Quantikine®

Human VEGF/P*l*GF Heterodimer Immunoassay

Catalog Number DVPH00

For the quantitative determination of human Vascular Endothelial Growth Factor/Placenta Growth Factor heterodimer (VEGF/P*l*GF) 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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MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems China Co. Ltd.
24A1 Hua Min Empire Plaza
726 West Yan An Road
Shanghai PRC 200050

TELEPHONE: +86 (21) 52380373
FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

The Vascular Endothelial Growth Factor (VEGF) family is best known for its important roles in regulating the growth of vascular and lymphatic endothelia. Family members include VEGF (VEGF-A), -B, -C, -D, -E (viral), and P*l*GF (Placenta Growth Factor) (1). P*l*GF shares approximately 42% amino acid (aa) sequence identity with VEGF (53% within the PDGF region), and the two proteins share significant structural similarity (2).

P*l*GF exists in at least 3 alternatively spliced forms: P*l*GF-1 ($PlGF_{131}$), P*l*GF-2 ($PlGF_{152}$), and P*l*GF-3 ($PlGF_{203}$) (2 - 4). Notable differences between these forms include the insertion of a heparin-binding domain in P*l*GF-2 that might result in increased association with the cell membrane or altered affinities for receptors (5). In mice, only the P*l*GF-2 ortholog has been described and shares 65% aa sequence identity with its human counterpart (6). As the name reflects, P*l*GF was first identified in human placenta where it is expressed prominently under normal conditions (4, 7 - 9). Other tissues and cell types expressing P*l*GF include microvascular and human umbilical vein endothelia, bone marrow, uterine natural killer cells, and keratinocytes (5). It is upregulated under certain pathological conditions including wound healing, tumor formation, and preeclampsia (5, 10 - 17).

Like P*l*GF, a number of alternatively spliced forms of VEGF have been reported and include variants of 121, 145, 165, 183, 189, and 206 aa (18 - 21). $VEGF_{165}$ appears to be the most abundant isoform (19). The 189 and 206 aa variants have stretches of basic residues that enhance heparin binding and anchor them to the extracellular matrix, while the 121 aa isoform is freely diffusible (22). VEGF can be detected in both human plasma and serum samples, with higher levels found in serum due to its release from platelets (23). VEGF is upregulated during conditions of low oxygen tension and is dependent upon the activities of the hypoxia-inducible transcription factors, HIF-1α and HIF-2α (24, 25). Transcription can also be activated by oncogenes including H-ras, and several cytokines and growth factors including EGF, FGF-4, PDGF, and TGF-β (26 - 29).

VEGF homodimers bind the pro-angiogenic receptor VEGF R2 (KDR or Flk-1), and both VEGF and P*l*GF homodimers are ligands for VEGF R1 (Flt-1), Neuropilin-1, and Neuropilin-2 (30 - 35). VEGF and P*l*GF appear to have different effects on VEGF R1. For instance, they stimulate autophosphorylation of different tyrosine residues in VEGF R1 and induce the expression of different downstream genes (36). Despite not binding directly to VEGF R2, P*l*GF stimulation may result in VEGF R1-mediated transactivation of VEGF R2 (36). Naturally occurring VEGF/P*l*GF heterodimers have also been described (37). Although the role of the VEGF/P*l*GF heterodimer in vessel growth is unclear, it may induce an active VEGF R1/R2 heterodimer (36, 38, 39). The VEGF/P*l*GF heterodimer has been shown to enhance endothelial cell survival and promote *in vitro* endothelial cell tube formation (40). In addition, the heterodimer suppresses blood vessel regression in retinal survival assays and enhances corneal neovascularization (38, 41). The VEGF/P*l*GF heterodimer has been described in the conditioned media of several tumor cell lines, synovial fluid, and plasma (37, 38, 42).

The Quantikine Human VEGF/P*l*GF Heterodimer Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human VEGF/P*l*GF in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human VEGF/P*l*GF and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human VEGF/P*l*GF showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Human VEGF/P*l*GF kit can be used to determine relative mass values for naturally occurring VEGF/P*l*GF.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF/P*l*GF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF/P*l*GF 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/P*l*GF 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 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.
- This assay is designed to eliminate interference by soluble receptors, 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.

MATERIALS PROVIDED

VEGF/P*l*GF Microplate (Part 892983) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against VEGF/P*l*GF.

VEGF/P*l*GF Conjugate (Part 892984) - 21 mL of a polyclonal antibody against VEGF conjugated to horseradish peroxidase with preservatives.

VEGF/P*l*GF Standard (Part 892985) - 40 ng of recombinant human VEGF/P*l*GF in a buffer with preservatives; lyophilized.

Assay Diluent RD1X (Part 895121) - 11 mL of a buffered protein base with preservatives. *May contain a precipitate. Warm to room temperature, and mix well before and during use.*

Calibrator Diluent RD5L (Part 895028) - 21 mL of a buffered protein base with preservatives. *For cell culture supernate samples.*

Calibrator Diluent RD6S (Part 895142) - 21 mL of diluted animal serum in buffer with preservatives. *For serum/plasma samples.*

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

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

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="10">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="9">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1X</td></tr>
<tr><td>Calibrator Diluent RD5L</td></tr>
<tr><td>Calibrator Diluent RD6S</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>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.*</td></tr>
</table>

*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.
- **Polypropylene** test tubes for serial dilution.
- Human VEGF/P*l*GF Controls (optional; available from R&D Systems).

PRECAUTION

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 - 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.*

REAGENT PREPARATION

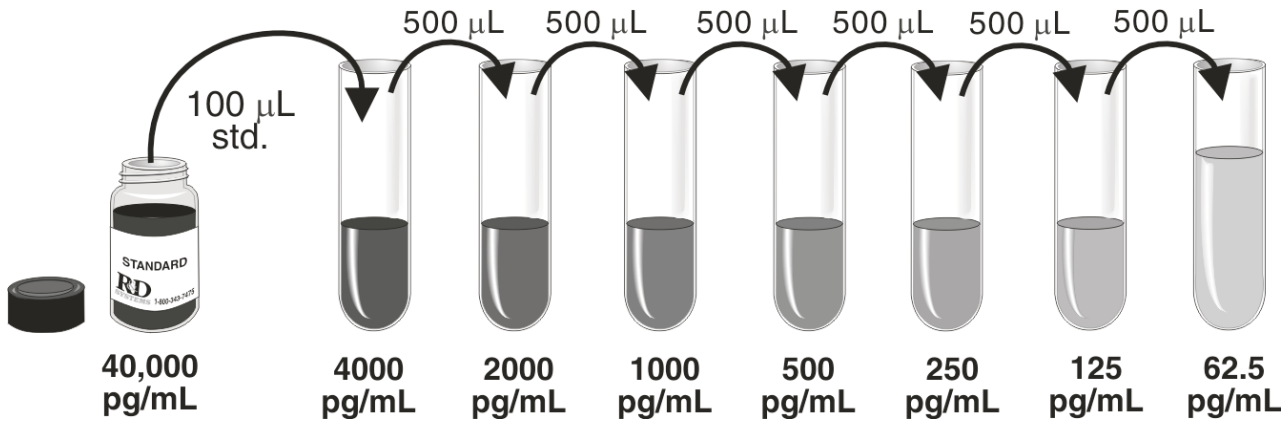
Bring all reagents to room temperature before use.

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.

VEGF/P*l*GF Standard - Reconstitute the VEGF/P*l*GF Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD5L (*for cell culture supernate samples*) or Calibrator Diluent RD6S (*for serum/plasma samples*) into the 4000 pg/mL tube. Pipette 500 mL 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 standard 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 samples, controls, and standards 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 RD1X to each well. Assay Diluent RD1X may contain a precipitate. Warm to room temperature, and mix well before and during use.

4. Add 50 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

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 VEGF/P*l*GF 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. Incubate for 30 minutes at room temperature. **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.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.

2. Add 100 µL Assay Diluent RD1X to each well.

3. Add 50 µL Standard, control, or sample to each well. Incubate 2 hours at RT.

4. Aspirate and wash 4 times.

5. Add 200 µL Conjugate to each well. Incubate 2 hours at RT.

6. Aspirate and wash 4 times.

7. Add 200 µL Substrate Solution to each well. **Protect from light.** Incubate 30 minutes.

8. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 minutes.
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.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

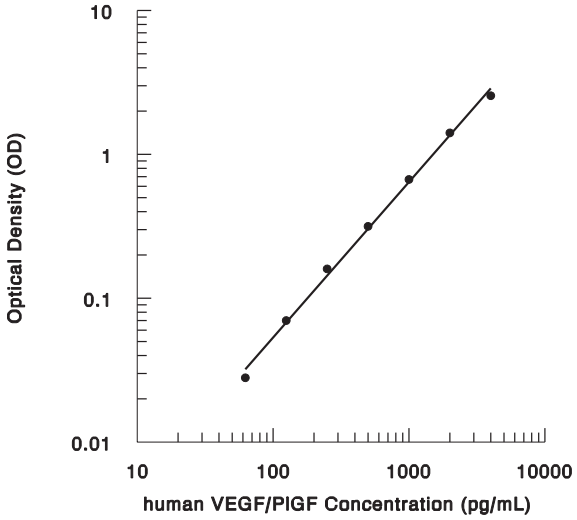
To determine the VEGF/P*l*GF concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding VEGF/P*l*GF concentration.

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

TYPICAL DATA

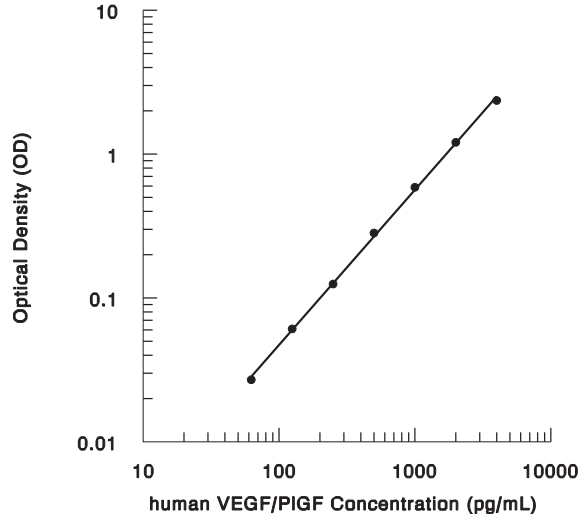
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Calibrator Diluent RD5L



pg/mL	O.D.	Average	Corrected
0	0.012 0.013	0.013	——
62.5	0.040 0.042	0.041	0.028
125	0.081 0.085	0.083	0.070
250	0.169 0.177	0.173	0.160
500	0.324 0.333	0.329	0.316
1000	0.680 0.684	0.682	0.669
2000	1.411 1.435	1.423	1.410
4000	2.562 2.578	2.570	2.557

Calibrator Diluent RD6S



pg/mL	O.D.	Average	Corrected
0	0.010 0.011	0.011	——
62.5	0.038 0.038	0.038	0.027
125	0.070 0.073	0.072	0.061
250	1.133 0.139	0.136	0.125
500	0.292 0.295	0.294	0.283
1000	0.590 0.608	0.599	0.588
2000	1.209 1.227	1.218	1.207
4000	2.368 2.370	2.369	2.358

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.
- 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.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

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.

Serum/Plasma Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	494	997	2612	443	907	2586
Standard deviation	12.2	16.7	104	40.7	73.3	154
CV (%)	2.5	1.7	4.0	9.2	8.1	6.0

Cell Culture Supernate Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	371	780	2180	402	829	2352
Standard deviation	12.8	24.9	106	32.1	50.1	119
CV (%)	3.5	3.2	4.9	8.0	6.0	5.1

RECOVERY

The recovery of VEGF/P*l*GF spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample	Average % Recovery	Range
Cell culture supernates (n=4)	93	85 - 99%
Serum (n=4)	98	86 - 114%
EDTA plasma (n=4)	106	97 - 114%
Heparin plasma (n=4)	102	91 - 112%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of VEGF/P*l*GF were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture samples (n=4)	Serum (n=4)	Heparin plasma (n=4)	EDTA plasma (n=4)
1:2	Average % of Expected	104	92	97	95
	Range (%)	98 - 107	86 - 99	86 - 107	86 - 102
1:4	Average % of Expected	101	98	100	100
	Range (%)	95 - 107	94 - 104	94 - 107	94 - 103
1:8	Average % of Expected	93	103	99	101
	Range (%)	88 - 105	100 - 105	100 - 109	95 - 103
1:16	Average % of Expected	92	99	99	101
	Range (%)	87 - 101	94 - 102	92 - 106	99 - 102

SENSITIVITY

Fifty-one assays were evaluated and the minimum detectable dose (MDD) of VEGF/P*l*GF ranged from 0.66 - 10.8 pg/mL. The mean MDD was 3.35 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 *E. coli*-expressed recombinant human VEGF/P*l*GF produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of VEGF/P*l*GF 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=35)	98.8	2.9	ND - 98.8
EDTA plasma (n=35)	105	2.9	ND - 105
Heparin plasma (n=35)	86.4	2.9	ND - 86.4

ND = Non-detectable

Cell Culture Supernates -
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 5 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. Aliquots of the cell culture supernate were removed and assayed for levels of natural VEGF/P*l*GF. No detectable levels were observed.

JE-3 (human choriocarcinoma) cells were grown to confluency in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. The cells were stimulated overnight with 10 ng/mL TNF-α.

Condition	Day 1 (pg/mL)
Unstimulated	322
Stimulated	237

JAR (human choriocarcinoma) cells were grown to confluency in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Aliquots of the cell culture supernate were removed, assayed for levels of natural VEGF/P*l*GF, and measured 836 pg/mL.

HepG2 (human liver) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were grown to 100% confluency, assayed for levels of natural VEGF/P*l*GF, and measured 228 pg/mL.

4MBr-5 (rhesus macaque bronchus) cells were grown to 100% confluency in F12 supplemented with 10% fetal calf serum, 50 ng/mL rhEGF, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Aliquots of the cell culture supernate were removed, assayed for levels of natural VEGF/P*l*GF, and measured 154 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human VEGF/P*l*GF. 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 recombinant human VEGF/P*l*GF standard were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
β-ECGF
EGF
EG-VEGF
FGF acidic
FGF basic$_{146}$
FGF-4
FGF-5
FGF-6
FGF-9
FGF-10
FGF-18
Flt-3/Flk-2 ligand
Flt-4
G-CSF
GM-CSF
HB-EGF
HGF
HRG-α
IGF-I
IGF-II
KGF
M-CSF
MSP
MSPβ
β-NGF
PDGF
PDGF-AA
PDGF-AB
PDGF-BB
PD-ECGF
P*l*GF
VEGF$_{121}$
VEGF$_{165}$
VEGF-B
VEGF-C
VEGF-D
VEGF R1
VEGF R2
VEGF R3

Recombinant mouse:
FGF-8b
FGF-8c
Flt-3/Flk-2 ligand
G-CSF
GM-CSF
M-CSF
P*l*GF-2
VEGF$_{120}$
VEGF$_{164}$
VEGF-B
VEGF-D
VEGF R1
VEGF R2
VEGF R3

Recombinant rat:
GM-CSF
β-NGF
PDGF-BB
VEGF

Recombinant canine:
VEGF

Recombinant porcine:
GM-CSF

Recombinant zebrafish:
VEGF

Natural proteins:
bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.