Quantikine® ELISA

Mouse Lipocalin-2/NGAL Immunoassay

Catalog Number MLCN20

For the quantitative determination of mouse Lipocalin-2/NGAL concentrations in cell culture supernates, serum, platelet-poor plasma, and urine.

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

Members of the Lipocalin family have limited sequence identity but share a highly conserved fold with an eight-stranded anti-parallel β barrel motif that encloses an internal ligand-binding site. They are known for their actions as transporters that carry small hydrophobic molecules such as steroid hormones, vitamins, odorants, and metabolic products (1). Lipocalin-2, also known as Neutrophil Gelatinase-associated Lipocalin (NGAL), Siderocalin, and 24p3, was originally identified as a component of neutrophil granules (2). Its synthesis is induced in macrophages, glial cells, and epithelial cells during inflammation (3-5). Lipocalin-2 has been implicated in a variety of cellular processes including the innate immune response, differentiation, tumorigenesis, and cell survival. It is a 25 kDa glycoprotein that exists in monomeric and homodimeric forms and associates covalently with MMP-9 (2, 6). Its association with MMP-9 may modulate protease activity by protecting MMP-9 from degradation (6, 7). Mature mouse Lipocalin-2 shares 62% and 81% amino acid sequence identity with human and rat Lipocalin-2, respectively.

Lipocalin-2 binds catecholate siderophores which are secreted by bacteria and required for bacterial iron uptake. Lipocalin-2 functions as a bacteriostatic agent in the innate immune response by limiting the bacterial iron supply (8, 9). TLR4 activation induces its production in immune and epithelial cells, and Lipocalin-2 knockout mice are impaired in resisting bacterial infection (3, 9-11). Some virulent bacterial strains evade immune clearance by producing modified siderophores that are not bound by Lipocalin-2 (12, 13). Iron uptake by Lipocalin-2 into mammalian cells is important for the regulation of iron-sensitive gene transcription (14). In the kidney, Lipocalin-2-mediated iron trafficking is required for protection from renal injury (15). Megalin, a member of the LDL receptor family, and 24p3 R/NGALR/BOCT have been reported as endocytic receptors for Lipocalin-2 (16, 17). In apparent contradiction, Lipocalin-2 has been shown to act as both a survival factor and a pro-apoptotic factor (5, 17-20). Its induction by pro-inflammatory cytokines may vary between mouse and human (21).

Lipocalin-2 is also upregulated in non-bacterial inflammatory pathologies such as psoriasis, ulcerative colitis, and adipose tissue in obesity where it promotes insulin resistance in hepatocytes (22-25). It is upregulated under conditions of anemia or hypoxia and inhibits the differentiation of erythrocytes (26, 27). Lipocalin-2 levels in the urine and serum are elevated following acute renal injury (28). Lipocalin-2 is upregulated in several cancers or tumor-associated stroma, but its association with cancer is complex and may depend on tumor type or the local microenvironment (29). Lipocalin-2 has been shown to promote tumor angiogenesis, epithelial-mesenchymal transition, and metastasis in some cases (30-33) but suppress them in others (34-36).

The Quantikine Mouse Lipocalin-2 Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse Lipocalin-2 in cell culture supernates, serum, platelet-poor plasma, and urine. It contains NS0-expressed recombinant mouse Lipocalin-2 and antibodies raised against the recombinant factor. Natural mouse Lipocalin-2 showed dose-response curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards, indicating that this kit can be used to determine relative levels of natural mouse Lipocalin-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Lipocalin-2 has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse Lipocalin-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for mouse Lipocalin-2 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 Lipocalin-2 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.
- 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 Lipocalin-2 Microplate	893772	One 96 well microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse Lipocalin-2.	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 Lipocalin-2 Conjugate	893773	12 mL of monoclonal antibody against mouse Lipocalin-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse Lipocalin-2 Standard	893774	25 ng of recombinant mouse Lipocalin-2 in a buffered protein base with preservatives; lyophilized.	
Mouse Lipocalin-2 Control	893775	Recombinant mouse Lipocalin-2 in a buffered protein base with preservatives; lyophilized. The expected range of the mouse Lipocalin-2 control is shown on the vial label.	
Assay Diluent RD1-34	895265	11 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-24	895325	21 mL of a concentrated 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.
- 100 mL and 500 mL graduated cylinders.
- 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 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - To measure circulating Lipocalin-2 levels, platelet-poor plasma is recommended. Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. For complete platelet removal, an additional centrifugation step is recommended. Centrifuge the separated plasma at 2-8 °C for 10 minutes at 10,000 x g. 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.*

Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

SAMPLE PREPARATION

Cell culture supernate samples may require dilution.

Serum and platelet-poor plasma samples require a 100-fold dilution. A suggested 100-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-24 (1X). Complete the 100-fold dilution by adding 20 µL of the diluted sample to 180 µL of Calibrator Diluent RD5-24 (1X).

Urine samples require a 20-fold dilution. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-24 (1X).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse Lipocalin-2 Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

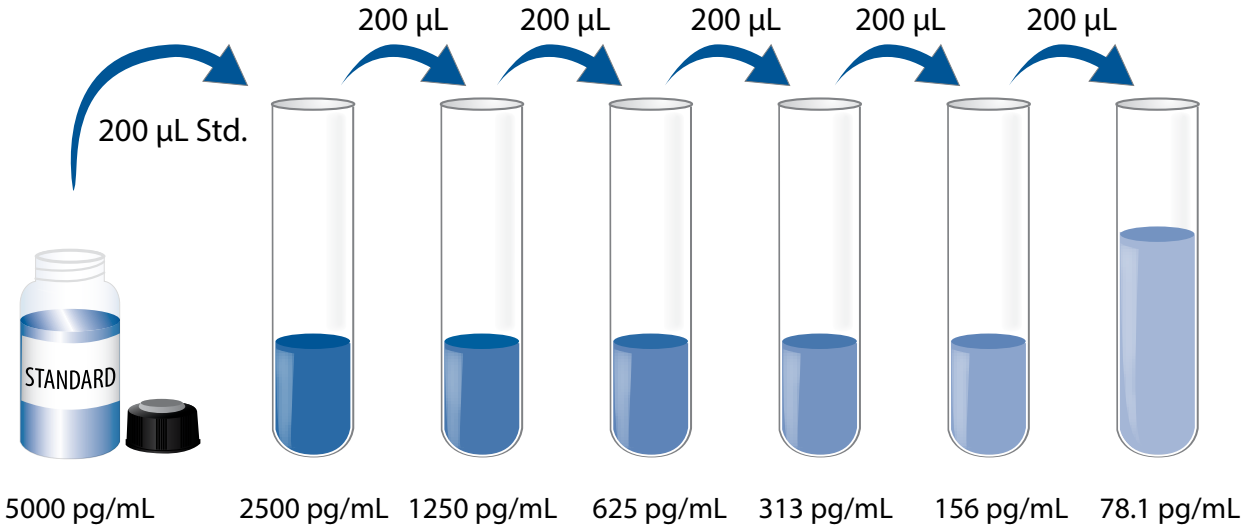
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5-24 (1X) - Add 20 mL of Calibrator Diluent RD5-24 to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-24 (1X).

Mouse Lipocalin-2 Standard - Reconstitute the Mouse Lipocalin-2 Standard with 5.0 mL of Calibrator Diluent RD5-24 (1X). This reconstitution produces a stock solution of 5000 pg/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 RD5-24 (1X) into each of six tubes. Use the Standard stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 5000 pg/mL tube serves as the high standard. Calibrator Diluent RD5-24 (1X) 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 reagents and standard dilutions as directed in the previous section.
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 on the benchtop. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four 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 Lipocalin-2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the benchtop.
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. If color change does not appear uniform, 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

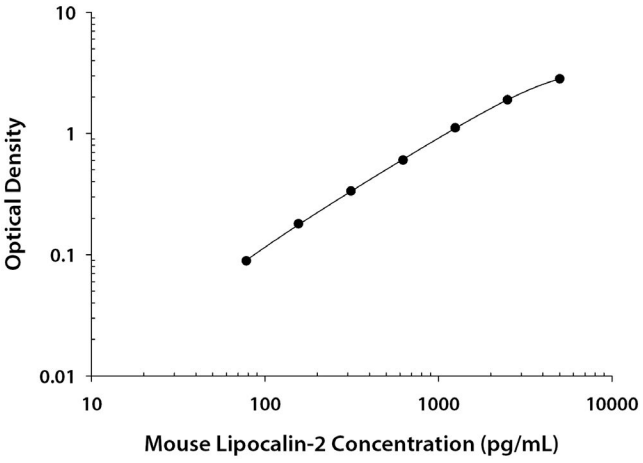
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Lipocalin-2 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.



(pg/mL)	O.D.	Average	Corrected
0	0.014 0.016	0.015	—
78.1	0.099 0.109	0.104	0.089
156	0.193 0.197	0.195	0.180
313	0.340 0.360	0.350	0.335
625	0.610 0.625	0.618	0.603
1250	1.120 1.135	1.128	1.113
2500	1.897 1.922	1.910	1.895
5000	2.810 2.870	2.840	2.825

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 forty separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	196	476	1534	211	477	1564
Standard deviation	13.2	19.6	65.9	15.2	28.6	98.5
CV (%)	6.7	4.1	4.3	7.2	6.0	6.3

RECOVERY

The recovery of mouse Lipocalin-2 spiked to three levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture samples (n=4)	93	82-101%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse Lipocalin-2 were 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		Urine* (n=4)
				EDTA plasma* (n=4)	Heparin plasma* (n=4)	
1:2	Average % of Expected	100	101	106	103	103
	Range (%)	96-103	99-103	100-110	98-108	93-116
1:4	Average % of Expected	103	106	104	101	102
	Range (%)	98-106	101-115	96-114	91-109	93-112
1:8	Average % of Expected	108	105	105	101	102
	Range (%)	100-112	99-114	91-117	90-110	95-111
1:16	Average % of Expected	106	101	104	98	105
	Range (%)	94-118	95-118	90-118	91-108	96-111

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

SENSITIVITY

Fifty-four assays were evaluated and the minimum detectable dose (MDD) of mouse Lipocalin-2 ranged from 1.40-8.80 pg/mL. The mean MDD was 3.18 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.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-derived recombinant mouse Lipocalin-2 produced at R&D Systems.

SAMPLE VALUES

Serum/Platelet-poor Plasma/Urine - Samples were evaluated for the presence of Lipocalin-2 in this assay. Samples were diluted prior to assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	141	58.3-290	60.2
Platelet-poor heparin plasma (n=20)	76.0	40.7-143	30.1
Platelet-poor EDTA plasma (n=20)	54.1	32.5-74.7	12.3
Urine (n=20)	48.6	12.9-168	34.1

Cell Culture Supernates:

J774A.1 mouse reticulum cell sarcoma macrophage cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin sulfate. Cells were either untreated or stimulated with 100 ng/mL of recombinant mouse IFN-γ and 1 µg/mL of lipopolysaccharide (LPS) for 2 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse Lipocalin-2.

Condition	Observed Values (ng/mL)
Unstimulated	0.817
Stimulated	161

WEHI-3 myelomonocytic leukemia cells (2 x 10^5 cells/mL) were and cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin sulfate. Cells were either untreated or stimulated with 1 µg/mL of LPS for 4 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse Lipocalin-2.

Condition	Observed Values (ng/mL)
Unstimulated	83.7
Stimulated	178

Two mouse brains and kidneys were homogenized and seeded in RPMI 1640 supplemented with 10% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin sulfate. The resulting supernates were either untreated or stimulated with 1 µg/mL of LPS for 3 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse Lipocalin-2.

Condition	Observed Values (ng/mL)
Brain unstimulated	0.334
Brain stimulated	2.01

Condition	Observed Values (ng/mL)
Kidney unstimulated	1.28
Kidney stimulated	4.69

SPECIFICITY

This assay recognizes natural and recombinant mouse Lipocalin-2.

The factors listed below were prepared at 200 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 200 ng/mL in a mid-range mouse Lipocalin-2 standard were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-12

Recombinant rat:

Lipocalin-2/NGAL
MMP-9

Recombinant human:

Lipocalin-1
Lipocalin-2/NGAL
MMP-9

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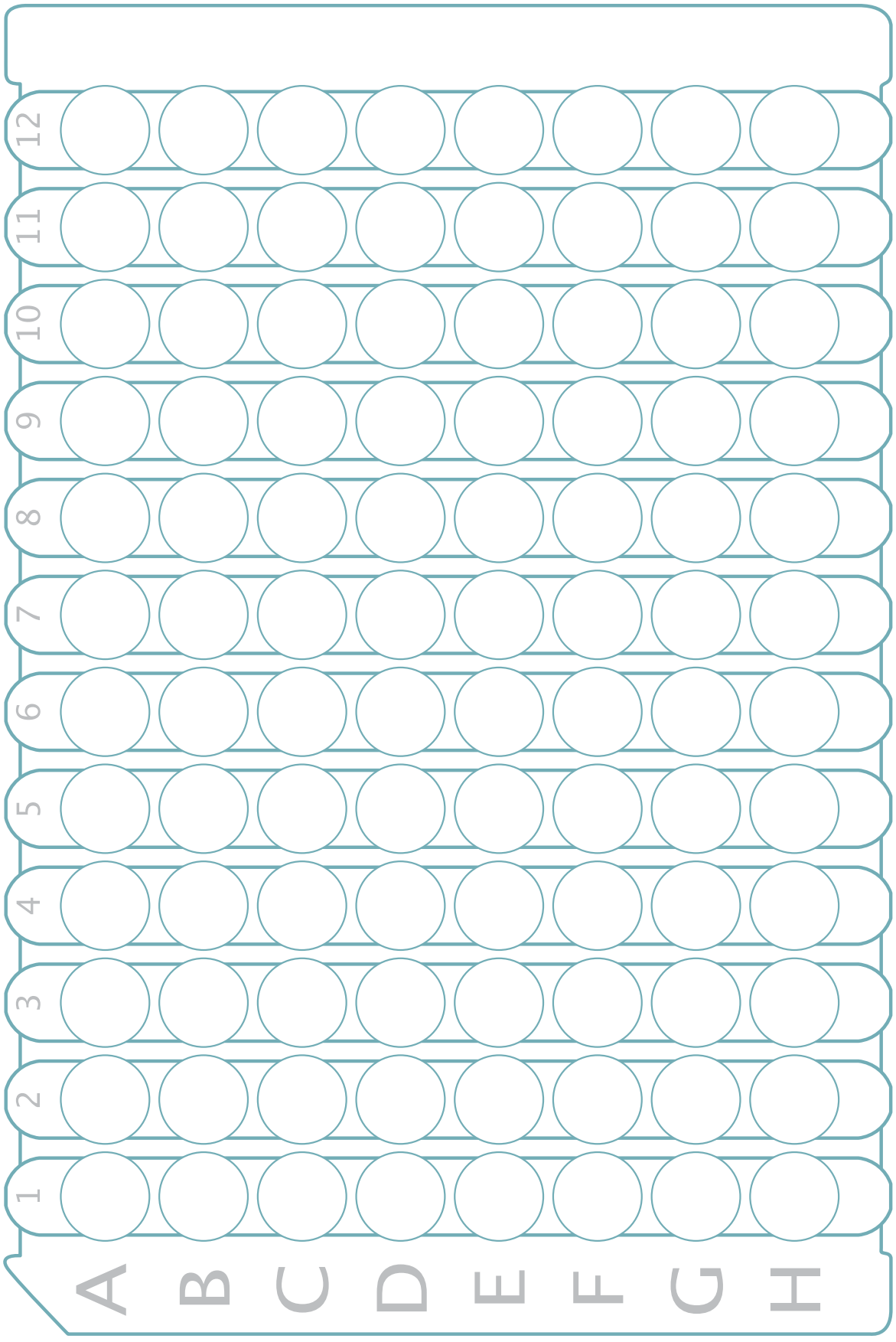
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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