Quantikine® ELISA

Human VEGF-C Immunoassay

Catalog Number DVEC00

For the quantitative determination of human Vascular Endothelial Growth Factor C (VEGF-C) concentrations in cell culture supernates, serum, platelet-poor 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

The Vascular Endothelial Growth Factors (VEGFs) are well known for their important roles in vascular and lymphatic vessel growth. The family includes VEGF-A, -B, -C, -D, -E (Orf virus-encoded), and Placenta Growth Factor (PlGF). VEGF-C and VEGF-D constitute a sub-family that shares the conserved VEGF homology domain (VHD) with other mammalian VEGF family members and are also characterized by long N- and C-terminal extensions (1-4). The VEGF-C C-terminal pro-region is cysteine-rich with two copies of a $C_6C_{10}CRC$ motif and five nearly complete copies of a $C_{10}CXCXC$ motif (characteristic of the Balbiani 3 Ring protein) (1, 2, 5). Both VEGF R2 and VEGF R3 act as receptors for VEGF-C (3, 6). The VEGF-C propeptide undergoes stepwise proteolytic processing to generate ligands with increasing affinity for VEGF R3 (7), however, only the fully processed VEGF-C isoform appears to bind VEGF R2 (7). The proteases Plasmin, Furin, PC5, and PC7 are shown to mediate VEGF-C processing (8, 9). The Integrin $\alpha_9\beta_1$ and the Semaphorin/VEGF family receptor Neuropilin-2 may also act as receptors or co-receptors for VEGF-C (10, 11). VEGF-C is expressed in multiple adult human tissues, most predominantly in lymph nodes, heart, placenta, ovary, and small intestine (3). VEGF-C is also detected in the brain, liver, thymus, skeletal muscle, spleen, prostate, testis, and colon (3).

VEGF-C can act on both vascular and lymphatic vessels. It induces vascular permeability through the activation of VEGF R2 (12, 13). It has also been shown to induce the proliferation and migration of endothelial cells *in vitro*, as well as stimulating vascular growth in certain model systems *in vivo* (4, 14-18). The VEGF-C/VEGF R3 signaling pathway is known to be important for lymphangiogenesis. VEGF-C and VEGF R3 are co-expressed at sites of lymphatic vessel sprouting in the embryo (19). *In vitro*, VEGF-C/VEGF R3 promotes the survival, proliferation, and migration of lymphatic endothelial cells and induces signaling cascades that include PKC, MAP kinase, PI3K, and Akt (20). It also stimulates lymphangiogenesis in several model systems including the avian chorioallantoic membrane model (21), transgenic mice (22, 23), and in corneal angiogenesis assays (14). Knockout of the VEGF-C gene is embryonic lethal late in development, and although cells differentiate into the lymphatic lineage, they fail to sprout and form lymphatic vessels (24). Inactivation of a single VEGF-C allele results in the development of cutaneous lymphatic hypoplasia and lymphedema (24).

In addition to their primary physiological roles in leukocyte trafficking and fluid homeostasis, the lymphatics also act as pathways for tumor cell metastases (25, 26). Elevated VEGF-C levels have been correlated with many human cancers, and expression may be a predictor of lymph node metastases (27-40). Whether VEGF-C promotes tumor-associated lymphangiogenesis in human tumors remains unclear (26); however, increased VEGF-C-mediated lymphangiogenesis and metastases are observed in several models of human cancer (41-44). Dysfunction of lymphatic vessels can result in lymphedema characterized by interstitial fluid accumulation, impaired immune responses, and enhanced risk of infection (45, 46). Primary lymphedema can be caused by rare developmental disorders, while secondary lymphedema may be associated with radiation therapy, surgery, or infection. Several animal models of lymphedema suggest that VEGF-C can restore lymphatic function and, therefore, might be of therapeutic benefit to lymphedema patients (10, 47, 48).

The Quantikine Human VEGF-C Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human VEGF-C in cell culture supernates, serum, plasma, and saliva. It contains NS0-expressed recombinant human VEGF-C (Cys156Ser substituted) and has been shown to accurately quantitate the recombinant factor. Results obtained using recombinant human VEGF-C (wild type) and natural human VEGF-C 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 naturally occurring human VEGF-C.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human VEGF-C has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF-C present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human VEGF-C 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-C 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-C Microplate	892740	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against VEGF-C.	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-C Standard	892742	70 ng of recombinant human VEGF-C in a buffer with preservatives; lyophilized.	Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 1 month.*
Calibrator Diluent RD6U	895148	21 mL of animal serum with preservatives. *May contain a precipitate. Mix well before and during use.*	Prepare fresh daily. Discard diluted diluent after use.
VEGF-C Conjugate	892741	21 mL of polyclonal antibody against VEGF-C conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as a Salivette® or equivalent.
- Test tubes for dilution of standards.
- Human VEGF-C 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-C 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.

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 ≤ -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.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

VEGF-C is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of VEGF-C, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood.

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

SAMPLE PREPARATION

Serum samples require a 5-fold dilution. A suggested 5-fold dilution is 40 µL of sample + 160 µL of Calibrator Diluent RD6U (1:2).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of VEGF-C are found in saliva. It is recommended that a face mask and gloves be used 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.

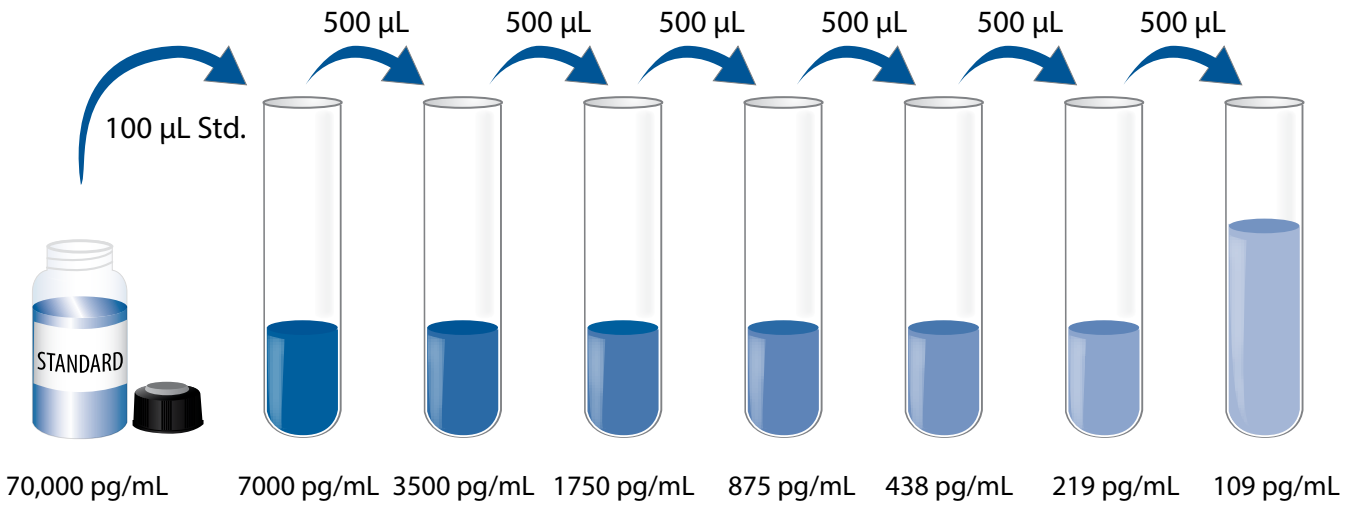
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD6U:
Note: *Prepare only as much Calibrator Diluent as needed per day. Discard after use.*

> **For Cell Culture Supernate/Saliva samples** - Dilute 4.0 mL of Calibrator Diluent RD6U into 16 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD6U (1:5).
>
> **For Serum/Plasma samples** - Dilute 10 mL of Calibrator Diluent RD6U into 10 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD6U (1:2).

VEGF-C Standard - Reconstitute the VEGF-C Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 70,000 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.

Pipette 900 µL of Calibrator Diluent RD6U (1:5) (*for cell culture supernate/saliva samples*) or Calibrator Diluent RD6U (1:2) (*for serum/plasma samples*) into the 7000 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 7000 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 standards, samples, and controls be assayed in duplicate.

Note: *High concentrations of VEGF-C are found in saliva. It is recommended that a face mask and gloves be used 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 50 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 ± 50 rpm. 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-C 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.

*Serum samples 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.).

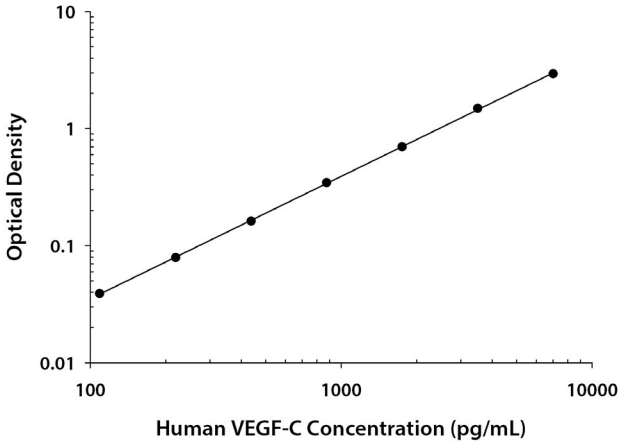
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points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

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

TYPICAL DATA

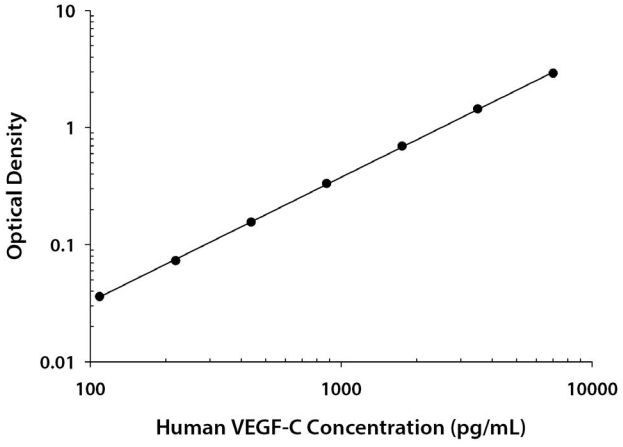
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/SALIVA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.071 0.075	0.073	—
109	0.108 0.116	0.112	0.039
219	0.148 0.156	0.152	0.079
438	0.233 0.236	0.235	0.162
875	0.416 0.420	0.418	0.345
1750	0.748 0.796	0.772	0.699
3500	1.495 1.622	1.559	1.486
7000	2.973 3.040	3.007	2.934

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.067 0.070	0.069	—
109	0.103 0.106	0.105	0.036
219	0.138 0.145	0.142	0.073
438	0.222 0.227	0.225	0.156
875	0.395 0.408	0.402	0.333
1750	0.739 0.786	0.763	0.694
3500	1.421 1.598	1.510	1.441
7000	2.946 2.997	2.972	2.903

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/SALIVA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	572	1622	3243	520	1527	3069
Standard deviation	39.3	54.4	136	50.1	102	200
CV (%)	6.9	3.4	4.2	9.6	6.7	6.5

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)	512	1543	3195	514	1540	3066
Standard deviation	33.8	54.2	136	43.6	110	198
CV (%)	6.6	3.5	4.3	8.5	7.2	6.4

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture supernate (n=4)	107	93-115%
Serum (n=4)	99	89-106%
EDTA plasma (n=4)	101	96-110%
Heparin plasma (n=4)	97	88-103%

LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of human VEGF-C were 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)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva (n=4)
1:2	Average % of Expected	94	103	103	105	96
	Range (%)	87-97	96-108	99-107	95-109	87-112
1:4	Average % of Expected	94	105	103	107	106
	Range (%)	94-94	96-112	98-108	98-114	100-111
1:8	Average % of Expected	98	105	101	107	———
	Range (%)	93-103	94-113	99-105	101-114	———
1:16	Average % of Expected	97	105	98	106	———
	Range (%)	91-105	89-112	85-106	102-108	———

*Samples were diluted 2-fold prior to assay.

SENSITIVITY

Ninety-four assays were evaluated and the minimum detectable dose (MDD) of human VEGF-C ranged from 4.0-48.4 pg/mL. The mean MDD was 13.3 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 NS0-expressed recombinant human VEGF-C produced at R&D Systems.

SAMPLE VALUES

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

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=30)	4847	2459-6651	1110
Platelet-poor EDTA plasma (n=30)	332	185-1231	225
Platelet-poor heparin plasma (n=30)	356	222-819	135
Saliva (n=10)	513	195-851	206

Cell Culture Supernates - Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 50 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 supernates were removed and assayed for natural human VEGF-C. No detectable levels were observed.

Other cell lines tested:

Cell Line	Value (pg/mL)
IMR-90	1340
PC-3 stimulated	2477
PC-3 unstimulated	1570
Hs294-T	210
HUVEC stimulated with IL-4	518
HUVEC unstimulated	246
HT-1080	2193

SPECIFICITY

This assay recognizes natural human VEGF-C, as well as recombinant human VEGF-C (wild type) and recombinant human VEGF-C (Cys156Ser substituted).

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD6U and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human VEGF-C 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-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/FGF-7
M-CSF
MSP
MSPβ
β-NGF
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PlGF
VEGF
$VEGF_{121}$
$VEGF_{165}$
VEGF/PlGF
VEGF-B
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
PlGF-2
VEGF
$VEGF_{120}$
$VEGF_{164}$
VEGF-B
VEGF-D
VEGF R1
VEGF R2
VEGF R3

Recombinant rat:

GM-CSF
β-NGF
PDGF-BB
VEGF

Other recombinants:

porcine GM-CSF
zebrafish VEGF

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF

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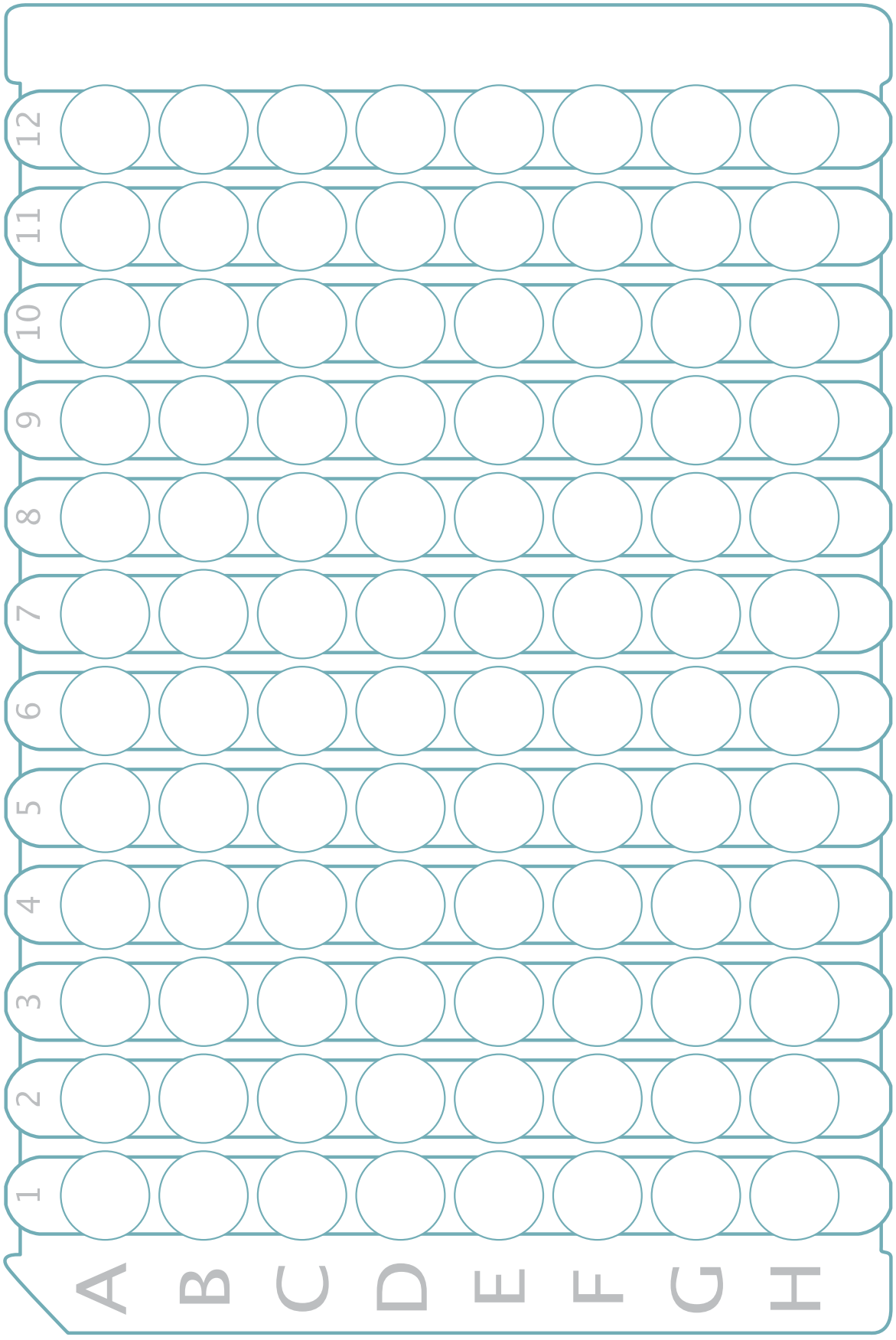
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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