Quantikine® ELISA

Human VEGF Immunoassay

Catalog Number DVE00
SVE00
PDVE00

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

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
PRINCIPLE OF THE ASSAY	2
LIMITATIONS OF THE PROCEDURE	2
TECHNICAL HINTS	2
MATERIALS PROVIDED & STORAGE CONDITIONS	3
OTHER SUPPLIES REQUIRED	4
PRECAUTIONS	4
SAMPLE COLLECTION & STORAGE	4
REAGENT PREPARATION	5
ASSAY PROCEDURE	6
CALCULATION OF RESULTS	7
TYPICAL DATA	7
PRECISION	8
RECOVERY	8
SENSITIVITY	8
LINEARITY	9
CALIBRATION	9
SAMPLE VALUES	10
SPECIFICITY	11
REFERENCES	12
PLATE LAYOUT	13

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INTRODUCTION

Vascular endothelial growth factor (VEGF or VEGF-A), also known as vascular permeability factor (VPF), is a potent mediator of both angiogenesis and vasculogenesis in the fetus and adult (1-3). It is a member of the PDGF family that is characterized by the presence of eight conserved cysteine residues in a cystine knot structure and the formation of antiparallel disulfide-linked dimers (4). Humans express alternately spliced isoforms of 121, 145, 165, 183, 189, and 206 amino acids (aa) in length (4). $VEGF_{165}$ appears to be the most abundant and potent isoform, followed by $VEGF_{121}$ and $VEGF_{189}$ (3, 4). Isoforms other than $VEGF_{121}$ contain basic heparin-binding regions and are not freely diffusible (4). Human $VEGF_{165}$ shares 88% aa sequence identity with corresponding regions of mouse and rat VEGF. VEGF is expressed in multiple cells and tissues including skeletal and cardiac muscle (5, 6), hepatocytes (7), osteoblasts (8), neutrophils (9), macrophages (10), keratinocytes (11), brown adipose tissue (12), $CD34^+$ stem cells (13), endothelial cells (14), fibroblasts, and vascular smooth muscle cells (15). VEGF expression is induced by hypoxia and cytokines such as IL-1, IL-6, IL-8, oncostatin M and TNF-α (3, 4, 9, 16). VEGF isoforms are differentially expressed during development and in the adult (3).

VEGF dimers bind to two related receptor tyrosine kinases, VEGF R1 (also called Flt-1) and VEGF R2 (Flk-1/KDR), and induce their homodimerization and autophosphorylation (3, 4, 7, 17, 18). These receptors have seven extracellular immunoglobulin-like domains and an intracellular split tyrosine kinase domain. They are expressed on vascular endothelial cells and a range of non-endothelial cells. Although VEGF affinity is highest for binding to VEGF R1, VEGF R2 appears to be the primary mediator of VEGF angiogenic activity (3, 4). $VEGF_{165}$ also binds the semaphorin receptor, neuropilin-1, which promotes complex formation with VEGF R2 (19).

VEGF is best known for its role in vasculogenesis. During embryogenesis, VEGF regulates the proliferation, migration, and survival of endothelial cells (3, 4), thus regulating blood vessel density and size, but playing no role in determining vascular patterns. VEGF promotes bone formation through osteoblast and chondroblast recruitment and is also a monocyte chemoattractant (20-22). In postnatal life, VEGF maintains endothelial cell integrity and is a potent mitogen for micro- and macro-vascular endothelial cells. In adults, VEGF functions mainly in wound healing and the female reproductive cycle (3). In diseased tissues, VEGF promotes vascular permeability. It is thus thought to contribute to tumor metastasis by promoting both extravasation and tumor angiogenesis (23, 24). Various strategies have been employed therapeutically to antagonize VEGF-mediated tumor angiogenesis (25). Circulating VEGF levels correlate with disease activity in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (26).

The Quantikine Human VEGF Immunoassay is a 4.5 hour solid phase ELISA designed to measure $VEGF_{165}$ levels in cell culture supernates, serum, and plasma. It contains *Sf* 21-expressed recombinant human $VEGF_{165}$ and antibodies raised against the recombinant protein. Results obtained for naturally occurring human VEGF and recombinant human $VEGF_{121}$ showed linear curves that were parallel to the standard curves obtained using the Quantikine Human VEGF Immunoassay 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.
- 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 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.

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 # DVE00	CATALOG # SVE00	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
VEGF Microplate	890218	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against VEGF.	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.*
VEGF Standard	890220	3 vials	18 vials	2000 pg/vial of recombinant $VEGF_{165}$ in a buffered protein base with preservatives; lyophilized.	Discard the VEGF stock solution and dilutions after 4 hours. Use a fresh standard for each assay.
VEGF Conjugate	890219	1 vial	6 vials	21 mL/vial of a polyclonal antibody against VEGF 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 base with preservatives.	
Calibrator Diluent RD5K	895119	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD6U	895148	1 vial	6 vials	21 mL/vial of animal serum with preservatives. *For serum/plasma samples.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	
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.

DVE00 contains sufficient materials to run an ELISA on one 96 well plate.
SVE00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDVE00). 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.
- **Polypropylene** test tubes for dilution of standards.
- Human VEGF Controls (optional; available from R&D Systems).

PRECAUTIONS

Calibrator Diluent RD6U contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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 - 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 ≤ -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 EDTA, heparin, or citrate 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.

REAGENT PREPARATION

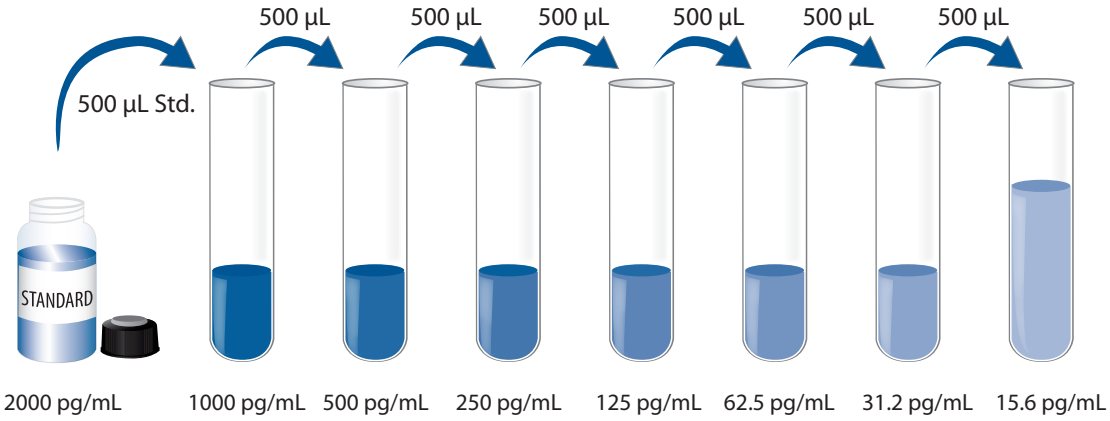
Bring all reagents to room temperature before use.

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.

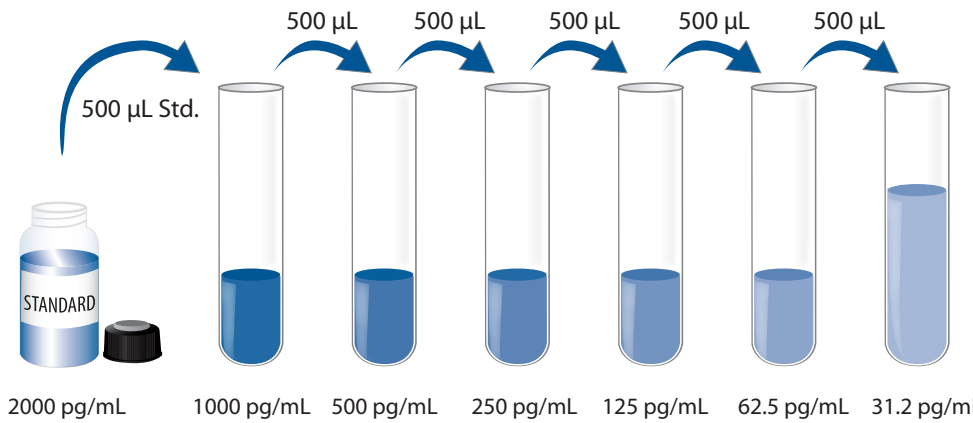
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

VEGF Standard - Reconstitute the VEGF Standard with 1.0 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 standards, samples, and controls 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. **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, control, or sample per well.
   For Serum/Plasma Samples: Add 100 µL of Standard, control, 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, 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 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. **Protect from light.**
   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. 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.

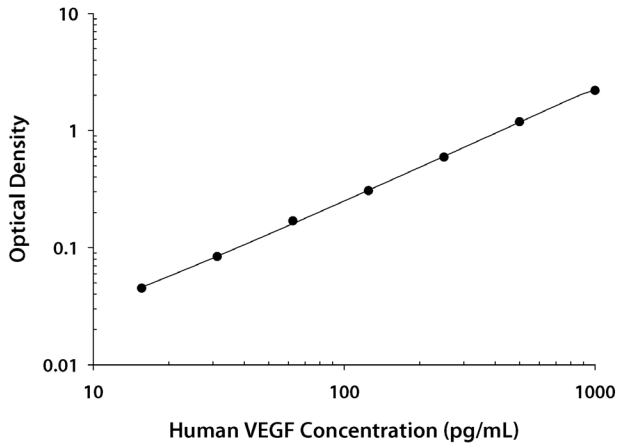
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best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

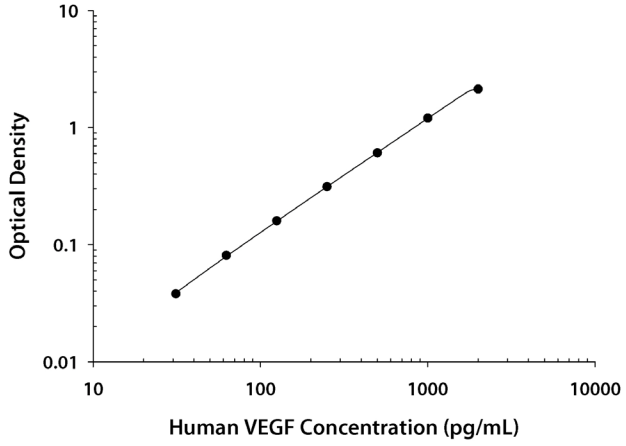
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CALIBRATOR DILUENT RD5K



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

CALIBRATOR DILUENT RD6U



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

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.

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)	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

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)	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

RECOVERY

The recovery of VEGF 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=5)	102	95-111%
Serum (n=5)	102	92-115%
EDTA plasma (n=5)	97	82-113%
Heparin plasma (n=5)	93	82-102%
Citrate plasma (n=5)	100	88-113%

SENSITIVITY

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

LINEARITY

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

		Cell culture media (n=5)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)	Citrate plasma (n=5)
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

CALIBRATION

This immunoassay is calibrated against a highly purified *Sf* 21-expressed recombinant human $VEGF_{165}$ produced at R&D Systems.

The NIBSC/WHO $VEGF_{165}$ preparation 02/286 (recombinant human DNA) was evaluated in this kit. The dose response curve of the standard 02/286 parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine Human VEGF kit to approximate NIBSC/WHO 02/286 Units, use the equation below.

NIBSC/WHO (02/286) approximate value (U/mL) = 0.002 x Quantikine VEGF value (pg/mL)

Note: *Based on data generated in April 2011.*

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of VEGF 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=37)	220	100	62-707
EDTA plasma (n=37)	61	24	ND-115
Heparin plasma (n=37)	41	22	ND-55
Citrate plasma (n=37)	——	0	ND

ND=Non-detectable

Cell Culture Supernates - Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of natural VEGF.

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

SPECIFICITY

This assay recognizes natural and recombinant human VEGF. This assay also recognizes recombinant human $VEGF_{165b}$.

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 control were assayed for interference. The following factors showed no cross-reactivity or interference.

Recombinant human:

ANG
AR
CNTF
β-ECGF
EGF
Epo
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
FGF-7
G-CSF
GM-CSF
sgp130
GROα
GROβ
GROγ
HB-EGF
HGF
IFN-γ
IGF-I
IGF-II
IL-1α
IL-1β
IL-1ra
IL-1 sRI
IL-1 sRII
IL-2
IL-2 sRα
IL-3
IL-3 sRa
IL-3 sRβ
IL-4
IL-5
IL-5 sRα
IL-6
IL-6 sR
IL-7
IL-8
IL-9
IL-10
IL-11
IL-12
IL-13
LAP (TGF-β1)
LIF
M-CSF
MCP-1
MIP-1α
MIP-1β
β-NGF
OSM
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PlGF
PTN
RANTES
SCF
SLPI
TGF-α
TGF-β1
TGF-β3
TGF-β sRII
TNF-a
TNF-β
sTNF RI
sTNF RII
$VEGF_{165}$/PlGF
$VEGF\text{-}B_{167}$
VEGF-C
VEGF-D
VEGF R3

Recombinant mouse:

GM-CSF
IL-1α
IL-1β
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-13
LIF
MIP-1α
MIP-1β
PlGF-2
SCF
TNF-α
$VEGF_{120}$
$VEGF_{164}$
VEGF R3

Other recombinants:

chicken TGF-β3
amphibian TGF-β5
rat VEGF
zebrafish VEGF

Natural proteins:

mouse EGF
bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
porcine TGF-β1

VEGF-related factors showing cross-reactivity or interference.

Recombinant human VEGF R1 (Flt-1)/Fc Chimera	Interference at levels ≥ 500 pg/mL
Recombinant human VEGF R2 (KDR)/Fc Chimera	Interference at levels ≥ 2000 pg/mL
Recombinant mouse VEGF R1	Interference at levels ≥ 500 pg/mL
Recombinant mouse VEGF R2	Interference at levels ≥ 4000 pg/mL
Recombinant canine VEGF	Cross-reacts at about 67%

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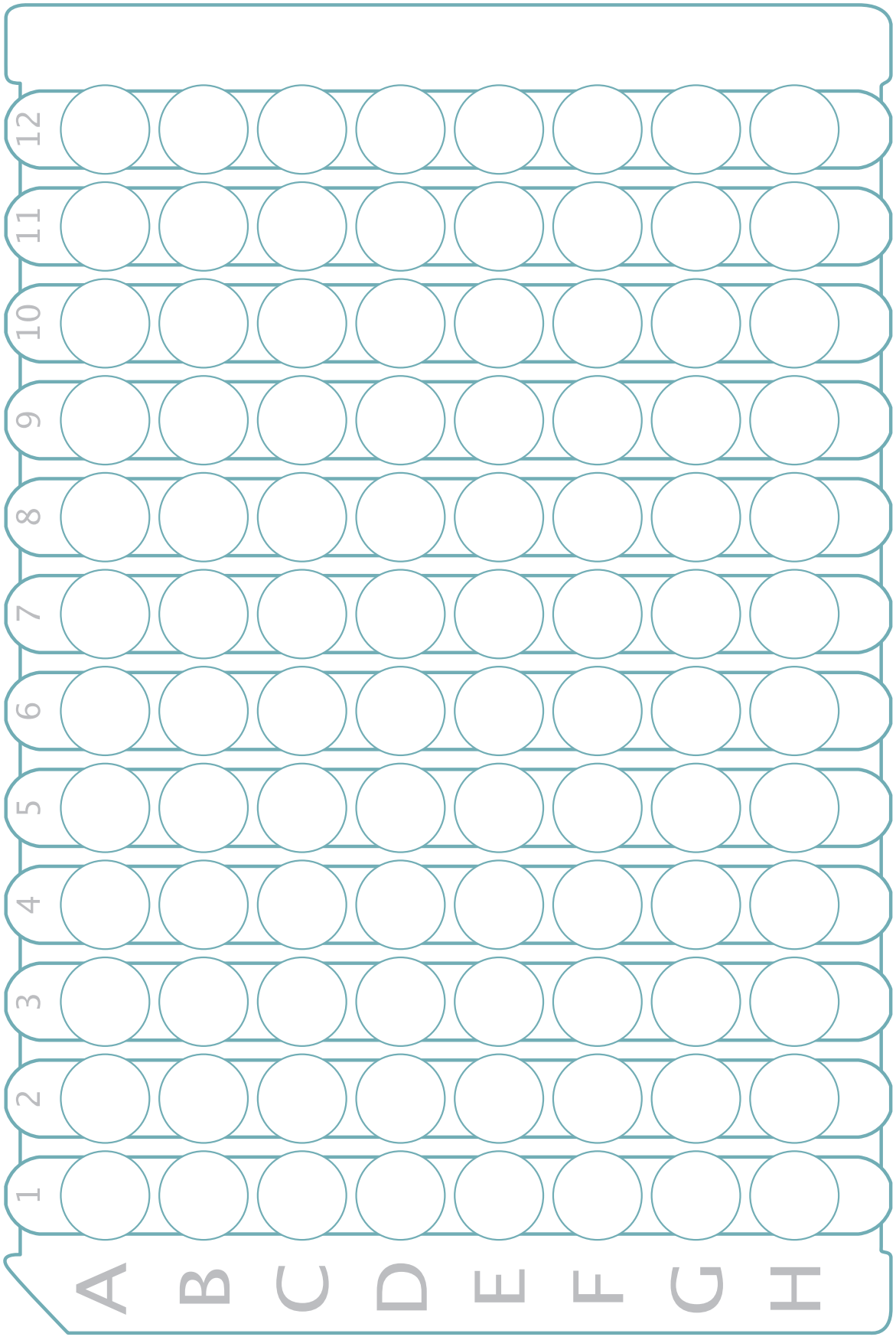
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES