Quantikine® ELISA

Rat Total MMP-9 Immunoassay

Catalog Number RMP900

For the quantitative determination of rat Matrix Metalloproteinase 9 (MMP-9) concentrations in cell culture supernates, serum, and platelet-poor 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

Matrix metalloproteinases (MMPs), also called matrixins, constitute a family of zinc and calcium dependent endopeptidases that function in the breakdown of the extracellular matrix (ECM) and in the processing of a variety of molecules in different subcellular environments. They play an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction, and tissue remodeling (1, 2). They also participate in inflammatory and autoimmune disorders such as arthritis, cancer, and cardiovascular disease (3-5). While the amounts of newly synthesized MMPs are regulated mainly at the levels of transcription, the proteolytic activities of existing MMPs are controlled through both the activation of proenzymes or zymogens and the inhibition of active enzymes by endogenous inhibitors, α2-Macroglobulin, and tissue inhibitors of metalloproteinases (TIMPs) (6).

MMP-9 (also referred to as gelatinase B, 92 kDa type IV collagenase, 92 kDa gelatinase, and type V collagenase) is secreted as a glycosylated proenzyme (6-8). Activation of the proenzyme involves proteolytic removal of the N-terminal pro region, resulting in the 82 kDa active enzyme (9, 10). In addition to the zinc-binding site, the catalytic domain also contains three contiguous fibronectin type II homology units responsible for binding gelatin (11). A proline-rich hinge region links the catalytic domain to the C-terminal hemopexin-like domain. *In vitro* treatment of the proenzyme with 4-aminophenylmercuric acetate (APMA) produces not only the active enzyme but also a C-terminal truncated form with activity comparable to that of the active form (12). MMP-9 degrades components of the ECM with high specific activity for denatured collagens (gelatin). It can cleave native collagens of type III, IV, V, and XI, as well as Elastin, Nidogen-1, and Vitronectin (2, 3). MMP-9 can also cleave a variety of chemokines and growth factors, Amyloid β peptide, Substance P, and Myelin Basic Protein (3, 13-15). This action can increase or decrease the biological activity of soluble factors and can also liberate them from association with the ECM (16, 17). MMP-9 can also trigger signaling through various transmembrane proteins or inhibit signaling by inducing their shedding from the cell surface (3, 18-20).

MMP-9 is produced by a variety of normal and transformed cells including neutrophils, monocytes, macrophages, astrocytes, fibroblasts, osteoclasts, chondrocytes, keratinocytes, endothelial and epithelial cells. It exerts physiological and pathological angiogenic and remodeling effects on the vasculature (21-25). Activated neutrophils release TIMP-1-free proMMP-9 which can be activated *in vivo*, allowing the liberation of pro-angiogenic FGF-2 from the ECM (17). MMP-9 in complex with TIMP-1 does not induce FGF-basic release (17). Neutrophil-derived MMP-9 exacerbates the inflammatory response, in part by generating collagen-derived peptides that induce the release of additional neutrophil MMP-9 (26). MMP-9 also plays a role in bone formation and remodeling (1, 21, 27), methamphetamine-induced behavioral sensitization and reward (28), the regulation of neuronal synapse remodeling (29), trophoblast invasion during implantation (30), and the inactivation of Serpin α1-Proteinase Inhibitor (31). The shedding of adhesion proteins by MMP-9 has a direct effect on tumor cell invasiveness (18-20).

Circulating levels of MMP-9 are increased in many inflammatory disorders including intraluminal thrombus formation (32), atherosclerosis (33), Crohn's disease (34), hepatitis C virus infection (35), colorectal cancer (36), and Duchenne muscular dystrophy (37). The ratio of MMP-9 to TIMP-1 is also increased in multiple sclerosis serum (38) and cystic fibrosis sputum (39), but it is decreased in the serum during cytomegalovirus infection (40). Levels of free MMP-9 and complexes of MMP-9 with Lipocalin-2/NGAL are elevated in the urine of ovarian cancer and uterine tract infection patients, respectively (41, 42).

The Quantikine Rat Total MMP-9 Immunoassay is a 4.5 hour solid phase ELISA designed to measure total rat MMP-9 (Pro-, active, and TIMP-complexed MMP-9) in cell culture supernates, serum, and platelet-poor plasma. It contains NS0-expressed recombinant rat MMP-9 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant protein. Results obtained using natural rat MMP-9 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 rat MMP-9.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat MMP-9 has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any rat MMP-9 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for MMP-9 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 rat MMP-9 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.
- It is recommended that the samples be pipetted within 15 minutes.
- 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
Rat Total MMP-9 Microplate	894142	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody specific for rat MMP-9.	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 Total MMP-9 Conjugate	894143	12 mL of a polyclonal antibody specific for MMP-9 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Rat Total MMP-9 Standard	894144	50 ng of recombinant rat MMP-9 in a buffered protein base with preservatives; lyophilized.	
Rat Total MMP-9 Control	894145	1 vial of recombinant rat MMP-9 in a buffered protein base with preservatives; lyophilized. The concentration range of rat MMP-9 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 RD1-34	895265	11 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-10	895266	2 vials (21 mL/vial) 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.
- 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.

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.

Platelet-poor Plasma - Collect plasma on ice using heparin as an anticoagulant. Centrifuge for 20 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *EDTA and Citrate are not recommended anticoagulants for use in this assay due to their chelating properties.*

MMP-9 is released upon platelet activation. To measure circulating levels of MMP-9, platelet-free plasma should be used for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory and Standards Institute (CLSI), result in incomplete removal of platelets or platelet activation. This may cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

SAMPLE PREPARATION

Serum and platelet-poor heparin plasma samples require a 10-fold dilution. A suggested 10-fold dilution can be achieved by adding 20 µL of sample + 180 µL of Calibrator Diluent RD5-10.

REAGENT PREPARATION

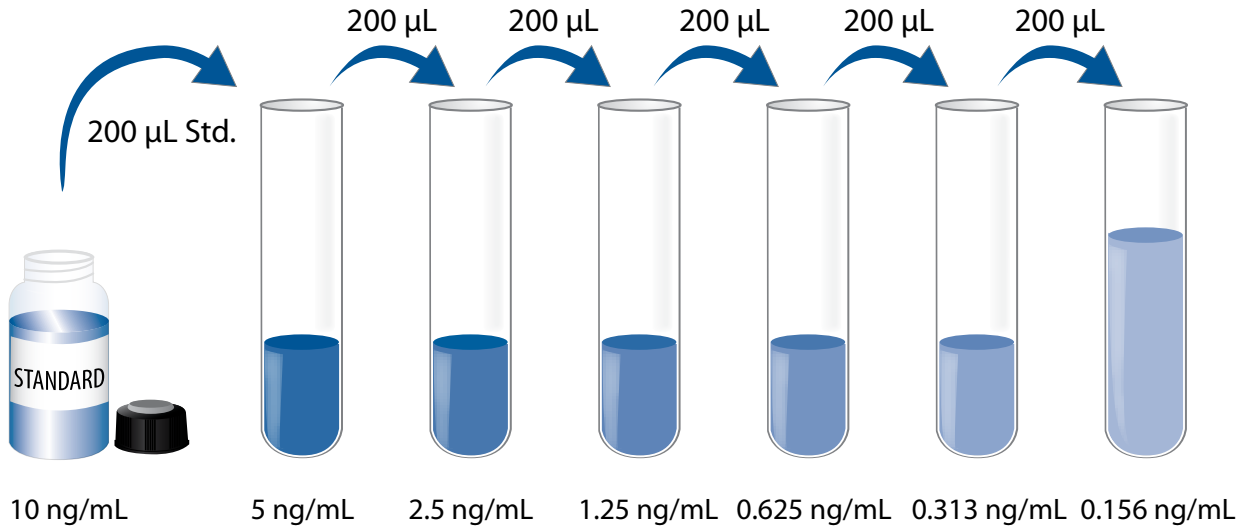
Bring all reagents to room temperature before use.

Rat MMP-9 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.

Rat MMP-9 Standard - Reconstitute the Rat MMP-9 Standard with 5.0 mL of Calibrator Diluent RD5-10. This reconstitution produces a stock solution of 10 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-10 into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard serves as the high standard. Calibrator Diluent RD5-10 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, 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 RD1-34 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 the 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 Rat Total MMP-9 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

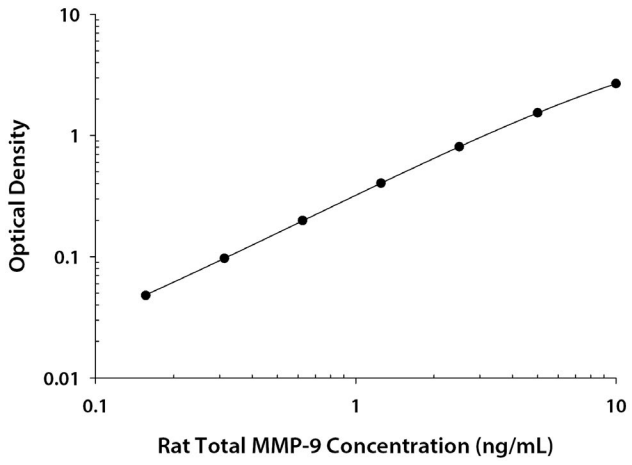
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 rat MMP-9 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	0.032 0.037	0.035	—
0.156	0.081 0.084	0.083	0.048
0.313	0.132 0.132	0.132	0.097
0.625	0.232 0.235	0.234	0.199
1.25	0.438 0.439	0.439	0.404
2.5	0.827 0.856	0.842	0.807
5	1.549 1.594	1.572	1.537
10	2.658 2.763	2.711	2.676

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	0.39	1.22	5.27	0.51	1.18	3.55
Standard deviation	0.027	0.068	0.238	0.035	0.046	0.153
CV (%)	6.9	5.6	4.5	6.9	3.9	4.3

RECOVERY

The recovery of rat MMP-9 spiked into various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture samples (n=4)	94	87-107%
Serum* (n=4)	97	90-115%
Platelet-poor heparin plasma* (n=4)	92	80-103%

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

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of rat MMP-9 were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=4)	Serum* (n=4)	Platelet-poor Heparin plasma* (n=4)
1:2	Average % of Expected	106	99	109
	Range (%)	98-116	96-103	106-117
1:4	Average % of Expected	103	96	109
	Range (%)	103-104	93-101	104-111
1:8	Average % of Expected	102	98	110
	Range (%)	93-109	91-110	103-116
1:16	Average % of Expected	108	97	106
	Range (%)	103-113	90-106	98-112

*Samples were diluted prior to assay.

SENSITIVITY

Fifty-three assays were evaluated and the minimum detectable dose (MDD) of Rat MMP-9 ranged from 0.005-0.028 ng/mL. The mean MDD was 0.013 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-derived recombinant rat MMP-9 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Rat samples were evaluated for the presence of MMP-9 in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=15)	33.4	16.5-48.7	9.03
Platelet-poor heparin plasma (n=10)	14.5	9.41-24.1	4.86

Cell Culture Supernates:
Tissue from rats was removed, rinsed in 1X PBS, and kept on ice. The tissue was then 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 for 18 hours. Aliquots of the cell culture supernates were removed and assayed for levels of natural rat MMP-9.

Tissue Type	(ng/mL)
Lung	1.81
Spleen	3.33

NRK-49F rat fibroblasts (1 x 10^6 cell/mL) were cultured in DMEM supplemented with 10% bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 1µg/mL lipopolysaccharide for 2 days. Aliquots of the cell culture supernates were removed, assayed for levels of natural rat MMP-9, and measured 0.261 ng/mL and 1.49 ng/mL respectively.

SPECIFICITY

This assay recognizes natural and recombinant rat MMP-9 (Pro-, active, and TIMP-complexed).

The factors listed below were prepared at 100 ng/mL in Calibrator Diluent RD5-10 and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant rat MMP-9 standard were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant rat:
Lipocalin-2/NGAL
MMP-8
TIMP-1

Recombinant mouse:
Lipocalin-2/NGAL
MMP-2
MMP-3
MMP-7
MMP-8
MMP-12
TIMP-1

Recombinant human:
Lipocalin-2/NGAL
MMP-1
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-9/NGAL Complex
MMP-9/TIMP-1 Complex
MMP-10
MMP-12 Hemopexin Domain
Pro-MMP-12
MMP-13
MMP-14
MMP-16
Pro-MMP-16
MMP-19
MMP-24
TIMP-1

Recombinant mouse MMP-9 cross-reacts at approximately 1.3%.

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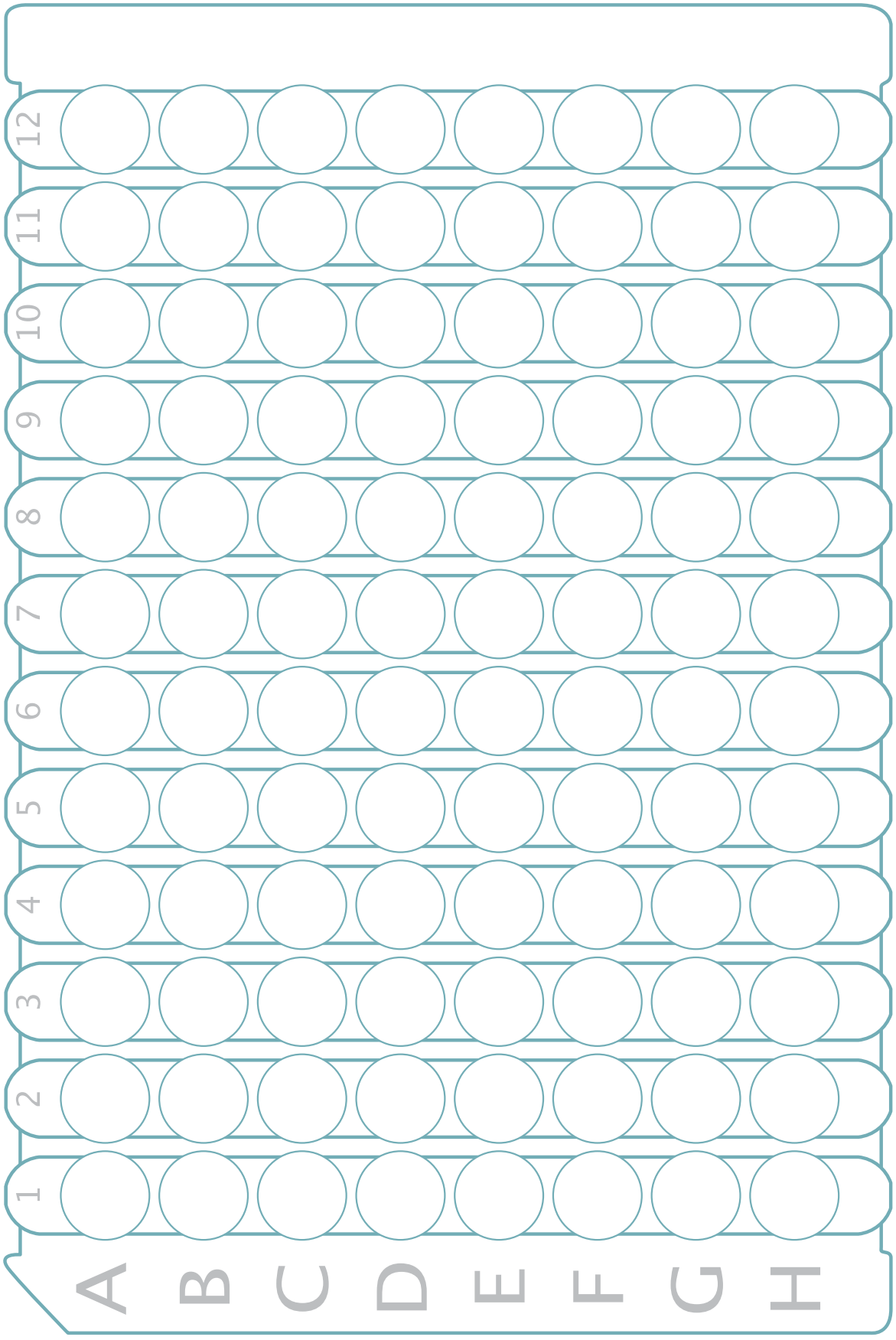
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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