Quantikine® ELISA

Mouse sVEGF R2/Flk-1 Immunoassay

Catalog Number MVR200B

For the quantitative determination of mouse soluble Vascular Endothelial Growth Factor Receptor 2 (sVEGF R2) 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

The vascular endothelial growth factor (VEGF) family comprises five members including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) (1-7). These proteins exist as homodimers. In addition, naturally occurring heterodimers of PlGF/VEGF and VEGF/VEGF-B have been reported (8-10). The biological activities of these ligands are mediated by neuropilin-1 and three receptor tyrosine kinases (RTKs): VEGF R1 (Flt1), VEGF R2 (mouse Flk-1 and human KDR) and VEGF R3 (Flt4) (1, 2, 11, 12). These three kinases are characterized by the presence of seven immunoglobulin (Ig)-like domains in their extracellular regions as well as a split kinase domain in their cytoplasmic regions. They constitute a subfamily within the RTK superfamily.

Mouse VEGF R2, also known as fetal liver kinase-1 (Flk-1), is a 200-230 kDa type I transmembrane glycoprotein (1, 7, 13-15). It is synthesized as a 1367 amino acid (aa) precursor with a 19 aa signal sequence, a 743 aa extracellular region containing seven Ig-like domains, a 22 aa transmembrane segment, and a 583 aa cytoplasmic region (13, 14). Mouse VEGF R2 shares approximately 94% and 85% aa sequence identity with rat and human VEGF R2, respectively (14-16). The first three amino-terminal Ig-like domains of VEGF R2 are important for high-affinity ligand binding, while Ig-like domains 4-7 act to prevent ligand-independent receptor dimerization and signaling (17-19). Although mRNA encoding a secreted form of VEGF R2 has not been reported, soluble VEGF R2 has been detected in various biological fluids including human and mouse serum/plasma, as well as conditioned media. It is likely that the soluble VEGF R2 is released from the transmembrane protein as a result of proteolytic cleavage.

VEGF R2 is expressed primarily on vascular endothelial cells and endothelial cell progenitors (6, 15, 20). It is also expressed on endometrial epithelium (21), $CD34^+$ hematopoietic stem cells (20, 22), liver sinusoidal endothelial cells (15), Sertoli cells and Leydig cells (23), platelets and megakaryocytes (22), sensory and autonomic neurons (24), Schwann cells (24), osteoblasts (20) and retinal progenitors plus Muller glial cells (25).

The VEGF family of ligands and receptors are key mediators of angiogenesis and may contribute to inflammation (1, 26). In mice, VEGF R2 binds only VEGF-A and -C, and tranduces signals for blood vessel growth. VEGF R3 binds VEGF-C and -D and mediates lymphangiogenesis (1, 2, 11). VEGF R1 binds VEGF-A and PlGF, however, its role in angiogenesis is not clear (2, 17). VEGF R1 and VEGF R2 can also undergo ligand-induced heterodimerization in addition to homodimerization (2). The activating ligands for and the signals transduced by the heteromeric receptors are not well understood. In addition to angiogenesis, VEGF R2 mediates a signal that protects certain cell types, including $CD34^+$ hematopoietic stem cells and sensory and sympathetic neurons (22, 24), against apoptosis. Mice deficient in VEGF R2 have been shown to die *in utero* as a result of an early defect in the development of endothelial cells as well as hematopoietic cells (27).

The Quantikine Mouse sVEGF R2/Flk-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure soluble mouse VEGF R2 in cell culture supernates, tissue homogenates, serum, and plasma. It contains NS0-expressed recombinant mouse VEGF R2 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural mouse sVEGF R2 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 mouse sVEGF R2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse sVEGF R2 has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse VEGF R2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse sVEGF R2 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 mouse soluble VEGF R2 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 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.
- 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse sVEGF R2 Microplate	893030	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse sVEGF R2.	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 sVEGF R2 Conjugate	893031	12 mL of a polyclonal antibody against mouse sVEGF R2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse sVEGF R2 Standard	893032	20 ng of recombinant mouse sVEGF R2 in a buffered protein base with preservatives; lyophilized.	
Mouse sVEGF R2 Control	893033	1 vial of recombinant mouse sVEGF R2 in a buffered protein base with preservatives; lyophilized. The concentration range of mouse sVEGF R2 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.	
Assay Diluent RD1-21	895215	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	2 vials (21 mL/vial) of a buffered protein solution 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	895174	23 mL of diluted hydrochloric 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.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Homogenates - Prior to assay, tissues must be prepared according to the directions in the Sample Values section.

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 room temperature 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

Serum and plasma samples require a 15-fold dilution prior to assay. A suggested 15-fold dilution is 10 µL of sample + 140 µL of Calibrator Diluent RD5-3.

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

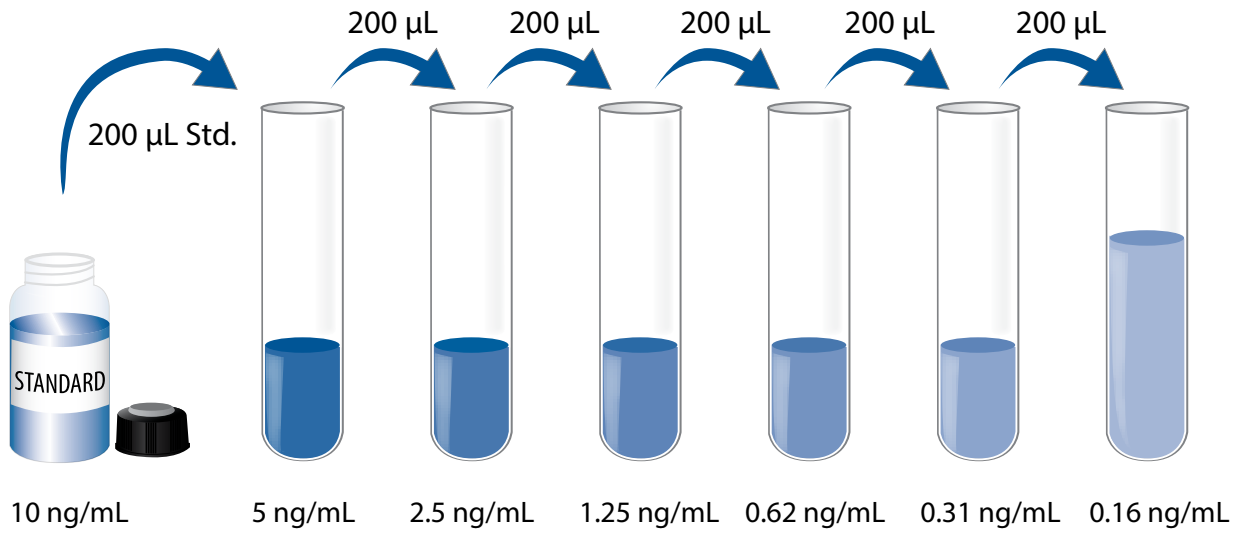
Mouse sVEGF R2 Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. 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. 100 µL of the resultant mixture is required per well.

Mouse sVEGF R2 Standard - Reconstitute the Mouse sVEGF R2 Standard with 2.0 mL of Calibrator Diluent RD5-3. Do not substitute other diluents. This reconstitution produces a stock solution of 10 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse sVEGF R2 Standard serves as the high standard (10 ng/mL). Calibrator Diluent RD5-3 serves as the zero standard (0 ng/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, standard dilutions, 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 RD1-21 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. 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 sVEGF R2 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

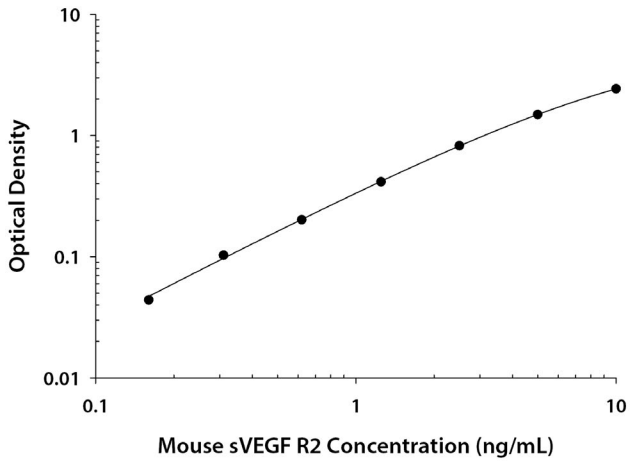
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 sVEGF R2 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.



(ng/mL)	O.D.	Average	Corrected
0	0.086 0.086	0.086	—
0.16	0.127 0.133	0.130	0.044
0.31	0.189 0.189	0.189	0.103
0.62	0.283 0.293	0.288	0.202
1.25	0.486 0.515	0.501	0.415
2.5	0.878 0.942	0.910	0.824
5	1.474 1.676	1.575	1.489
10	2.434 2.588	2.511	2.425

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-four separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	24	24	24
Mean (ng/mL)	0.63	1.40	3.83	0.57	1.28	3.50
Standard deviation	0.04	0.07	0.21	0.06	0.10	0.21
CV (%)	6.3	5.0	5.5	10.5	7.8	6.0

RECOVERY

The recovery of mouse sVEGF R2 spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	105	100-111%
Serum* (n=3)	104	82-120%
Plasma* (n=4)	108	89-116%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse sVEGF R2 in each matrix were diluted with Calibrator Diluent and then assayed.

		Cell culture supernates (n=6)	Tissue homogenates (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	99	101	101	98	98
	Range (%)	93-106	96-105	100-102	95-101	95-100
1:4	Average % of Expected	97	101	100	99	97
	Range (%)	90-102	94-107	99-102	94-101	95-101
1:8	Average % of Expected	98	95	100	95	98
	Range (%)	83-103	87-104	97-103	93-98	96-99
1:16	Average % of Expected	102	93	97	92	100
	Range (%)	101-102	86-107	94-102	89-101	97-101

*Samples were diluted prior to assay.

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SENSITIVITY

Ninteen assays were evaluated and the minimum detectable dose (MDD) of mouse sVEGF R2 ranged from 0.012-0.049 ng/mL. The mean MDD was 0.027 ng/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 mouse sVEGF R2/Fc Chimera produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse sVEGF R2 in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	103	44-143	21
EDTA plasma (n=20)	66	27-96	17
Heparin plasma (n=20)	72	50-115	15

Cell Culture Supernates:
Mouse splenocytes (1 x 10^6 cells/mL) were cultured for 3 days in RPMI supplemented with 10% fetal calf serum, 100 ng/mL of recombinant mouse IFN-γ and 1 µg/mL of LPS. The cell culture supernate was removed, assayed for mouse sVEGF R2, and measured 0.3 ng/mL.

Mouse hearts or kidneys were homogenized using a tissue homogenizer. Cells (1 x 10^6 cells/mL) were stimulated with 1 µg/mL of LPS and 7 µg/mL of PHA for 3 days in RPMI supplemented with 10% fetal calf serum and 10 ng/mL of recombinant human IL-2. The cell culture supernates were removed, assayed for mouse sVEGF R2, and measured 0.56 ng/mL and 3.2 ng/mL, respectively.

Tissue Homogenates - Mouse heart, spleen, and kidney tissue were rinsed with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS and stored overnight at ≤ -20 °C. Homogenates were thawed and centrifuged for 5 minutes at 5000 x g. Homogenates were assayed for mouse sVEGF R2 and measured 3 ng/mL, 5 ng/mL, and 5 ng/mL, respectively.

SPECIFICITY

This assay recognizes natural and recombinant mouse sVEGF R2.

The factors listed below were prepared at 100 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range mouse sVEGF R2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

PlGF-2
$VEGF_{115}$
$VEGF_{120}$
$VEGF_{164}$
$VEGF\text{-}B_{186}$
VEGF-D
VEGF R1
VEGF R3

Recombinant human:

$VEGF_{121}$
$VEGF_{165}$
$VEGF_{206}$
VEGF/PlGF
$VEGF\text{-}B_{167}$
VEGF-C
VEGF-D
VEGF R1
VEGF R3

Other recombinants:

canine VEGF
zebrafish $VEGF_{165}$

Recombinant human sVEGF R2 cross-reacts approximately 1.4% in this assay.

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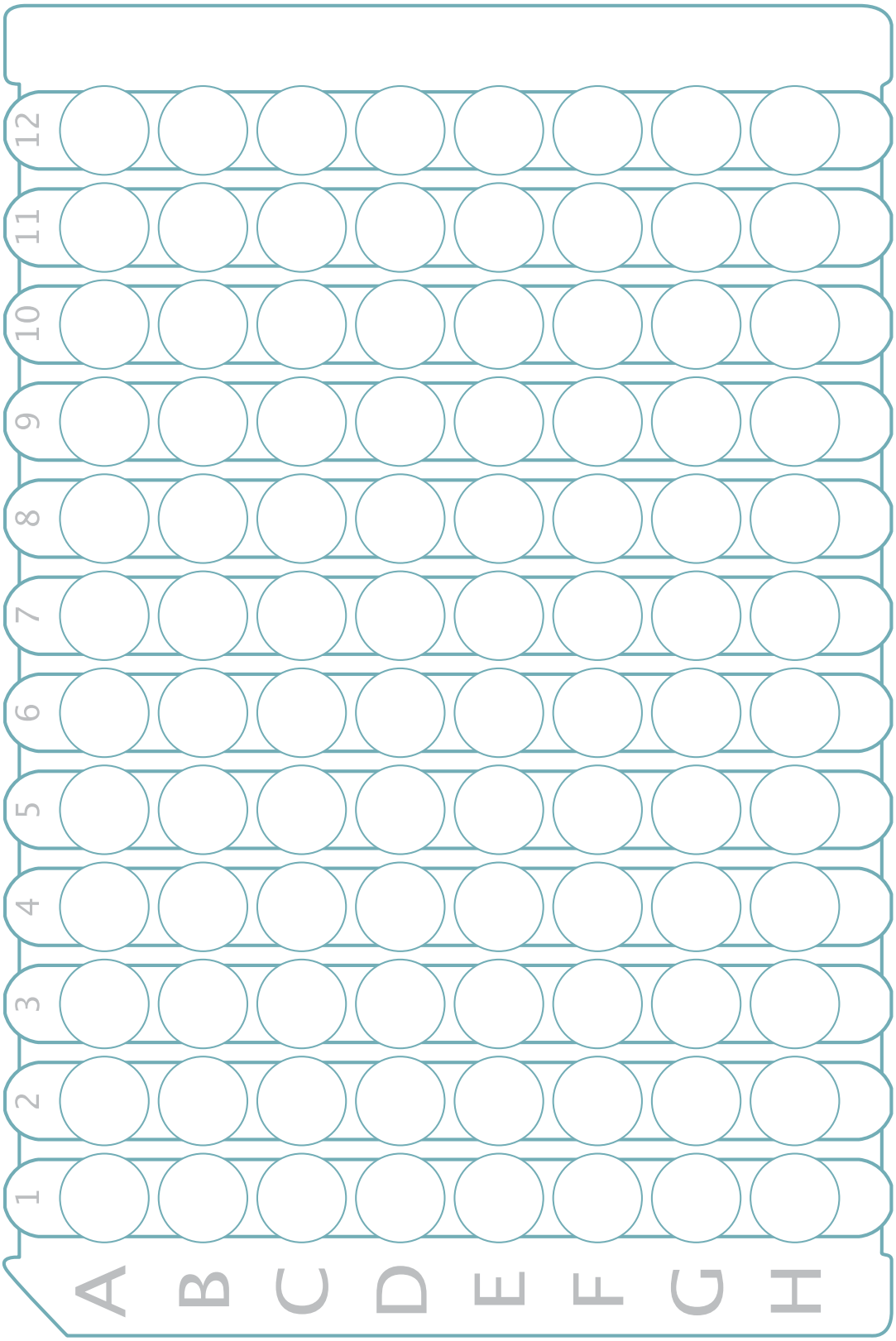
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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