Quantikine™ ELISA

Mouse VEGF Immunoassay

Catalog Number MMV00
SMMV00
PMMV00

For the quantitative determination of mouse Vascular Endothelial Growth Factor (VEGF) concentrations in cell culture supernates, tissue homogenates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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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 in adults (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 anti-parallel disulfide-linked dimers (4). Alternately spliced isoforms of 120, 164 and 188 amino acids (aa) have been found in mice, while 121, 145, 165, 183, 189, and 206 aa isoforms have been identified in humans (2, 4). In humans, $VEGF_{165}$ appears to be the most abundant and potent isoform, followed by $VEGF_{121}$ and $VEGF_{189}$ (3, 4). The same pattern may exist in mice. Isoforms other than $VEGF_{120}$ and $VEGF_{121}$ contain basic heparin-binding regions and are not freely diffusible (4). Mouse $VEGF_{164}$ shares 97% aa sequence identity with corresponding regions of rat VEGF. It also shares 89% aa sequence identity with human and porcine VEGF, 88% with bovine VEGF, and 90% with feline, equine, and canine 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). The 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™ Mouse VEGF Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse VEGF in cell culture supernates, tissue homogenates, mouse serum, and plasma. It contains *Sf* 21-expressed mouse VEGF and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant mouse VEGF accurately. Results obtained using natural mouse VEGF showed dose-response curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse VEGF.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse VEGF has been pre-coated onto a microplate. Standards, control, 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 mouse VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of VEGF bound in the initial step. The sample values are then read off the standard curve.

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 calibrator diluent and repeat the assay.
- Any variation in 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.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # MMV00	CATALOG # SMMV00	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse VEGF Microplate	890783	2 plates	6 plates	96 well polystyrene microplates (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse 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.*
Mouse VEGF Standard	890784	1 vial	3 vials	Recombinant mouse VEGF in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Mouse VEGF Control	890785	1 vial	3 vials	Recombinant mouse VEGF in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse VEGF Conjugate	892667	1 vial	3 vials	23 mL/vial of a polyclonal antibody specific for mouse VEGF conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1N	895488	1 vial	3 vials	12 mL/vial of a buffered protein solution with preservative.	
Calibrator Diluent RD5T	895175	2 vials	6 vials	21 mL/vial of a buffered protein solution with preservatives.	
Wash Buffer Concentrate	895003	2 vials	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	3 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	3 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	1 vial	3 vials	23 mL/vial of diluted hydrochloric acid.	
Plate Sealers	N/A	8 strips	24 strips	Adhesive strips.	

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

MMV00 contains sufficient materials to run ELISAs on two 96 well plates.
SMMV00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PMMV00). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 microplates (96 wells/plate). The package inserts supplied are the same as those supplied in the single kit packs and because of this, a few minor differences related to the number of reagents and their container sizes should be noted.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
  Note: *Additional wash buffer is available for purchase (R&D Systems®, Catalog # WA126).*

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
Mouse VEGF Microplate	890783	50 plates
Mouse VEGF Standard	890784	25 vials
Mouse VEGF Control	890785	25 vials
Mouse VEGF Conjugate	892667	25 vials
Assay Diluent RD1N	895488	25 vials
Calibrator Diluent RD5T	895175	50 vials
Wash Buffer Concentrate	895126	9 bottles
Color Reagent A	895000	25 vials
Color Reagent B	895001	25 vials
Stop Solution	895174	25 vials
Plate Sealers	N/A	100 sheets
Package inserts	752523	2 booklets

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 and samples

PRECAUTIONS

Assay Diluent RD1N contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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.

Tissue Homogenates - The preparation of tissue homogenates will vary depending upon the tissue type. For this assay, heart, kidney, and spleen tissue from three mice and lung tissue from eight mice were rinsed with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS, and stored overnight at ≤ -20 °C.

After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. Samples can be assayed immediately or stored at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 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 20 minutes at 2000 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.*

SAMPLE PREPARATION

Use polypropylene tubes.

Serum and plasma samples require a 5-fold dilution into Calibrator Diluent RD5T. A suggested 5-fold dilution is 50 µL of sample + 200 µL of Calibrator Diluent RD5T.

Cell culture supernates and tissue homogenates may require dilution. The dilution factor is dependent upon sample values.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

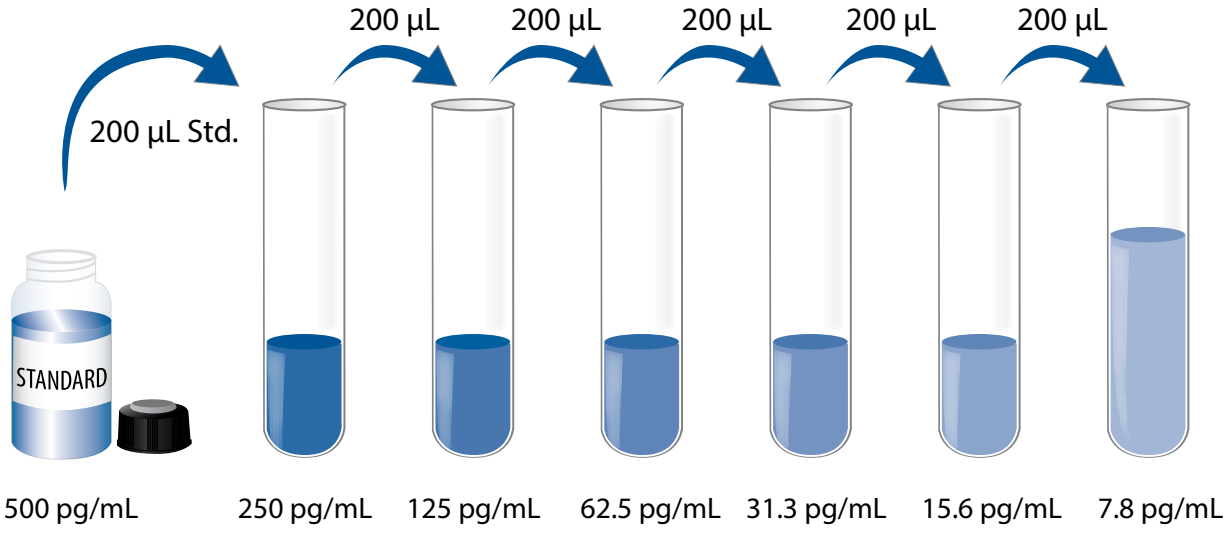
Mouse VEGF Control - Reconstitute the control with 1.0 mL deionized or distilled water. Mix thoroughly. Assay the control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, 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.

Mouse VEGF Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse VEGF Standard with Calibrator Diluent RD5T. Do not substitute other diluents. This reconstitution produces a stock solution of 500 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5T into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse VEGF Standard (500 pg/mL) serves as the high standard. Calibrator Diluent RD5T 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, control, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, control, 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 50 µL of Assay Diluent RD1N to each well.
4. Add 50 µL of standard, control, or sample* to each well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five 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 Mouse 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 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

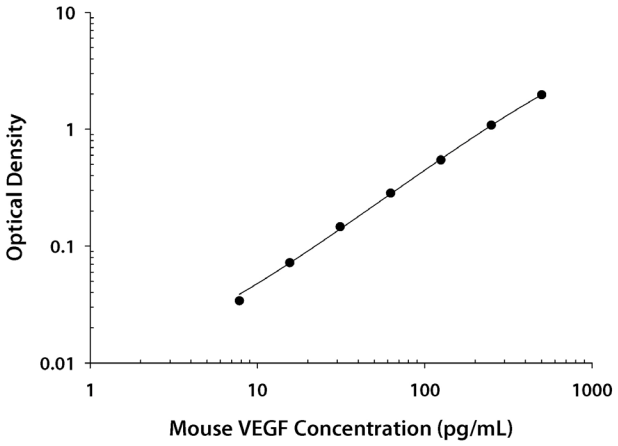
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse 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 must be read from the standard curve 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	0.061 0.070	0.066	—
7.8	0.099 0.102	0.100	0.034
15.6	0.139 0.136	0.138	0.072
31.3	0.213 0.211	0.212	0.146
62.5	0.358 0.342	0.350	0.284
125	0.624 0.600	0.612	0.546
250	1.141 1.156	1.148	1.082
500	1.969 2.103	2.036	1.970

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	37.7	144	277	38.3	138	291
Standard deviation	3.1	6.2	12.9	3.2	7.8	18.5
CV (%)	8.2	4.3	4.7	8.4	5.7	6.4

RECOVERY

The recovery of mouse VEGF spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=9)	103	95-114%
Tissue homogenates (n=3)	91	76-107%
Serum* (n=9)	101	83-115%
EDTA plasma* (n=5)	91	82-103%
Heparin plasma* (n=4)	89	87-94%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

The minimum detectable dose (MDD) of mouse VEGF is typically less than 3.0 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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with various concentrations of mouse VEGF in each matrix were diluted with calibrator diluent and then assayed.

		Cell culture supernates (n=2)	Tissue homogenates (n=2)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	101	106	101	101	113
	Range (%)	93-109	101-113	95-108	98-102	111-115
1:4	Average % of Expected	98	113	104	107	115
	Range (%)	92-103	105-124	87-120	98-112	112-117
1:8	Average % of Expected	96	115	106	105	112
	Range (%)	86-101	105-117	89-115	94-112	109-118
1:16	Average % of Expected	97	118	98	101	95
	Range (%)	81-106	103-130	90-106	85-120	82-119

*Samples were diluted prior to assay as directed in the Sample Preparation section.

CALIBRATION

This immunoassay is calibrated against a highly purified *Sf* 21-expressed recombinant mouse $VEGF_{164}$ produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Forty individual mouse serum samples and nine individual mouse plasma samples were evaluated for detectable levels of mouse VEGF in this assay. All samples measured less than the lowest mouse VEGF standard, 7.8 pg/mL.

Tissue Homogenates - Tissue homogenates from mouse heart, lung, kidney, and spleen tissue were assayed for mouse VEGF and measured 111 pg/mL, 3680 pg/mL, 157 pg/mL, and 30 pg/mL, respectively.

Cell Culture Supernates:
P388D1 mouse lymphoma cells (1 x 10^5 cells/mL) were cultured for 4 days in RPMI supplemented with 5% fetal bovine serum. The cell culture supernate was assayed for mouse VEGF and measured 7600 pg/mL.

Mouse lung conditioned media (1 lung, 1-2 mm pieces in 10 mL of RPMI supplemented with 10% fetal bovine serum) was collected after culturing for 5 days. The cell culture supernate was assayed for mouse VEGF and measured 4660 pg/mL.

Mouse heart conditioned media (1 heart, 1-2 mm pieces in 10 mL of RPMI supplemented with 10% fetal bovine serum) was collected after culturing for 5 days. The cell culture supernate was assayed for mouse VEGF and measured 378 pg/mL.

SPECIFICITY

This assay recognizes both the 164 and 120 amino acid residue forms of mouse VEGF.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range mouse VEGF control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

C10
G-CSF
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-10 sR
IL-12
IL-13
KC
JE/MCP-1
LIF
M-CSF
MIP-1α
MIP-1β
MIP-2
PlGF-2
SCF
TNF-α
Tpo
VEGF-B
VEGF-D
VEGF R2
VEGF R3

Recombinant human:

Flt-3 Ligand
PlGF
PDGF-AA
PDGF-AB

Natural proteins:

human PDGF

Some cross-reactivity was observed with the following:

Factor	Concentration Tested (pg/mL)	Observed Value (pg/mL)	% Cross-Reactivity
$rhVEGF_{165}$	50,000	106	0.2
$rhVEGF_{165}$/PlGF	50,000	27	0.05
rrVEGF	1000	250	25
rm $VEGF_{188}$	5000	457	9

Recombinant mouse VEGF R1 interferes in this assay at concentrations > 2500 pg/mL.

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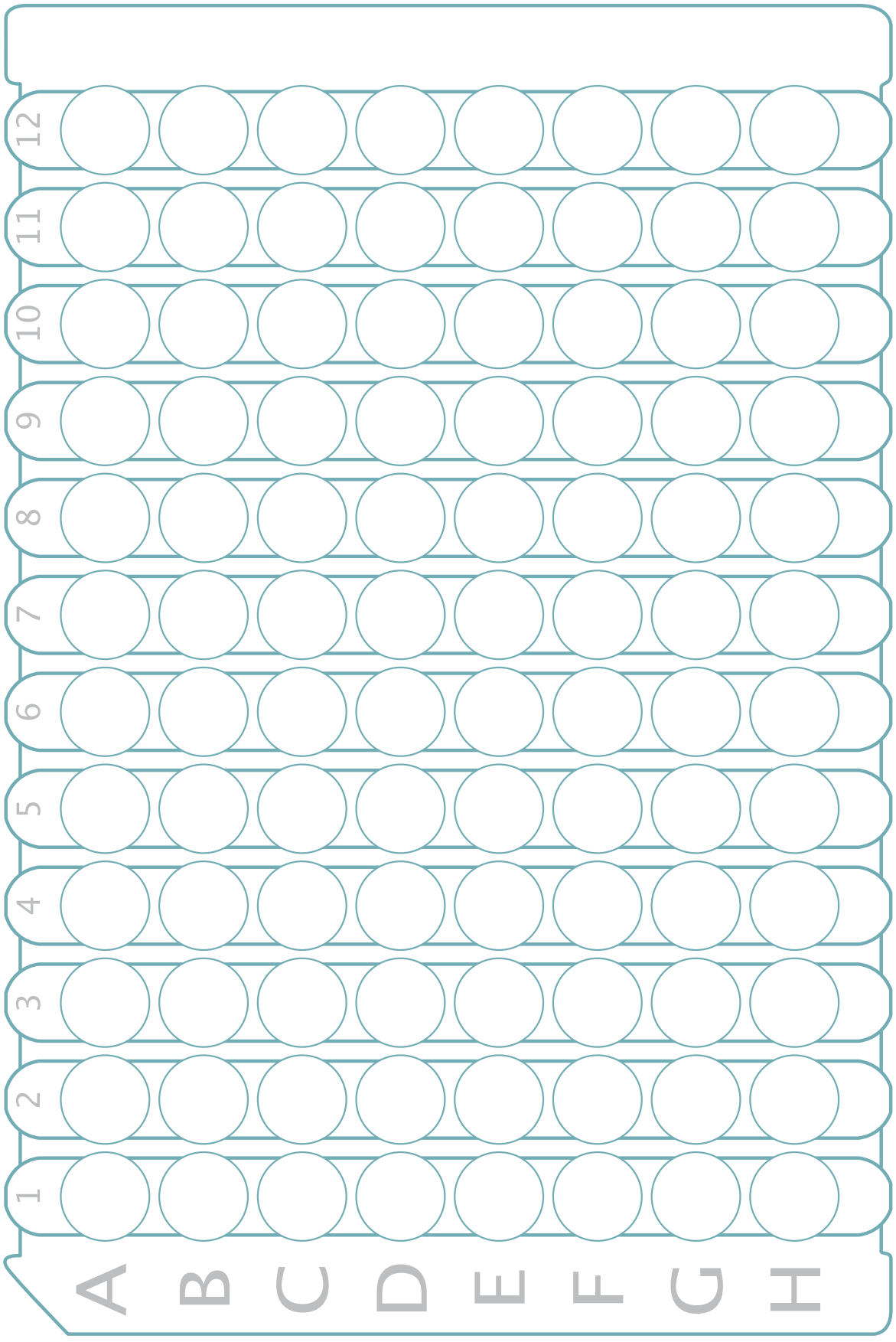
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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