Quantikine® ELISA

Mouse/Rat RGM-A Immunoassay

Catalog Number MRGMA0

For the quantitative determination of mouse and rat Repulsive Guidance Molecule A (RGM-A) concentrations in cell culture supernates, tissue homogenates, serum, and plasma.

Note: The standard reconstitution method has changed. Read this package insert in its entirety before using this product.

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

Mouse Repulsive Guidance Molecule (RGM-A) is a 33 kDa member of the RGM family of GPI-linked membrane associated proteins (1, 2). It is synthesized as a 454 amino acid (aa) prepro-protein that contains a 47 aa N-terminal signal sequence and a 27 aa C-terminal GPI attachment signal (3, 4). As a result of intramolecular cleavage, mature mouse RGM-A is a disulfide-linked dimer of the 122 aa N-terminal and 258 C-terminal segments. The N-terminal segment contains an RGD tripeptide and two potential N-linked glycosylation sites, while the C-terminal segment contains an abbreviated von Willebrand factor domain (3-5). Mouse RGM-A shares 86%, 94%, and 100% aa sequence identity with chicken, human, and rat RGM-A, respectively. It also shares 56% and 48% aa sequence identity with RGM-B and -C, respectively.

RGM-A is expressed in the developing central nervous system in a pattern that is complementary to the expression of RGM-B (3, 4). It is widely expressed on neuronal and glial cells of the mouse nervous system, particularly in the developing forebrain, the periventricular layers of the cerebrum, the optic tectum/superior colliculus of the midbrain, the retina, and in enteric ganglia of the fetal and adult gut (3-9). Among non-neuronal tissues, RGM-A mRNA is expressed in the gut, heart, lung, liver, skin, kidney, and testes (6, 8, 10).

RGM-A regulates neuronal survival and development through interactions with a receptor complex that includes Neogenin and UNC5H2 (11, 12). The pattern of Neogenin expression overlaps tissue regions that exhibit RGM-A responsiveness (11, 13). RGM-A acts as a nervous system morphogen and as an axon guidance molecule. It promotes neuronal survival, inhibits neurite outgrowth, and induces growth cone collapse (5, 9, 12-15). RGM-A regulates the layer-specific termination of entorhinal complex axons into the hippocampus (16). It also guides retinal ganglion cell axons through the optic fissure to their accurate termination in the optic tectum (5, 17-20). Following tissue damage, RGM-A exerts prosurvival and neuroprotective effects on injured retinal ganglion cells (14). Its upregulation in the vicinity of spinal cord injuries can also hinder neurological regeneration by inhibiting axonal regeneration and synapse formation (7, 21).

Like other RGM family members, RGM-A also functions as a co-receptor that enhances signaling by BMP-2 and BMP-4 (10). The exact BMP signaling function that is mediated by RGM-A remains to be determined. Recently, RGM-B has been shown to be an important negative regulator of IL-6 expression in immune cells (22). The RGM-A gene has been shown to be associated with experimental inflammation and multiple sclerosis, suggesting that variants of the RGM-A gene can differentially affect immunoregulation and be associated with pro-inflammatory cytokines and antibody responses (23). In colorectal cancer, downregulation of RGM-A correlates with increased tumor cell proliferation and invasion (24).

The Quantikine® Mouse/Rat RGM-A Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse and rat RGM-A in cell culture supernates, tissue homogenates, serum, and plasma. It contains NS0-expressed recombinant mouse RGM-A and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant factor accurately. Results obtained using natural RGM-A showed dose-response 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 natural mouse/rat RGM-A.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse/rat RGM-A/C has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any RGM-A present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat RGM-A 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 RGM-A 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 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.
- 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/Rat RGM-A/C Microplate	893968	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse RGM-A/C.	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/Rat RGM-A Conjugate	893969	12 mL of a polyclonal antibody specific for mouse RGM-A conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse/Rat RGM-A Standard	893970	Recombinant mouse RGM-A in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse/Rat RGM-A Control	893971	Recombinant mouse RGM-A in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	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	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.
- **Polypropylene** 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 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 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 - Brain tissue from individual mice was removed, rinsed in PBS, and kept on ice. Using a tissue homogenizer, the tissue was homogenized in PBS and frozen at ≤ -70 °C. Cells were thawed and then frozen again for a total of two freeze-thaw cycles. After the second freeze-thaw cycle, the cells were centrifuged at 5000 x g and the supernate was removed.

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.*
Grossly hemolyzed samples are not suitable for use in this assay.

SAMPLE PREPARATION

Serum and plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 40 µL of sample + 120 µL of Calibrator Diluent RD5-3.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

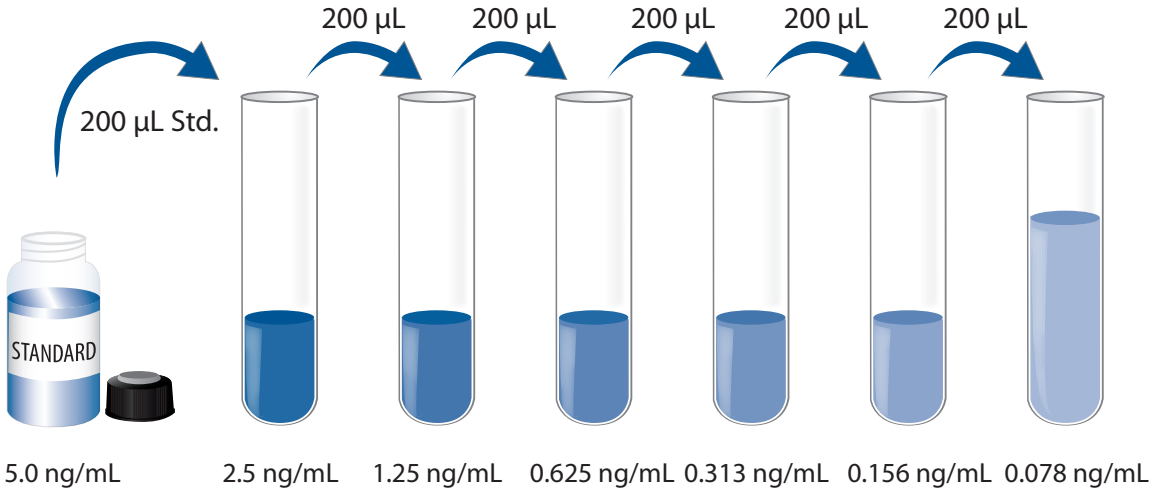
Mouse/Rat RGM-A Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

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/Rat RGM-A Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse/Rat RGM-A Standard with Calibrator Diluent RD5-3. This reconstitution produces a stock solution of 5.0 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse/Rat RGM-A Standard (5.0 ng/mL) serves as the high standard. 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, standards, control, and samples as directed by 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 RD1W 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. Gently tap the plate to ensure thorough mixing. 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/Rat RGM-A 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 the Sample Preparation section.

CALCULATION OF RESULTS

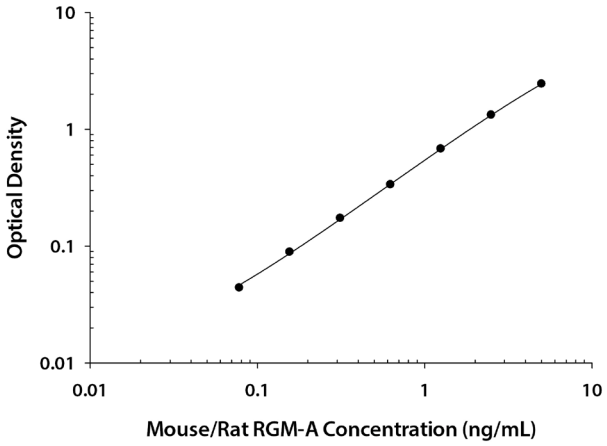
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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/rat RGM-A 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	0.017 0.019	0.018	—
0.078	0.060 0.063	0.062	0.044
0.156	0.106 0.107	0.107	0.089
0.313	0.182 0.200	0.191	0.173
0.625	0.343 0.364	0.354	0.336
1.25	0.675 0.720	0.698	0.680
2.5	1.326 1.356	1.341	1.323
5.0	2.428 2.494	2.461	2.443

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 kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	0.190	0.742	2.12	0.195	0.538	2.07
Standard deviation	0.010	0.018	0.087	0.015	0.029	0.147
CV (%)	5.3	2.4	4.1	7.7	5.4	7.1

RECOVERY

The recovery of mouse/rat RGM-A spiked to levels throughout the range of the assay in various matrices was evaluated.

Mouse Samples	Average % Recovery	Range
Cell culture supernates (n=4)	101	91-112%
Tissue homogenates (n=4)	100	93-106%
Serum* (n=4)	113	103-120%
EDTA plasma* (n=4)	106	91-119%
Heparin plasma* (n=4)	102	95-117%

Rat Samples	Average % Recovery	Range
Cell culture supernates (n=4)	100	90-113%
Serum* (n=4)	107	94-119%
EDTA plasma* (n=4)	107	103-115%
Heparin plasma* (n=4)	99	91-106%

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

SENSITIVITY

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of mouse/rat RGM-A ranged from 0.004-0.019 ng/mL. The mean MDD was 0.010 ng/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 high concentrations of mouse/rat RGM-A were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

Mouse Samples		Cell culture supernates (n=4)	Tissue homogenates (n=3)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	96	99	98	99	98
	Range (%)	95-98	91-104	88-105	93-105	92-102
1:4	Average % of Expected	98	94	98	104	96
	Range (%)	94-100	84-100	87-103	96-113	92-100
1:8	Average % of Expected	97	90	96	101	94
	Range (%)	94-100	81-96	89-100	90-116	86-100
1:16	Average % of Expected	97	93	89	92	88
	Range (%)	92-100	93-94	86-95	87-97	85-93

Rat Samples		Cell culture supernates (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	97	98	97	100
	Range (%)	94-104	94-104	93-102	94-104
1:4	Average % of Expected	97	102	98	100
	Range (%)	90-106	98-114	89-104	89-108
1:8	Average % of Expected	98	103	97	99
	Range (%)	89-109	96-112	91-101	85-117
1:16	Average % of Expected	98	103	96	98
	Range (%)	88-108	94-109	83-102	80-116

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

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse RGM-A produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse/rat RGM-A in this assay.

Mouse Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	8.78	6.84-11.9	1.42
EDTA plasma (n=20)	8.14	6.28-11.6	1.36
Heparin plasma (n=20)	7.36	6.08-10.0	1.06

Rat Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=10)	5.80	3.26-7.24	1.12
EDTA plasma (n=10)	4.54	2.84-5.44	0.73
Heparin plasma (n=10)	4.74	4.04-6.04	0.63

Cell Culture Supernates:
Tissues from individual mice or rats were removed and rinsed in PBS and kept on ice. The tissue was homogenized using a tissue homogenizer and seeded into media containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 1.0 µg/mL lipopolysaccharide (LPS) for the times indicated. Aliquots of the cell culture supernates were removed and assayed for levels of mouse/rat RGM-A.

Tissue Type	Unstimulated (ng/mL)	Stimulated (ng/mL)
Mouse Brain (3 days)	0.859	0.779
Rat Brain (1 day)	2.96	——
Rat Lung (1 day)	0.274	0.269
Rat Heart (1 day)	0.336	0.392

3T3-L1 mouse embryonic fibroblast adipose-like cells (2 x 10^6 cells per flask) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and incubated for 3 days. The media was removed and 50 mL of fresh DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate was added and incubated for 4 additional days. An aliquot of the cell culture supernate was removed, assayed for mouse RGM-A, and measured 0.728 ng/mL.

Tissue Homogenates - Mouse brain tissue was prepared as described in the Sample Collection and Storage section. An aliquot of the resulting supernate was removed, assayed for mouse RGM-A, and measured 15.9 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat RGM-A.

The factors listed below were prepared at 500 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 500 ng/mL in a mid-range mouse/rat RGM-A control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:
BMP-4
BMPR-IB
BMP-4 + BMPR-IB
Neogenin
RGM-B

Recombinant rat:
UNC5H2

Recombinant human:
BMP-2

Recombinant human RGM-A cross-reacts approximately 22.8% in this assay.

Recombinant mouse RGM-C cross-reacts approximately 0.02% in this assay.

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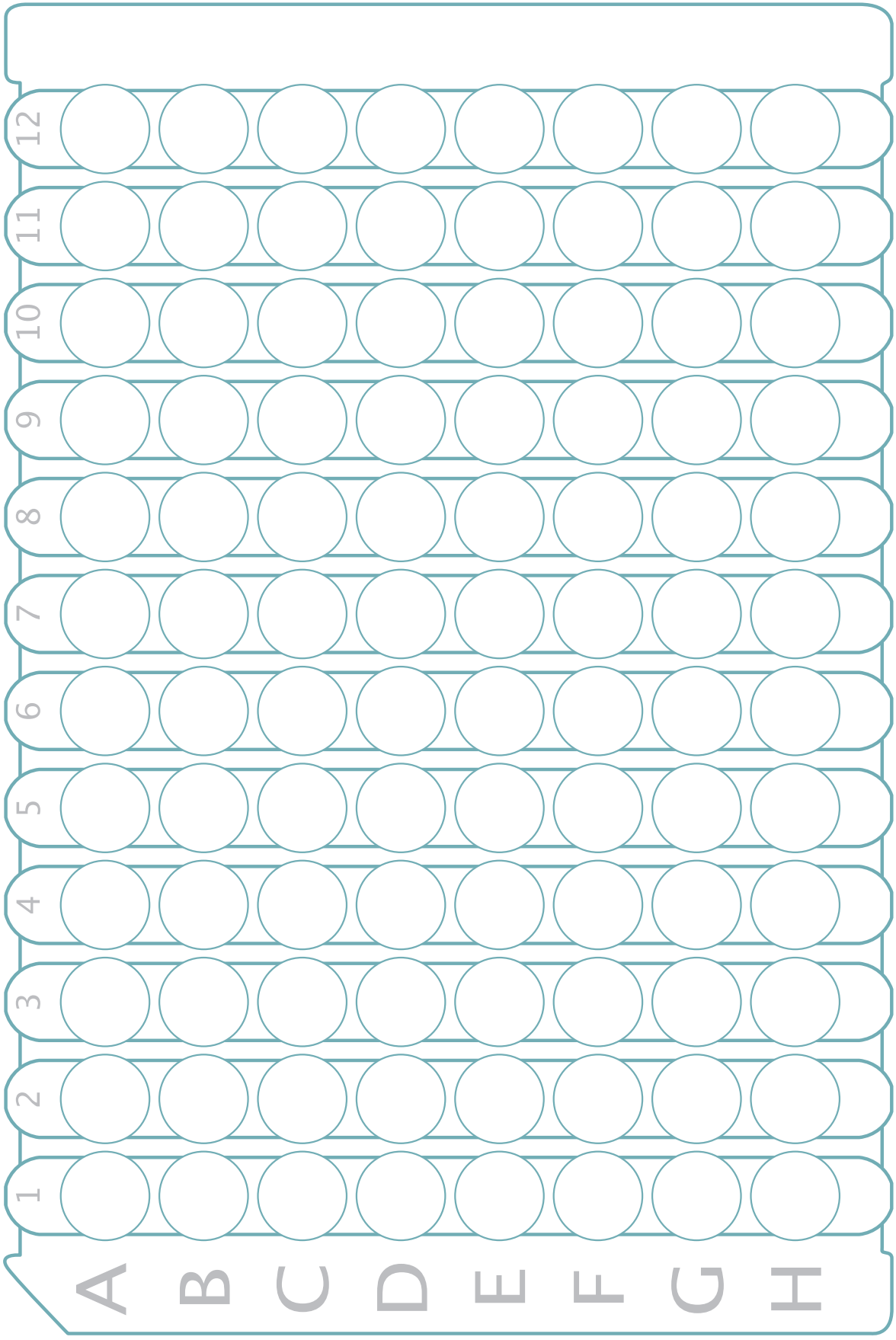
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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