

# Quantikine<sup>®</sup> ELISA

## Human VEGF-D Immunoassay

Catalog Number DVED00

For the quantitative determination of human Vascular Endothelial Growth Factor D (VEGF-D) 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

The vascular endothelial growth factor (VEGF) family of proteins is important for the development of blood vessels during embryogenesis and in pathological conditions such as tumorigenesis (see reference 1 for a review). VEGF (VEGF-A) is a homodimeric, heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells. In addition to splice variants of VEGF-A, several other members of the VEGF family have been cloned, including VEGF-B, -C and -D (see references 2 and 3 for reviews). Placenta growth factor (PlGF) is also closely related to VEGF-A. VEGF-A, -B, -C, -D and PlGF exhibit limited amino acid (aa) sequence homology with the A and B chains of platelet-derived growth factor (PDGF). Eight cysteine aa residues involved in intra- and inter-chain disulfide bonds are conserved among these growth factors.

VEGF-D (also known as c-fos-induced growth factor or FIGF (4)) is most closely related to VEGF-C (5, 6). It shares structural homology and receptor specificity with VEGF-C, thus suggesting that VEGF-C and VEGF-D represent a subfamily of the VEGFs. VEGF-D is initially synthesized as a precursor protein containing unique N- and C-terminal propeptides in addition to a central receptor-binding VEGF homology domain (VHD) (6). The N- and C-terminal propeptides are not found within other VEGF family members. These propeptides are proteolytically cleaved during biosynthesis to generate a mature, secreted form consisting of noncovalent dimers of the VHD (7).

Like VEGF-C, VEGF-D binds the cell surface receptor tyrosine kinases VEGF receptor 2 (VEGF R2/Flk-1/KDR) and VEGF R3 (Flt-4) (6). VEGF R2 (8, 9) and VEGF R3 (10, 11) are localized on vascular and lymphatic endothelial cells and signal for angiogenesis and lymphangiogenesis. The mature, secreted form of VEGF-D binds to both VEGF R2 and VEGF R3 with much higher affinity than unprocessed VEGF-D (7). Monoclonal antibodies generated against VEGF-D block its interactions with both VEGF R2 and VEGF R3, thus suggesting that the regions of VEGF-D that interact with these two receptors may be very similar (12). VEGF-D binding and activating of VEGF R2 has no vascular permeability activity, indicating that VEGF R2 binding does not necessarily correlate with permeability activity for all VEGF family members (7).

The gene for VEGF-D maps to the X chromosome in both mouse and human (5, 13). VEGF-D gene expression occurs at many sites within the developing embryo, particularly lung mesenchyme (7, 14, 15). VEGF-D is also localized in tumor cells (16, 17). In adult human tissues, VEGF-D mRNA is expressed in the heart, lung, skeletal muscle, colon, and small intestine (6).

The Quantikine Human VEGF-D Immunoassay is a 4.5 hour solid phase ELISA designed to measure VEGF-D levels in cell culture supernates, serum, and plasma. It contains Sf 21-expressed, recombinant human VEGF-D and antibodies raised against the recombinant protein. Results obtained for naturally occurring human VEGF-D showed linear curves that were parallel to the standard curves obtained using the kit standards. These results indicate that this kit can be used to determine relative mass values for natural human VEGF-D.

## PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human VEGF-D has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF-D present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human VEGF-D 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-D 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.
- 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.
- 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
VEGF-D Microplate	890816	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human VEGF-D.	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.*
VEGF-D Standard	890818	40 ng of recombinant human VEGF-D 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.*
VEGF-D Conjugate	890817	21 mL of monoclonal antibody specific for human VEGF-D conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1X	895121	11 mL of a buffered protein base with preservatives. <i>May contain crystals. Warm to room temperature and mix well to dissolve.</i>	
Calibrator Diluent RD5R	895190	21 mL of a buffered protein base with preservatives. <i>For cell culture supernate samples.</i>	
Calibrator Diluent RD6P	895118	21 mL of animal serum with preservatives. <i>For serum/plasma samples.</i>	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
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.
- Test tubes for dilution of standards.
- Human VEGF-D Controls (optional; available from R&D Systems).

## 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 and assay immediately or aliquot and store samples at  $\leq -20$  °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  $\leq -20$  °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  $\leq -20$  °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.*

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## REAGENT PREPARATION

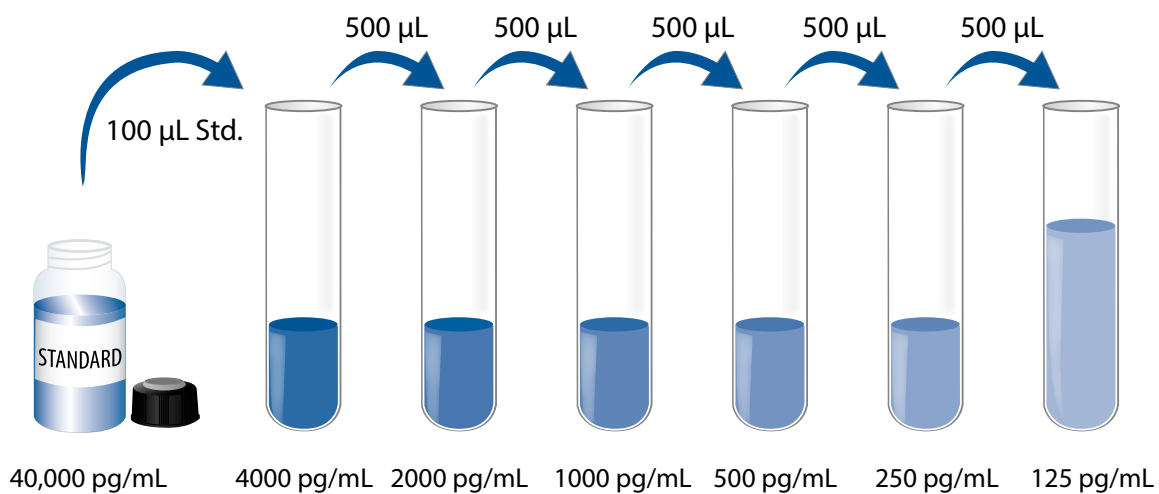
**Bring all reagents to room temperature before use.**

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. 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  $\mu$ L of the resultant mixture is required per well.

**VEGF-D Standard** - Reconstitute the VEGF-D Standard with 1 mL of deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900  $\mu$ L of Calibrator Diluent RD5R (*for cell culture supernate samples*) or Calibrator Diluent RD6P (*for serum/plasma samples*) into the 4000 pg/mL tube. Pipette 500  $\mu$ L 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 4000 pg/mL dilution serves as the high standard. The appropriate Calibrator Diluent 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.**

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, and reseal.
3. Add 100  $\mu\text{L}$  of Assay Diluent RD1X to each well. *May contain crystals. Warm to room temperature and mix well to dissolve.*
4. Add 50  $\mu\text{L}$  of Standard, sample, or control per well. Cover with the adhesive strip provided and incubate for 2 hours at room temperature.
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  $\mu\text{L}$ ) 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  $\mu\text{L}$  of VEGF-D 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  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50  $\mu\text{L}$  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.).

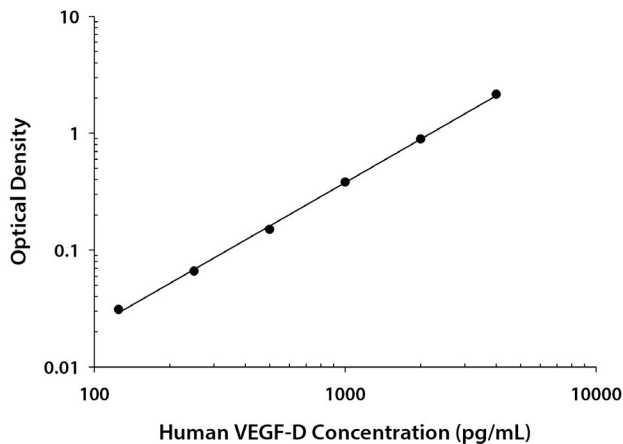
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human VEGF-D concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

## TYPICAL DATA

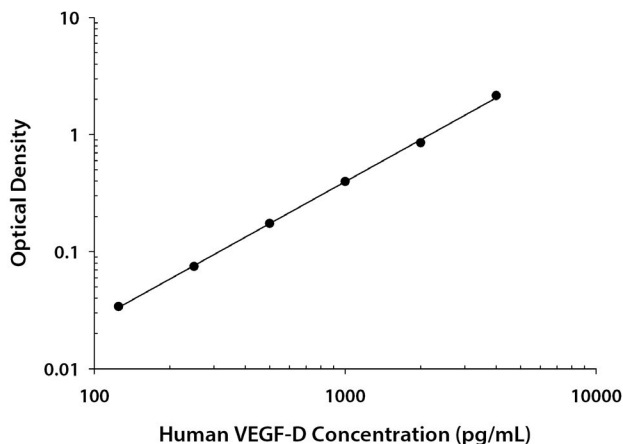
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

### CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.007 0.007	0.007	—
125	0.038 0.038	0.038	0.031
250	0.074 0.071	0.073	0.066
500	0.165 0.149	0.157	0.150
1000	0.404 0.373	0.389	0.382
2000	0.918 0.877	0.898	0.891
4000	2.201 2.119	2.160	2.153

### SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.011 0.012	0.012	—
125	0.047 0.045	0.046	0.034
250	0.088 0.086	0.087	0.075
500	0.183 0.188	0.186	0.174
1000	0.421 0.399	0.410	0.398
2000	0.883 0.847	0.865	0.853
4000	2.254 2.072	2.163	2.151

## 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 forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

## 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)	560	1098	2192	512	978	1978
Standard deviation	31.8	35.5	45.6	46.3	72.8	117
CV (%)	5.7	3.2	2.1	9.0	7.4	5.9

## 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)	448	970	2081	474	956	2013
Standard deviation	27.8	40.9	49.3	38.0	68.5	145
CV (%)	6.2	4.2	2.4	8.0	7.2	7.2

## RECOVERY

The recovery of human VEGF-D spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	100	97-104%
Serum (n=5)	106	94-113%
EDTA plasma (n=5)	105	89-114%
Heparin plasma (n=5)	104	88-114%

## LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human VEGF-D were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)
1:2	Average % of Expected	100	98	96	96
	Range (%)	97-104	94-103	94-98	92-102
1:4	Average % of Expected	94	95	94	94
	Range (%)	90-97	90-100	91-97	90-99
1:8	Average % of Expected	93	92	92	92
	Range (%)	89-98	89-96	91-93	88-97
1:16	Average % of Expected	91	91	90	90
	Range (%)	86-96	86-102	86-99	85-103

## SENSITIVITY

Thirty-four assays were evaluated and the minimum detectable dose (MDD) of human VEGF-D ranged from 4.7-31.3 pg/mL. The mean MDD was 11.4 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 Sf 21-expressed recombinant human VEGF-D produced at R&D Systems.

## SAMPLE VALUES

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

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=60)	297	100	153-642
EDTA plasma (n=35)	234	80	ND-437
Heparin plasma (n=35)	259	100	134-508

ND=Non-detectable

**Cell Culture Supernates** - Human peripheral blood mononuclear cells ( $1 \times 10^6$  cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10  $\mu$ g/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assay for levels of natural human VEGF-D. All samples measured less than the lowest VEGF-D standard, 125 pg/mL.

## SPECIFICITY

This assay recognizes natural and recombinant human VEGF-D.

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 VEGF-D control were assayed for interference. No significant cross-reactivity or interference was observed.

### Recombinant human:

EGF	PD-ECGF
FGF acidic	PDGF-AA
FGF basic	PDGF-AB
HB-EGF	PDGF-BB
HGF	PIGF
IGF I	VEGF
IGF II	VEGF-C
KGF	VEGF R2
LAP (TGF- $\beta$ 1)	VEGF R3/Flt-4
$\beta$ -NGF	

### Recombinant mouse:

VEGF-D
VEGF R3/Flt-4

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