QuantiGlo® ELISA

Human VEGF Immunoassay

Catalog Number QVE00B

For the quantitative determination of human Vascular Endothelial Growth Factor (VEGF) concentrations in cell culture supernates, serum, plasma, saliva, and urine.

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:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475 612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

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 QuantiGlo® Human VEGF Chemiluminescent Immunoassay is a 5.5 hour solid phase ELISA designed to measure human $VEGF_{165}$ levels in cell culture supernates, serum, plasma, saliva, and urine. 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 QuantiGlo® 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 human 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 human VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of VEGF bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted.

TECHNICAL HINTS

- 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.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the QuantiGlo® Immunoassay, the possibility of interference cannot be excluded.
- Variation in pipetting technique, washing technique, luminometers, incubation time or temperature can cause variations in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- 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.
- Relative light units (RLUs) may differ among luminometers. Adjust settings as recommended by the instrument manufacturer.
- Relative light units may vary within the 15 minute reading window.
- Due to limitations of many luminometers, it is recommended that the standards be assayed in duplicate from high to low beginning with the high standard in wells A_1 and A_2.

PRECAUTIONS

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

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

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
Human VEGF Microplate	892691	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human VEGF.	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.*
Human VEGF Standard	890678	2 vials of recombinant human $VEGF_{165}$ in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human VEGF Conjugate	890677	21 mL of a polyclonal antibody specific for human VEGF conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-8	895465	2 vials (11 mL/vial) of a buffered protein base with preservatives and blue dye.	
Calibrator Diluent RD5L	895028	21 mL of buffered protein base with preservatives. *Use undiluted for cell culture supernate/serum/plasma/saliva samples. Use diluted 1:2 for urine samples.*	
Wash Buffer Concentrate	895222	100 mL of a 10-fold concentrated solution of buffered surfactant with preservatives.	
Glo Reagent A	895868	4 mL of stabilized enhanced luminol.	
Glo Reagent B	895869	8 mL of stabilized hydrogen peroxide.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Luminometer set with the following parameters: 1.0 minute lag time; 0.5 sec/well read time; summation mode; auto gain on, or equivalent
- Pipettes and pipette tips
- 50 mL and 1000 mL graduated cylinders
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Collection device for saliva samples which has no protein binding or filtering capabilities such as Salivette® or equivalent
- Test tubes for dilution of standards
- Human VEGF Controls (optional; R&D Systems®, Catalog # QC198)

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 at room temperature 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 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 ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Hemolyzed samples are not suitable for use in this assay.

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.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter. Assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High levels of VEGF are found in saliva. Take necessary precautions (e.g. mask and gloves) to protect kit reagents.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 100 mL of Wash Buffer Concentrate to 900 mL of deionized or distilled water to prepare 1000 mL of Wash Buffer.

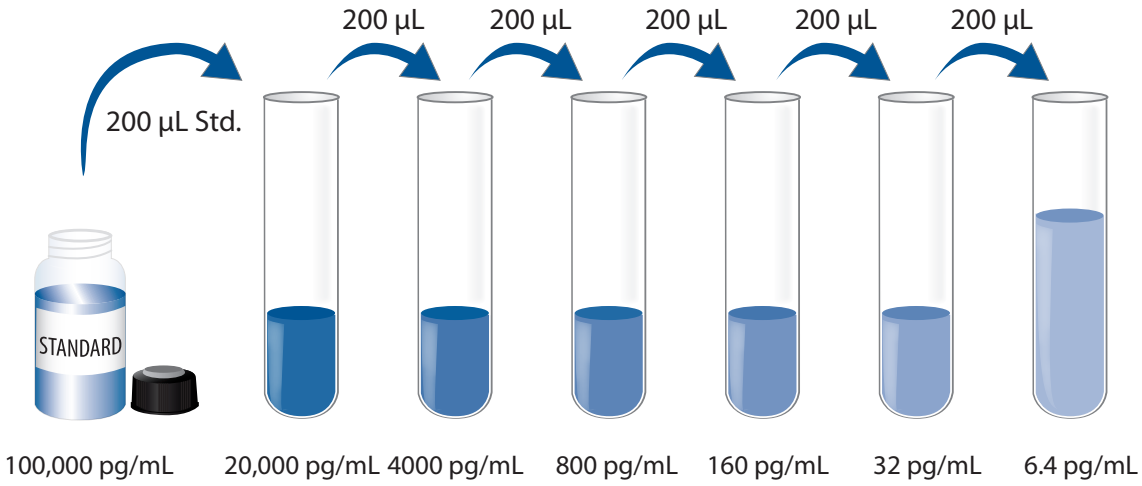
Calibrator Diluent RD5L (diluted 1:2) - **Urine samples only.** Add 10 mL of Calibrator Diluent RD5L to 10 mL of deionized or distilled water to prepare 20 mL of Calibrator Diluent RD5L (diluted 1:2).

Working Glo Reagent - 1 part Glo Reagent A (4.0 mL) and 2 parts Glo Reagent B (8.0 mL) should be mixed together 15 minutes to 4 hours before use in a capped plastic container and protected from light. 100 µL of the resultant mixture is required per well.

Note: *If running the assay in less than 96 wells, mix appropriate amounts Glo Reagent A and Glo Reagent B. To assay half a plate (48 wells), mix 2.0 mL of Glo Reagent A with 4.0 mL of Glo Reagent B. Working Glo Reagent should be discarded after use.*

Human VEGF Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human VEGF Standard with deionized or distilled water. This reconstitution produces a stock solution of 100,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 800 µL of Calibrator Diluent RD5L (*for cell culture supernates, serum, plasma and saliva samples*) or Calibrator Diluent RD5L (diluted 1:2) (*for urine samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20,000 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, controls, and samples be assayed in duplicate.

Note: *High levels of VEGF are found in saliva. Take necessary precautions (e.g. mask and gloves) to protect kit reagents.*

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 150 µL of Assay Diluent RD1-8 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 Human VEGF Conjugate to each well. Cover with a new adhesive strip. Incubate for 3 hours at room temperature on the shaker.

   Note: *Prepare Working Glo Reagent at this time.*
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Working Glo Reagent to each well. Incubate for 5-20 minutes at room temperature **on the benchtop. Protect from light.**
9. Determine the RLU of each well using a luminometer set with the following parameters: 1.0 min. lag time; 0.5 sec/well read time; summation mode; auto gain on, or equivalent.

CALCULATION OF RESULTS

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

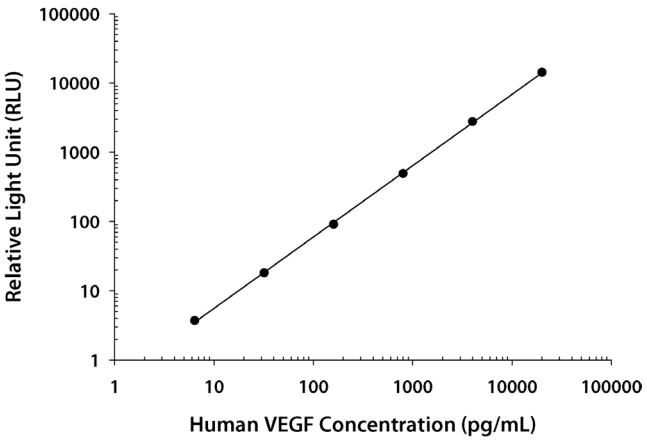
Create a standard curve by reducing the data using computer software capable of generating a cubic-spline curve fit.

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

TYPICAL DATA

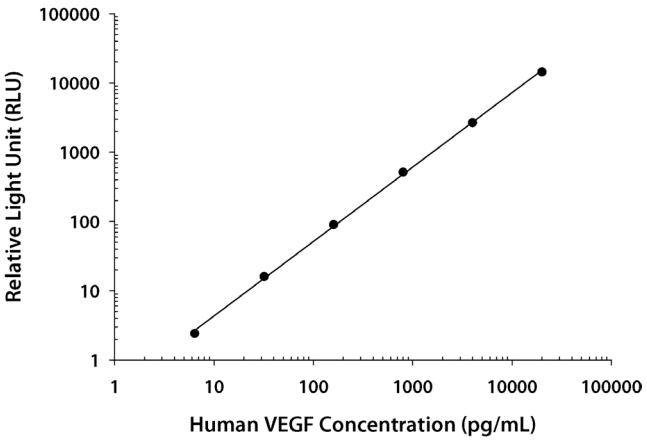
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/SERUM/PLASMA/SALIVA ASSAY



(pg/mL)	RLU	Average	Corrected
0	12.9 13.3	13.2	—
6.4	16.8 17.0	16.9	3.7
32	30.7 31.9	31.3	18.1
160	103 105	104	90.8
800	504 507	506	493
4000	2626 2940	2783	2770
20,000	12,811 15,712	14,262	14,249

URINE ASSAY



(pg/mL)	RLU	Average	Corrected
0	13.9 14.2	14.1	—
6.4	16.5 16.5	16.5	2.4
32	29.6 30.6	30.1	16
160	98 110	104	90
800	516 540	528	514
4000	2594 2779	2686	2672
20,000	13,520 15,334	14,427	14,413

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

CELL CULTURE SUPERNATE/SERUM/PLASMA/SALIVA ASSAY

	Intra-Assay Precision				Inter-Assay Precision			
Sample	1	2	3	4	1	2	3	4
n	20	20	20	20	20	20	20	20
Mean (pg/mL)	22.2	392	1857	13,972	22.7	389	1843	13,411
Standard deviation	1.76	12.9	52.3	557	2.0	27.6	131	562
CV (%)	7.9	3.3	2.8	4.0	8.8	7.1	7.1	4.2

URINE ASSAY

	Intra-Assay Precision				Inter-Assay Precision			
Sample	1	2	3	4	1	2	3	4
n	20	20	20	20	20	20	20	20
Mean (pg/mL)	24.4	417	1999	14,245	22.9	383	1823	12,825
Standard deviation	1.24	16.0	44.9	608	2.36	28.9	141	536
CV (%)	5.1	3.8	2.2	4.3	10.3	7.5	7.7	4.2

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	95-113%
Serum (n=4)	102	90-112%
EDTA plasma (n=4)	104	98-110%
Heparin plasma (n=4)	104	96-111%
Urine (n=4)	99	90-106%

SENSITIVITY

Sixty assays were evaluated. The minimum detectable dose (MDD) of human VEGF ranged from 1.61-5.99 pg/mL. The mean MDD was 3.30 pg/mL.

The MDD was determined by adding two standard deviations to the mean RLU of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples containing or spiked with high concentrations of human VEGF in various matrices were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva (n=4)	Urine (n=4)
1:2	Average % of Expected	98	102	103	101	102	100
	Range (%)	91-107	95-109	94-114	96-105	92-110	94-108
1:4	Average % of Expected	103	99	98	95	104	98
	Range (%)	100-105	95-107	89-112	92-100	97-110	90-109
1:8	Average % of Expected	99	93	96	94	101	103
	Range (%)	91-111	90-95	89-104	91-96	93-108	97-107
1:16	Average % of Expected	97	94	96	92	101	102
	Range (%)	91-105	92-95	88-104	88-97	91-108	97-111
1:32	Average % of Expected	102	96	94	93	101	96
	Range (%)	96-107	92-100	89-97	90-96	97-103	95-97

CALIBRATION

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

The NIBSC/WHO $VEGF_{165}$ 1st WHO Reference Reagent 02/286 (recombinant human DNA) was evaluated in this kit. The dose response curve of the standard 02/286 parallels the QuantiGlo® standard curve. To convert sample values obtained with the QuantiGlo® 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 QuantiGlo® VEGF value (pg/mL)

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

SAMPLES VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human VEGF 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=35)	239	47-666	155
EDTA plasma (n=35)	61	13-182	45
Heparin plasma (n=35)	49	20-143	30
Saliva (n=10)	1218	332-2928	775
Urine (n=27)	321	51-850	168

Cell Culture Supernates:
JEG-3 human epithelial choriocarcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum. After one day, the cell culture supernate was removed, assayed for levels of human VEGF, and measured 63.1 pg/mL. After seven days, the cell culture supernate was removed, assayed for human VEGF, and measured 512 pg/mL.

PC-3 human prostate cancer cells were cultured in RPMI supplemented with 5% fetal bovine serum. Cells were unstimulated or stimulated with 10 ng/mL recombinant human TNF-α. Cell culture supernates were removed, assayed for levels of human VEGF, and measured 303 pg/mL and 1079 pg/mL, respectively.

SPECIFICITY

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

The factors listed below were prepared at 200 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range VEGF control were assayed for interference. The following factors showed no cross-reactivity or interference.

Recombinant human:

EG-VEGF/PK1
PDGF-AA
PDGF-AB
PDGF-BB
PDGF-CC
PDGF-DD
PlGF
PlGF-2
$VEGF\text{-}B_{167}$
VEGF-C
VEGF-D
VEGF R3/Flt-4

Recombinant mouse:

PDGF-CC
PlGF-2
$VEGF_{164}$
VEGF-D
VEGF R3/Flt-4

Recombinant rat:

PDGF-AA
PDGF-AB
PDGF-BB
$VEGF_{120}$

Recombinant zebrafish:

$VEGF_{165}$

VEGF-related factors showing cross-reactivity or interference.

Recombinant human $VEGF_{165}$/PlGF	Cross-reacts approximately 5%
Recombinant human VEGF R1/Flt-1	Interference at levels ≥ 500 pg/mL
Recombinant human VEGF R2/KDR	Interference at levels ≥ 2500 pg/mL
Recombinant mouse $VEGF_{120}$	Cross-reacts approximately 0.13%
Recombinant mouse VEGF R1/Flk-1	Interference at levels ≥ 1000 pg/mL
Recombinant canine VEGF	Cross-reacts approximately 39%
Recombinant feline VEGF	Cross-reacts approximately 47%

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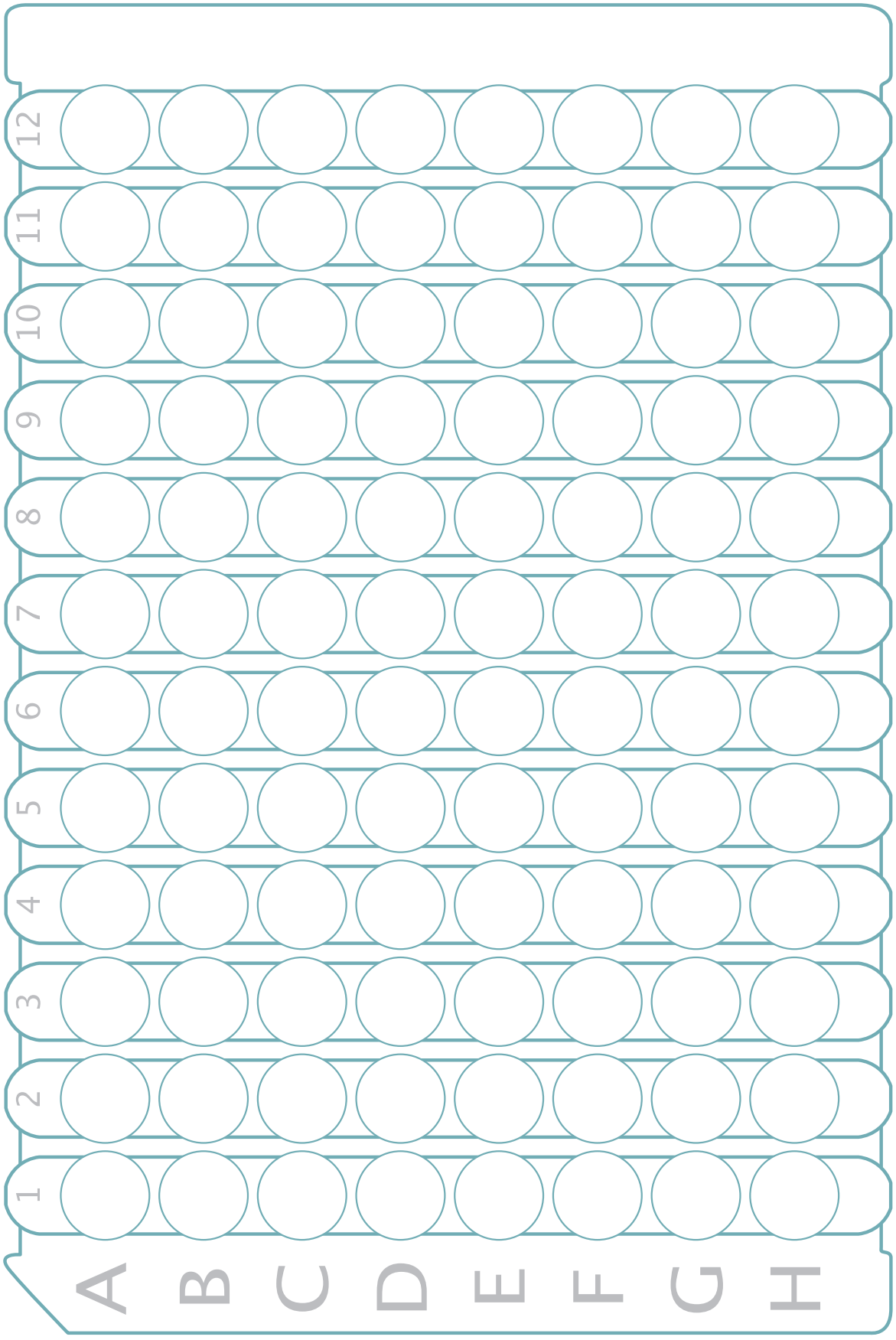
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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