Quantikine™ QuicKit™ ELISA

Human VEGF Immunoassay

Catalog Number QK293

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

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

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™ QuicKit™ Human VEGF Immunoassay is a one step, 80-minute solid phase ELISA designed to measure human VEGF levels in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains *Sf* 21-expressed recombinant human VEGF and antibodies raised against the recombinant protein. Results obtained using natural human VEGF showed linear curves that were parallel to the standard curves obtained using the QuicKit 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. An anti-tag antibody has been pre-coated onto a microplate. Standards and samples are pipetted into the wells followed by an antibody cocktail. The antibody cocktail consists of an affinity tag labeled monoclonal capture antibody and an enzyme-linked polyclonal detection antibody, specific for human VEGF. After washing away any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound. 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, further dilute the samples with the calibrator diluent and repeat the assay.
- Any variation in 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™ QuicKit™ 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.
- Ensure reagent addition to plate wells is uninterrupted.
- To ensure accurate results, proper adhesion of the plate sealer during the incubation step 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
QuicKit™ Coated Microplate	899063	96 well polystyrene microplate (12 strips of 8 wells) coated with an anti-tag monoclonal antibody.	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	899204	2 vials of recombinant human VEGF 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 Capture Ab Concentrate	899202	Lyophilized tagged monoclonal antibody specific for human VEGF.	May be stored for up to 1 month at 2-8 °C.*
Human VEGF Detection Ab Concentrate	899203	400 µL of a polyclonal antibody specific for human VEGF conjugated to horseradish peroxidase with preservatives.	
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5K	895119	21 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
- Test tubes for dilution of standards
- Human VEGF Controls (optional; R&D Systems®, Catalog # QC285)

PRECAUTIONS

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

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative 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. Refer to the SDS 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 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.*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Urine samples require a 4-fold dilution due to matrix effect. A suggested 4-fold dilution is 50 μL of sample + 150 μL of Calibrator Diluent RD5K.

Human milk samples require a 10-fold dilution due to high endogenous levels. A suggested 10-fold dilution is 20 μL of sample + 180 μL of Calibrator Diluent RD5K.

Saliva samples may require a 2-fold dilution due to high endogenous levels.

Cell culture supernate samples may require dilution due to high endogenous levels.

Multiple dilutions may be required for unknown samples.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *VEGF is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Human VEGF Capture Ab Concentrate - **Refer to the vial label for reconstitution volume.** Reconstitute the Human VEGF Capture Ab Concentrate with Assay Diluent RD1W. This reconstitution produces a 20X Capture Antibody stock. Allow the capture antibody to sit for a minimum of 5 minutes with gentle agitation prior to diluting. Once reconstituted, the 20X capture antibody stock can be stored for 4 weeks at 2-8 °C.

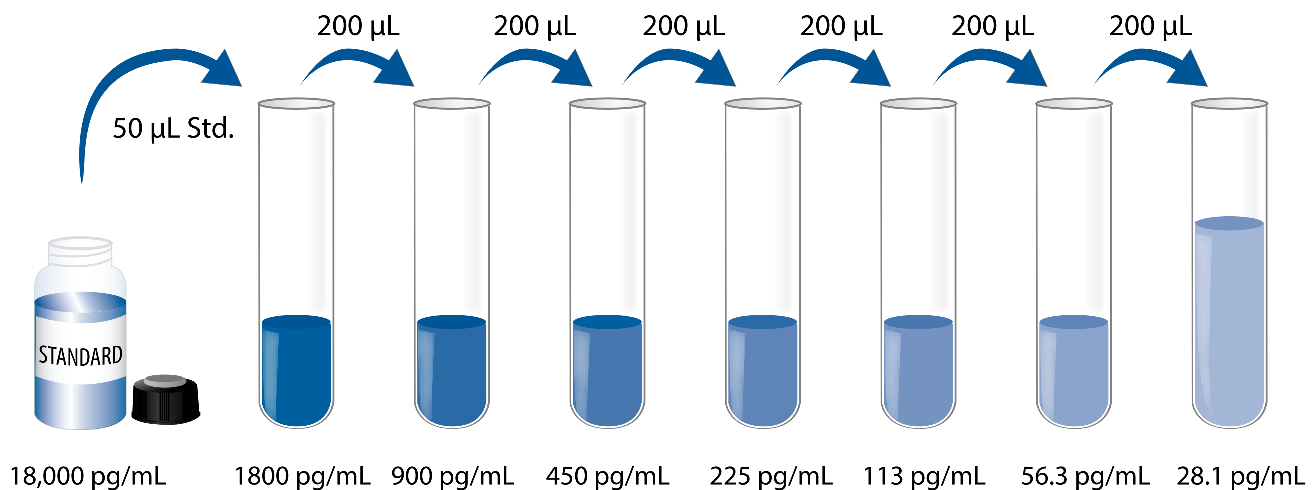
Antibody Cocktail - Dilute the reconstituted Capture Ab stock and the Detection Ab Concentrate 20-fold in Assay Diluent RD1W. For a full plate, add 300 µL of reconstituted Human VEGF Capture Ab stock and 300 µL of Human VEGF Detection Ab Concentrate to 5.4 mL of Assay Diluent RD1W to get 6.0 mL of Human VEGF Antibody Cocktail.

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 480 mL of 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. 100 µL of the resultant mixture is required per well.

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 18,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5K into the 1800 pg/mL tube. Pipette 200 µL into the remaining tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1800 pg/mL standard serves as the high standard. Calibrator Diluent RD5K serves as the zero standard (0 pg/mL).



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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: *VEGF is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, samples, and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of standard, control, or sample* per well. A plate layout is provided to record standards and samples assayed.
4. Add 50 µL Antibody Cocktail to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12″ orbit) set at 500 ± 50 rpm.
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 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature **on the benchtop. Protect from light.**
7. 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.
8. 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

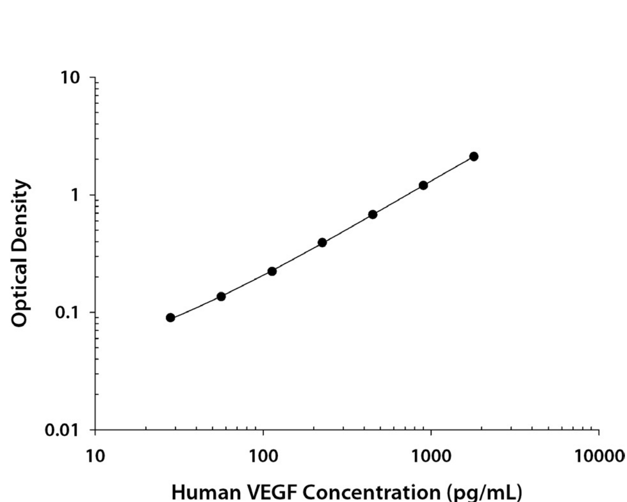
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 human 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

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.042 0.043	0.043	—
28.1	0.090 0.090	0.090	0.047
56.3	0.136 0.136	0.136	0.093
113	0.221 0.224	0.223	0.180
225	0.389 0.394	0.392	0.349
450	0.671 0.684	0.678	0.635
900	1.201 1.202	1.202	1.159
1800	2.106 2.119	2.113	2.070

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PRECISION

Intra-Assay Precision (Precision within an assay)
Two samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Two samples of known concentration were tested in ten separate assays to assess inter-assay precision. Assays were performed by at least three technicians.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	1	2	1	2
n	20	20	10	10
Mean (pg/mL)	209	1193	203	1216
Standard deviation	7.45	35.1	18.3	56.5
CV (%)	3.6	2.9	9.0	4.6

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	99	94-104%
Serum (n=2)	79	74-84%
EDTA plasma (n=2)	83	79-88%
Heparin plasma (n=2)	77	70-81%
Human milk (n=2)	102	98-105%
Saliva (n=2)	104	97-115%
Urine (n=2)	78	70-85%

LINEARITY

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

		Cell culture supernates* (n=5)	Serum (n=2)	EDTA plasma (n=2)	Heparin plasma (n=2)	Saliva* (n=2)	Urine* (n=2)	Human milk* (n=2)
1:2	Average % of Expected	96	94	106	112	100	104	100
	Range (%)	92-100	88-99	104-107	107-118	98-102	100-107	97-103
1:4	Average % of Expected	90	85	106	118	98	103	99
	Range (%)	86-97	82-87	101-110	103-132	94-102	100-106	95-102
1:8	Average % of Expected	87	90	103	115	95	107	95
	Range (%)	86-89	87-93	97-109	101-129	93-97	105-109	91-100
1:16	Average % of Expected	85	83	97	95	94	103	89
	Range (%)	84-86	77-89	88-106	90-100	90-98	101-106	82-96

*Samples were diluted prior to this assay.

SENSITIVITY

Sixteen assays were evaluated and the minimum detectable dose (MDD) of human VEGF ranged from 2.09-5.46 pg/mL. The mean MDD was 3.19 pg/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified *Sf* 21-expressed recombinant human VEGF produced at R&D Systems.

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SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - 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=10)	519	175-1093	352
EDTA plasma (n=10)	79.4	40.6-183	43.1
Heparin plasma (n=10)	69.8	38.9-105	23.7
Saliva (n=4)	3521	2055-4880	1374
Urine (n=4)	566	219-1184	429
Human milk (n=4)	27,535	23,100-31,888	4620

Cell Culture Supernates:

PBMCs were isolated from a single donor over a Ficoll-Paque PLUS density gradient. PBMCs were seeded at 1 x 10^6/mL and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. The cells were stimulated with 10 μg/mL PHA for 5 days. Aliquots of the cell culture supernatants were removed, assayed for human VEGF, and measured 169 pg/mL.

JEG-3 human epithelial choriocarcinoma cells were cultured in MEM Earle's Salts and supplemented with 10% FBS. Cells were stimulated with 100 ng/mL of LPS for 24 hours. An aliquot of the cell culture supernate was removed, assayed for human VEGF, and measured 11,100 pg/mL.

THP-1 acute monocytic leukemia cells were cultured in RPMI and supplemented with 10% fetal bovine serum. Cells were stimulated with 1 μg/mL Retionic Acid and 100 ng/mL PMA for 4 days. An aliquot of the cell culture supernate was removed, assayed for human VEGF, and measured 1829 pg/mL.

PC-3 human prostate cancer cells were cultured in RPMI and supplemented with 10% fetal bovine serum until confluent. An aliquot of the cell culture supernate was removed, assayed for human VEGF, and measured 2366 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human VEGF.

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 low level recombinant human VEGF control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

PlGF
PlGF-2
VEGF-B_{167}
VEGF-C
VEGF-D
VEGFR3/Flt-4

Recombinant mouse:

VEGF-B_{186}
VEGFR3/Flt-4

The following factors are also detectable in this assay.

Recombinant human:

$VEGF_{111}$
$VEGF_{111b}$
$VEGF_{121}$
$VEGF_{145}$
$VEGF_{162}$
$VEGF_{165b}$
$VEGF_{189}$

Other recombinants:

canine VEGF
feline VEGF

Cross-reactivity or interference was observed with the following factors:

Factor	Cross-reactivity	Interference
Recombinant human $VEGF_{111}$	Detectable	> 62.5 pg/mL
Recombinant human $VEGF_{206}$	2.52%	–
Recombinant human VEGF/PlGF	29.2%	–
Recombinant human VEGFR1	–	>156 pg/mL
Recombinant human KDR	–	>12.5 ng/mL
Recombinant mouse $VEGF_{120}$	2.63%	>6.25 ng/mL
Recombinant mouse $VEGF_{164}$	0.648%	>3.13 ng/mL
Recombinant mouse VEGFR1	–	>313 pg/mL
Recombinant mouse VEGFR2	–	>25 ng/mL
Recombinant rat $VEGF_{164}$	0.623%	>6.25 ng/mL

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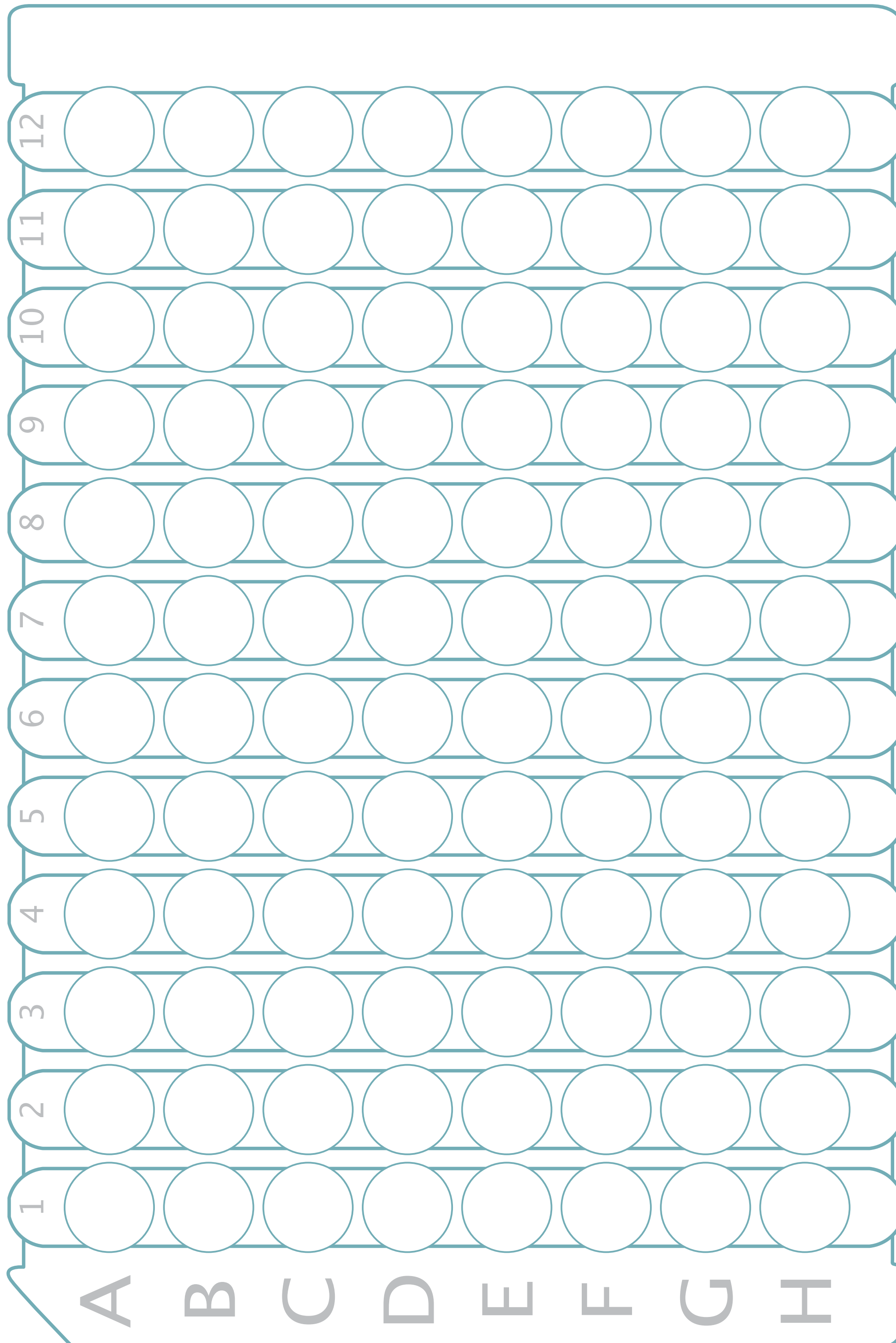
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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