of VEGF exhibits primary structural, as well as limited amino acid sequence, homology with that of the A and B chains of PDGF. All eight cysteine residues involved in intra- and inter-chain disulfide bonds are conserved among these growth factors. A cDNA encoding a protein having a 53% amino acid sequence homology in the PDGF-like region of VEGF has been isolated from a human placental cDNA library (4). This protein, named placenta growth factor (P/GF), is now recognized to be a member of the VEGF family of growth factors. Based on its homology with VEGF, P/GF was also proposed to be an angiogenic factor. A gene encoding a polypeptide with homology to VEGF has been discovered in the genome of the orf virus (OV), a parapoxvirus that affects sheep, goats and sometimes humans (5). For reviews on VEGF and P/GF, see references 6 - 10.

VEGF is expressed by numerous rodent and human tumor cells, including human lung adenocarcinoma, bladder carcinoma, fibrosarcoma, HL60 promyelocytic leukemia, GS-9L glioma, and U937 lymphoma cells (6 - 10). In normal tissues, VEGF expression has been found in activated macrophages (11), keratinocytes (12), renal glomerular visceral epithelium and mesangial cells (13, 14), hepatocytes (15), smooth muscle cells (16), Leydig cells (17), embryonic fibroblasts and bronchial and choroid plexus epithelium (18, 19). The expression of VEGF is upregulated by phorbol ester, TGF- β and in hypoxia (6 - 10, 18). In contrast to the widespread distribution of VEGF, the expression of P/GF mRNA is limited to placental tissue, choriocarcinoma cells and cultured endothelial cells (4, 20, 21). In the conditioned media of human choriocarcinoma cells (JAR and JE-3), the occurrence of VEGF/P/GF heterodimers has also been observed (22).

The gene for human VEGF is organized into 8 exons. As a result of alternative splicing, at least 4 transcripts encoding mature monomeric VEGF containing 121, 165, 189, and 206 amino acid residues (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆), each preceded by a 26 amino acid residue signal peptide, have been detected. VEGF₁₂₁ and VEGF₁₆₅ are diffusible proteins that are secreted into the medium. VEGF₁₈₉ and VEGF₂₀₆ have high affinity for heparin and are mostly bound to heparin-containing proteoglycans in the extracellular matrix. VEGF contains a potential N-linked glycosylation site and the natural protein is a glycoprotein. *E. coli*-expressed recombinant human VEGF is indistinguishable from natural VEGF in its *in vitro* biological actions, suggesting that the carbohydrate moiety may not be required for activities. VEGF is a highly conserved protein that has cross-species activity. Between human, rat or bovine VEGF, 84 - 94% sequence identity has been observed (6 - 10).

Two receptor tyrosine kinases have been described as putative VEGF receptors. Flt-1 (*fms*-like tyrosine kinase) (23), and KDR (kinase-insert-domain-containing receptor) proteins have been shown to bind VEGF with high affinity (24). The mouse homologue of KDR was named Flk-1, for fetal liver kinase-1. Mouse Flk-1 shares 85% amino acid sequence identity with human KDR (25). Flt-1 and KDR/Flk-1 are members of the superfamily of RTKs (receptor tyrosine kinases) that also include the receptors for PDGF, M-CSF and SCF. In addition to the membrane-spanning Flt-1, a cDNA encoding a soluble truncated form of Flt-1 has been cloned from a human vascular endothelial cell library. The mRNA for the soluble receptor is apparently generated by alternative splicing (26). Recombinant soluble Flt-1 binds VEGF with high affinity and inhibits VEGF actions on vascular endothelial cells. Thus, it is possible that natural soluble Flt-1 may act as a VEGF antagonist *in vivo*. Using a dominant-negative Flk-1 mutant, Flk-1 has been shown to be involved in the VEGF-mediated transduction of signals that are important for angiogenesis and vasculogenesis (27). As assessed by Northern blot analysis and/or *in situ* hybridization, Flt-1 was found to be expressed in both endothelial and non-endothelial cells, while KDR/Flk-1 expression was reported to be restricted to endothelial cells (28, 29, 30).

In vitro, VEGF is a potent endothelial cell mitogen (6 - 10). In cultured endothelial cells, VEGF can activate phospholipase C and induce rapid increases of free cytosolic Ca^{2+} . VEGF has been shown to stimulate von Willebrand factor release from endothelial cells and induce expression of tissue factor activity in endothelial cells as well as in monocytes. VEGF has also been shown to be chemotactic for monocytes and osteoblasts (31). *In vivo*, VEGF can induce angiogenesis as well as increase microvascular permeability. As a vascular permeability factor, VEGF acts directly on the endothelium and does not degranulate mast cells. It promotes extravasation of plasma fibrinogen, leading to fibrin deposition which alters the tumor extracellular matrix. The modified extracellular matrix subsequently promotes the migration of macrophages, fibroblasts and endothelial cells. Based on its *in vitro* and *in vivo* properties, VEGF is expected to play important roles in inflammation and during normal and pathological angiogenesis, a process that is associated with wound healing, embryonic development, and growth and metastasis of solid tumors. Elevated levels of VEGF have been reported in synovial fluids of rheumatoid arthritis patients and in sera from cancer patients (32 - 34).

Bioassays for VEGF, based on its proliferative effects on endothelial cells, are time-consuming and not completely specific for VEGF. The Quantikine VEGF Immunoassay is a 4.5 hour solid phase ELISA designed to measure VEGF₁₆₅ levels in cell culture supernates, serum, and plasma. It contains insect cell *Sf* 21-expressed recombinant human VEGF₁₆₅ and antibodies raised against the recombinant protein. Results obtained for naturally occurring human VEGF and recombinant human VEGF₁₂₁ 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.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF 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 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.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- As manufacturers we take great care to ensure that our products are suitable for use with all validated sample types, as designated in the product insert. However, it is possible that in some cases, high levels of interfering factors may cause unusual results.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Soluble receptors or other binding proteins present in biological samples do not necessarily interfere with the measurement of ligands in samples. However, until the factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

REAGENTS

VEGF Microplate (Part 890218) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against VEGF.

VEGF Conjugate (Part 890219) - 21 mL of polyclonal antibody against VEGF conjugated to horseradish peroxidase, with preservatives.

VEGF Standard (Part 890220) - 3 vials (2000 pg/vial) of recombinant human VEGF₁₆₅ in a buffered protein base with preservatives, lyophilized.

Assay Diluent RD1W (Part 895117) - 11 mL of a buffered protein base with preservatives.

Calibrator Diluent RD5K (Part 895119) - 21 mL of a buffered protein base with preservatives.
For cell culture supernate samples.

Calibrator Diluent RD6U (Part 895148) - 21 mL of animal serum with preservatives.
For serum/plasma samples.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Covers - 4 Adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Calibrator Diluent RD5K	
	Calibrator Diluent RD6U	
	Assay Diluent RD1W	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Discard the VEGF stock solution and dilutions after 4 hours. Use a fresh standard for each assay.
Microplate Wells	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.*	

*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.
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer.
- **12 mm x 75 mm polypropylene test tubes.**
- 500 mL graduated cylinder.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Cell culture supernates should contain at least 1% fetal calf serum for stability of the VEGF. Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 10 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin or citrate as an anticoagulant. Centrifuge at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

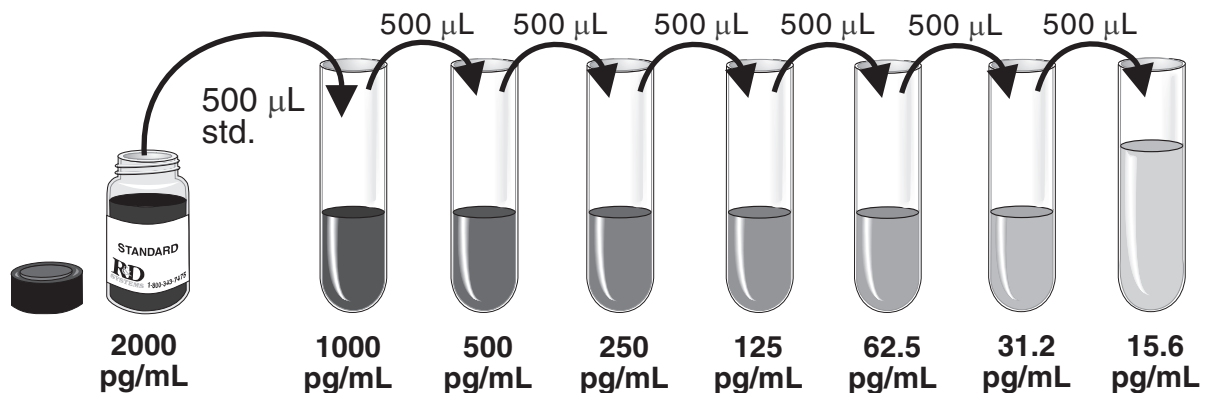
Bring all reagents to room temperature before use.

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.

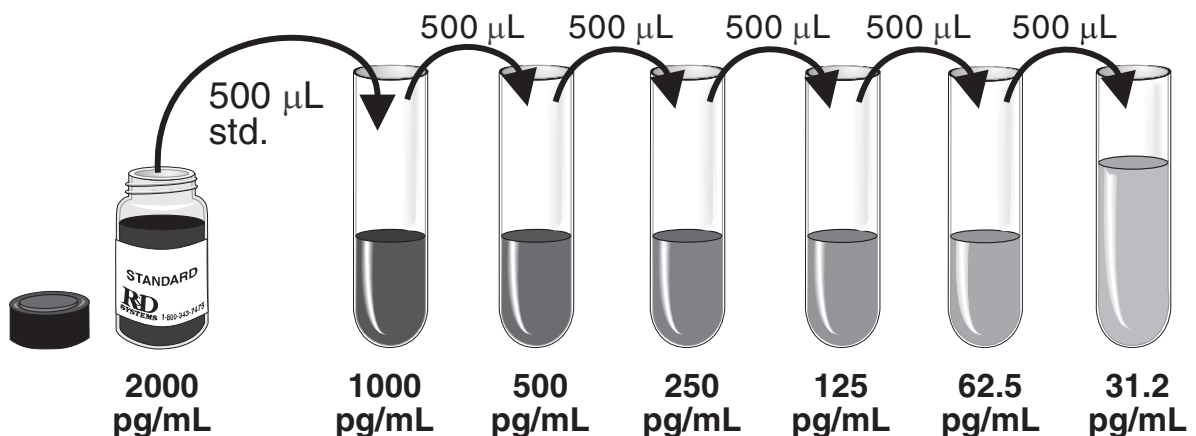
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. 200 μL of the resultant mixture is required per well.

VEGF Standard - Reconstitute the VEGF Standard with 1 mL of Calibrator Diluent RD5K (for cell culture supernate samples) or Calibrator Diluent RD6U (for serum/plasma samples). This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

For Cell Culture Supernate Samples: Use polypropylene tubes. Pipette 500 μL of Calibrator Diluent RD5K into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL dilution serves as the high standard. Calibrator Diluent RD5K serves as the zero standard (0 pg/mL).



For Serum/Plasma Samples: Use polypropylene tubes. Pipette 500 μL of Calibrator Diluent RD6U into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (2000 pg/mL). Calibrator Diluent RD6U 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 samples and standards be assayed in duplicate.

1. Prepare all reagents and working standards 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, reseal.
3. **For Culture Supernate Samples:** Add 50 μL of Assay Diluent RD1W to each well.
For Serum/Plasma Samples: Add 100 μL of Assay Diluent RD1W to each well.
4. **For Culture Supernate Samples:** Add 200 μL of Standard or sample per well.
For Serum/Plasma Samples: Add 100 μL of Standard or sample per well.
Cover with the adhesive strip provided and incubate for 2 hours at room temperature. A plate layout is provided to record the standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, multi-channel pipette, 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 toweling.
6. Add 200 μL of VEGF Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μL of Substrate Solution to each well.
For Culture Supernate Samples: Incubate for 20 minutes at room temperature.
For Serum/Plasma Samples: Incubate for 25 minutes at room temperature.
9. Add 50 μL of Stop Solution to each well. If 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.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and standards as directed.



2. Add Assay Diluent RD1W to each well.

Cell Culture Supernate Samples

Serum/Plasma Samples

Add 50 μ L

Add 100 μ L

3. Add Standard or sample to each well.

Cell Culture Supernate Samples

Serum/Plasma Samples

Add 200 μ L

Add 100 μ L

Incubate 2 hrs. RT



4. Aspirate and wash 3 times.



5. Add 200 μ L Conjugate to each well. Incubate 2 hrs. RT



6. Aspirate and wash 3 times.



7. Add 200 μ L Substrate Solution to each well.

Cell Culture Supernate Samples

Serum/Plasma Samples

Incubate 20 minutes RT

Incubate 25 minutes RT

8. Add 50 μ L Stop Solution to each well. Read at 450 nm within 30 min.
 λ correction 540 or 570 nm

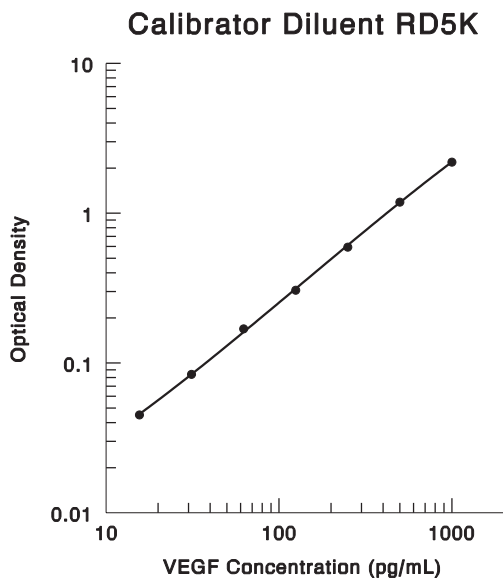
CALCULATION OF RESULTS

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

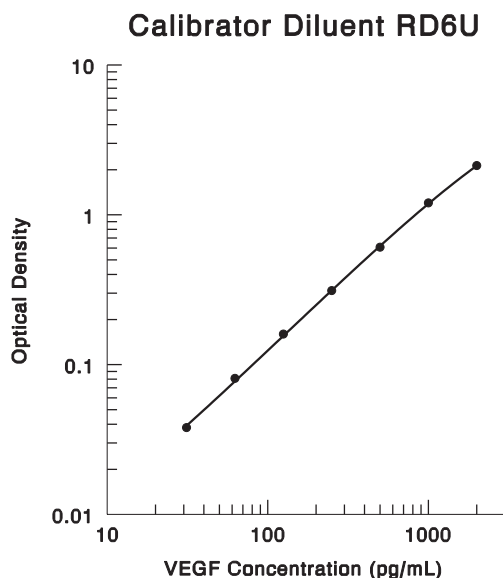
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the VEGF 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.



(pg/mL)	O.D.	Average	Corrected
0	0.074 0.076 0.118	0.075	
15.6	0.121 0.159	0.120	0.045
31.2	0.159 0.246	0.159	0.084
62.5	0.242 0.384	0.244	0.169
125	0.378 0.666	0.381	0.306
250	0.669 1.258	0.668	0.593
500	1.263 2.302	1.260	1.185
1000	2.233	2.268	2.193



(pg/mL)	O.D.	Average	Corrected
0	0.068 0.071 0.107	0.070	
31.2	0.110 0.149	0.108	0.038
62.5	0.153 0.230	0.151	0.081
125	0.230 0.377	0.230	0.160
250	0.387 0.657	0.382	0.312
500	0.699 1.261	0.678	0.608
1000	1.281 2.159	1.271	1.201
2000	2.246	2.202	2.132

TECHNICAL HINTS

- Substrate Solution should remain colorless until added to the plate. Substrate Solution should change from colorless to gradations of blue. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.
- 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.

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were assayed twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were assayed in forty separate assays to assess inter-assay precision.

Serum/Plasma Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	53.7	235	910	64.5	250	1003
Standard deviation	3.6	10.6	46.2	5.7	17.4	61.7
CV (%)	6.7	4.5	5.1	8.8	7.0	6.2

Cell Culture Supernate Assay

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	29.1	123	531	32.8	128	495
Standard deviation	1.9	5.0	18.4	2.8	6.4	33.0
CV (%)	6.5	4.1	3.5	8.5	5.0	6.7

RECOVERY

The recovery of VEGF spiked to three different levels in five samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media	102	95 - 111%
Serum	102	92 - 115%
EDTA plasma	97	82 - 113%
Heparin plasma	93	82 - 102%
Citrate plasma	100	88 - 113%

LINEARITY

To assess linearity of the assay, five samples were spiked with high concentrations of VEGF in various matrices and diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media	Serum	EDTA plasma	Heparin plasma	Citrate plasma
1:2	Average % of Expected	98	97	97	94	95
	Range (%)	94-100	91-103	82-107	87-99	90-100
1:4	Average % of Expected	96	97	98	93	94
	Range (%)	93-99	93-104	91-106	85-98	89-99
1:8	Average % of Expected	93	96	96	92	92
	Range (%)	88-102	93-103	89-106	85-101	85-97
1:16	Average % of Expected	93	94	94	94	92
	Range (%)	88-105	91-101	84-106	83-103	85-98

SENSITIVITY

Using Calibrator Diluent RD5K the minimum detectable dose of VEGF is typically less than 5.0 pg/mL. Using Calibrator Diluent RD6U the minimum detectable dose is typically less than 9.0 pg/mL.

The minimum detectable dose 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 VEGF₁₆₅ produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Thirty-seven matched human serum, EDTA plasma, heparin plasma, and citrate plasma samples were assayed.

Sample Type	% Detectable	Mean of Detectable (pg/mL)	Range (pg/mL)
Serum	100	220	62 - 707
EDTA plasma	24	61	ND - 115
Heparin plasma	22	41	ND - 55
Citrate plasma	0	-	ND

ND = Non-detectable

Cell culture supernates - Human peripheral blood mononuclear cells (1×10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 μ g/mL PHA for 1 and 5 days.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	356	332
Stimulated	14	1440

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
	A	B	C	D	E	F	G	H