Quantikine® ELISA

Rat VEGF Immunoassay

Catalog Number RRV00

For the quantitative determination of rat Vascular Endothelial Growth Factor (VEGF) concentrations in cell culture supernates, 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 formation of anti-parallel disulfide-linked dimers (4). Alternately spliced isoforms of 120, 164, and 188 amino acids (aa) have been found in rats and 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 rats and mice. Isoforms other than $VEGF_{120}$ and $VEGF_{121}$ contain basic heparin-binding regions and are not freely diffusible (4). Rat $VEGF_{164}$ shares 97% aa sequence identity with corresponding regions of mouse, 88% with human and bovine, 89% with porcine and canine, and 90% with feline and equine 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). 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 Rat VEGF Immunoassay is a 3.5 hour solid-phase ELISA designed to measure rat VEGF in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant rat VEGF and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural rat VEGF 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 rat VEGF.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for 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 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, 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.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Allow the plate to soak for 10-15 seconds between washes to 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.

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
Rat VEGF Microplate	892917	One 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for rat 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.*
Rat VEGF Conjugate	892918	12 mL of a polyclonal antibody conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Rat VEGF Standard	892919	2 vials of recombinant rat VEGF in a buffered protein base with preservatives; lyophilized.	
Rat VEGF Control	892920	1 vial of recombinant rat VEGF in a buffered protein base with preservatives; lyophilized. The concentration range of rat VEGF 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 RD1-41	895514	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-18	895335	21 mL of a buffered surfactant with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD5-3	895436	21 mL of a buffered protein solution with preservatives. *For serum/plasma samples.*	
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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling

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 - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 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 20 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.*
Grossly hemolyzed or lipemic samples may not be suitable for use in this assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

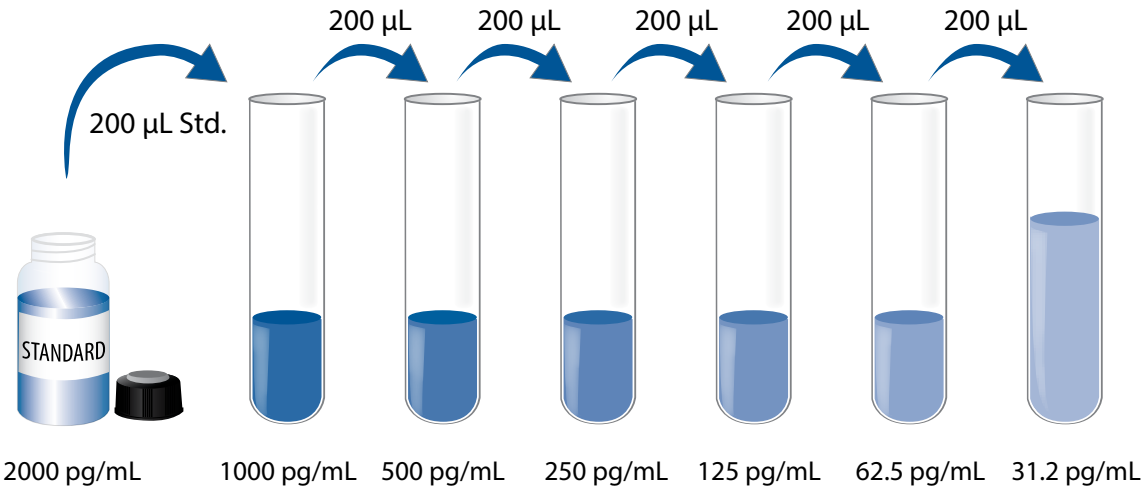
Rat VEGF Control - Reconstitute the Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

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.

Rat VEGF Standard - Reconstitute the Rat VEGF Standard with 2.0 mL of Calibrator Diluent RD5-18 (*for cell culture supernate samples*) or Calibrator Diluent RD5-3 (*for serum/plasma samples*). Do not substitute other diluents. This reconstitution produces a stock solution of 2000 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 the Calibrator Diluent RD5-18 (*for cell culture supernate samples*) or Calibrator Diluent RD5-3 (*for serum/plasma samples*) into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The undiluted rat VEGF Standard serves as the high standard (2000 pg/mL). 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 control 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 RD1-41 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, control, 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 Rat VEGF Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker.
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 **on the benchtop. 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.

CALCULATION OF RESULTS

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

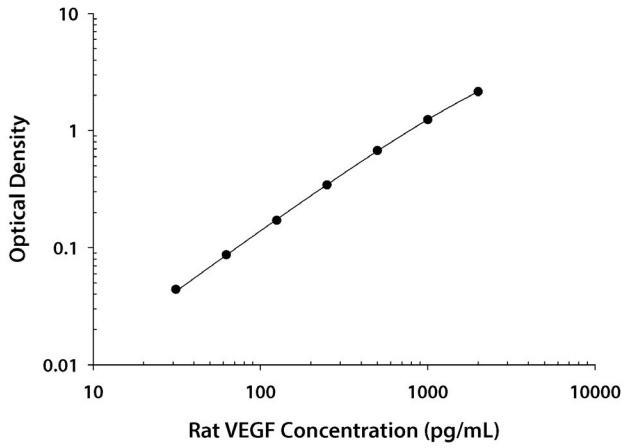
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the rat 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

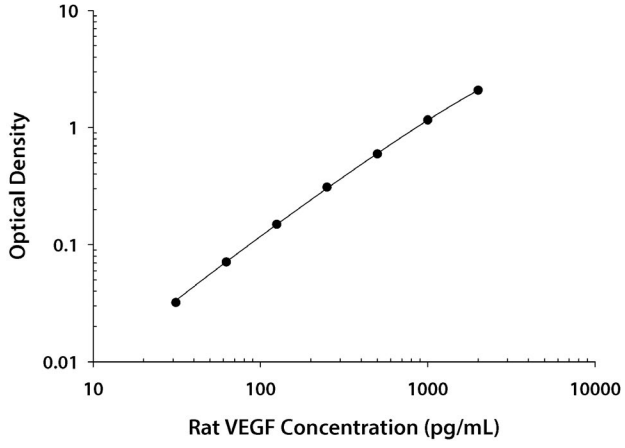
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CALIBRATOR DILUENT RD5-18



(pg/mL)	O.D.	Average	Corrected
0	0.118 0.119	0.119	—
31.2	0.162 0.164	0.163	0.044
62.5	0.201 0.210	0.206	0.087
125	0.287 0.293	0.290	0.171
250	0.462 0.464	0.463	0.344
500	0.791 0.795	0.793	0.674
1000	1.313 1.403	1.358	1.239
2000	2.237 2.296	2.267	2.148

CALIBRATOR DILUENT RD5-3



(pg/mL)	O.D.	Average	Corrected
0	0.112 0.112	0.112	—
31.2	0.140 0.147	0.144	0.032
62.5	0.179 0.187	0.183	0.071
125	0.260 0.262	0.261	0.149
250	0.415 0.426	0.421	0.309
500	0.698 0.716	0.707	0.595
1000	1.240 1.301	1.271	1.159
2000	2.128 2.266	2.197	2.085

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 separate assays to assess inter-assay precision.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	65	60	65
Mean (pg/mL)	79.4	174	797	87.6	204	828
Standard deviation	5.7	7.0	14.1	8.2	21.2	42.5
CV (%)	7.2	4.0	1.8	9.4	10.4	5.1

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	71	65	71
Mean (pg/mL)	103	191	891	107	233	900
Standard deviation	3.8	10.7	19.7	8.5	23.3	41.1
CV (%)	3.7	5.6	2.2	7.9	10.0	4.6

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture supernates (n=6)	103	94-108%
Serum (n=6)	99	90-116%
EDTA plasma (n=6)	102	95-108%
Heparin plasma (n=6)	92	80-100%

SENSITIVITY

Twenty-eight assays were evaluated and the minimum detectable dose (MDD) of rat VEGF ranged from 3.9-25.0 pg/mL. The mean MDD was 8.4 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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of rat VEGF were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=6)	Serum (n=6)	EDTA plasma (n=6)	Heparin plasma (n=6)
1:2	Average % of Expected	101	100	103	99
	Range (%)	98-104	94-103	101-106	94-102
1:4	Average % of Expected	103	101	104	102
	Range (%)	97-109	99-103	103-107	98-106
1:8	Average % of Expected	106	102	107	103
	Range (%)	99-115	97-105	102-111	96-111
1:16	Average % of Expected	105	104	115	95
	Range (%)	93-120	99-110	113-116	82-111

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant rat $VEGF_{164}$ produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Twelve individual serum and plasma samples were evaluated for detectable levels of VEGF in the assay. No detectable levels were observed.

Cell Culture Supernates:

Rat lung, cut into 1-2 mm pieces, was cultured for 4 days in 25-30 mL of RPMI supplemented with 10% fetal calf serum and stimulated with 2.5 ng/mL LPS. The cell culture supernate was removed, assayed for levels of rat VEGF, and measured 2264 pg/mL.

Rat spleen, cut into 1-2 mm pieces, was cultured for 3-4 days in 25-30 mL of RPMI supplemented with 10% fetal calf serum and stimulated with 10 µg/mL ConA. The cell culture supernate was removed, assayed for rat VEGF, and measured 1105 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant rat VEGF.

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

Recombinant rat:

CINC-2α
CINC-2β
CINC-3
CNTF
CNTF Rα
E-Selectin/Fc Chimera
EphA5/Fc Chimera
EphB1/Fc Chimera
Fractalkine
GDNF
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-4
IL-6
IL-10
IL-18
LIX
MAC/Fc Chimera
MIP-3α
β-NGF
Npn-1/Fc Chimera
Npn-2/Fc Chimera
PDGF-AA
PDGF-AB
PDGF-BB
TIMP-1
TNF-α

Recombinant mouse:

PlGF-2
$VEGF_{115}$
$VEGF\text{-}B_{167}$
$VEGF\text{-}B_{186}$
VEGF-D
VEGF R2
VEGF R3

Recombinant human:

$VEGF_{165}$
$VEGF\text{-}B_{167}$
VEGF-C
VEGF-D
VEGF R3

Other recombinants:

porcine PDGF
zebrafish VEGF

Some cross-reactivity was observed with the following:

Recombinant Factor	Concentration Tested (pg/mL)	% Cross-reactivity
Mouse $VEGF_{120}$ (*E. coli*-expressed)	2000	59
Mouse $VEGF_{164}$ (*Sf* 21-expressed)	500	73
Canine $VEGF_{164}$ (*E. coli*-expressed)	2000	14
Human $VEGF_{121}$ (*Sf* 21-expressed)	2000	26
Human $VEGF_{121}$ (*E. coli*-expressed)	2000	31
Human $VEGF_{162}$ (NS0-expressed)	2000	24
Human $VEGF_{206}$ (*E. coli*-expressed)	50,000	0.2
Human VEGF/PlGF	10,000	5.2

Some interference was observed with the following:

Recombinant mouse VEGF R1	At concentrations greater than 200 pg/mL
Recombinant human VEGF R1	At concentrations greater than 1500 pg/mL
Recombinant human VEGF R2	At concentrations greater than 25,000 pg/mL

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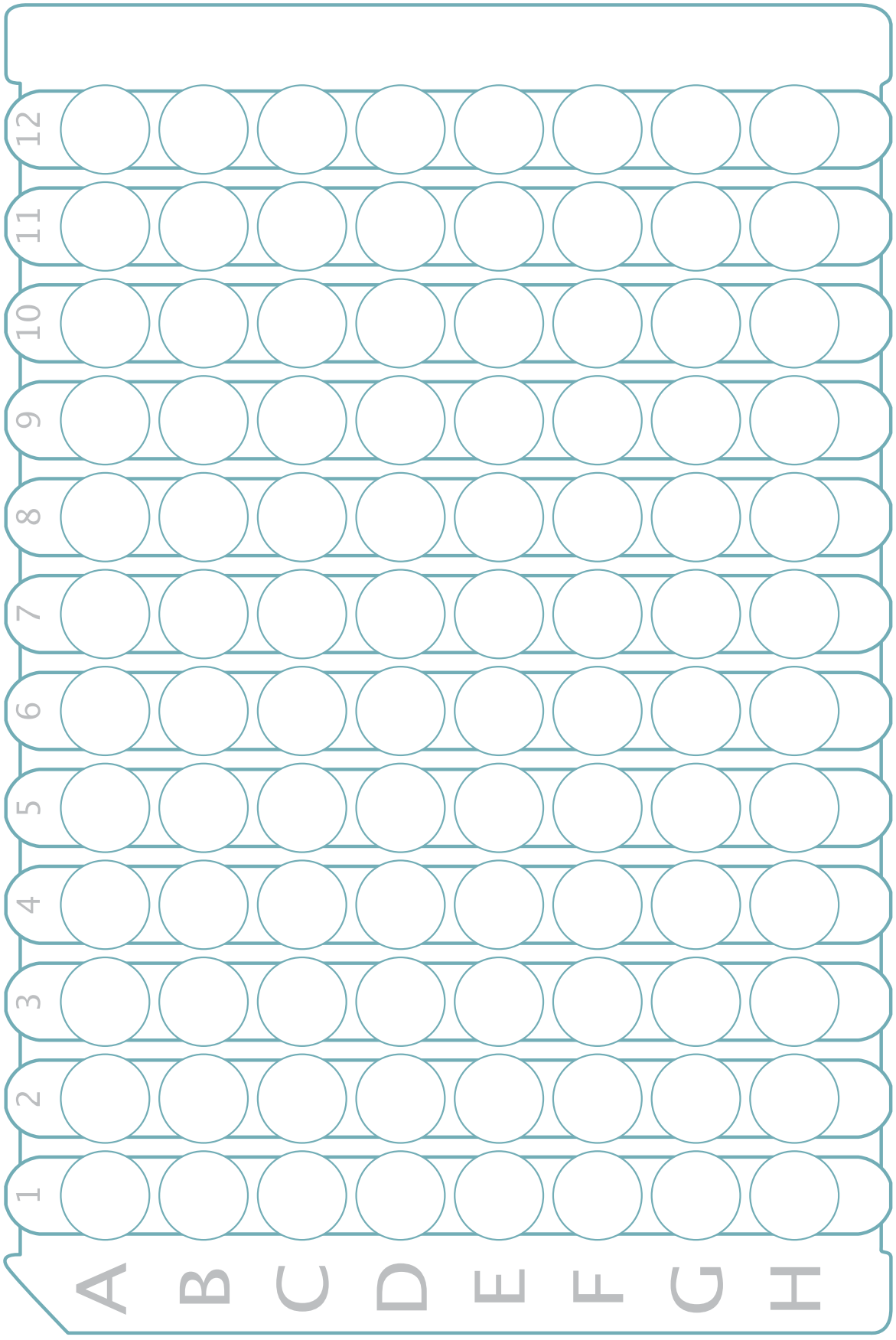
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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