Quantikine® ELISA

Mouse CXCL4/PF4 Immunoassay

Catalog Number MCX400

For the quantitative determination of mouse Platelet Factor 4 (PF4) 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

Platelet Factor 4 (PF4), also known as CXCL4, is an 8 kDa member of the CXC chemokine family that shares features with CXCL8/IL-8 and CXCL7/thymus chemokine-1/NAP-2/β-thromboglobulin (1-3). Mouse PF4 shares 64% and 89% amino acid identity with human and rat PF4, respectively. The active protein is a tetramer of PF4 subunits that forms a ring of heparin-binding positive charges from sites at the C-terminal region of each monomer (4). Megakaryocytes synthesize PF4 and store it in platelet α-granules (5, 6). Secretion from activated platelets can produce micromolar levels in serum and over 100-fold higher concentrations within clots (2). In contrast to other CXC chemokines, PF4 does not contain an ELR motif and lacks binding to nearly all chemokine receptors (2, 3). One potential high-affinity G-protein-coupled receptor for PF4, the CXCR3 isoform CXCR3B, is not expressed in mice (2, 3). Instead, it is likely that cell surface binding and signaling properties of PF4 are due to binding of glycosaminoglycan chains, particularly chondroitin sulfates (2, 7-9).

Release of PF4 from activated platelets affects coagulation. It binds and regulates thrombin/thrombomodulin complexes. It also regulates and enhances production of activated Protein C (APC), which limits the coagulation cascade (10-12). PF4 binds and influences the enzymatic activity of coagulation factor Xa (12). It binds fibrin and affects clot structure (14). PF4 is thought to aid platelet aggregation by adhering to the platelet surface after its release. Deletion of mouse PF4 impairs platelet aggregation in response to low doses of thrombin, however, transgenic over-expression of PF4 can inhibit platelet aggregation and thrombus formation (3, 15). Therapeutic doses of the anticoagulant heparin neutralize PF4 pro-coagulant effects (15). The complex between heparin and PF4 can be immunogenic, producing the pathological syndrome HITT (heparin-induced thrombocytopenia and thrombosis) (16). In addition, immunogenic complexes of PF4 with apolipoprotein H can contribute to anti-phospholipid syndrome (APS) (17).

Many other functions have been observed for PF4. In a feedback mechanism, PF4 interaction with megakaryocyte LDL receptor-related protein 1 (LRP-1) downregulates further platelet production (18, 19). In hematopoietic progenitors, heterodimers of PF4 and IL-8 can slow proliferation and promote adhesion (9, 20). PF4 can be pro-inflammatory. It is involved in monocyte survival, macrophage differentiation, TNF-α induction, and induction of endothelial cell apoptosis (7, 21-23). It promotes atherogenesis by downregulating macrophage CD163, and by binding endothelial cell LRP-1, which results in upregulation of E-selectin surface expression (24, 25). It also affects neutrophil transmigration through endothelial cells but is not chemotactic for neutrophils (2, 3, 26-29). PF4 can be anti-proliferative and anti-angiogenic, at least in part by interfering with FGF basic and VEGF heparin binding and thus inhibiting their signaling (30-32). Finally, an anti-microbial effect has been shown for PF4, both by direct binding of microbes and by stimulating macrophage respiratory bursts and cytokine production (33).

The Quantikine® Mouse CXCL4/PF4 Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse PF4 in cell culture supernates, serum, and platelet-poor plasma. It contains *E. coli*-expressed recombinant mouse PF4 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant factor accurately. Results obtained using natural mouse PF4 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 PF4.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse PF4 has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any PF4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse PF4 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 PF4 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.
- 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 PF4 Microplate	893843	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse PF4.	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 PF4 Standard	893845	2 vials of recombinant mouse PF4 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard and control for each assay. Discard after use.
Mouse PF4 Control	893846	2 vials of recombinant mouse PF4 in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse PF4 Conjugate	893844	12 mL of a polyclonal antibody specific for mouse PF4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-40	895513	12 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:4 in this assay.*	
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.
- **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. Cell culture supernates should be assayed at neutral pH, ~7.0.

Serum - Allow blood samples to clot for 2 hours at room temperature and centrifuge 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 - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at 2-8 °C for 20 minutes at 2000 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.*

SAMPLE PREPARATION

Serum samples require a 2000-fold dilution. A suggested dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-26 (diluted 1:4)*. Complete the 2000-fold dilution by adding 10 µL of the diluted sample to 990 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

Platelet-poor plasma samples require a 1000-fold dilution. A suggested dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-26 (diluted 1:4)*. Complete the 1000-fold dilution by adding 10 µL of the diluted sample to 490 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse PF4 Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

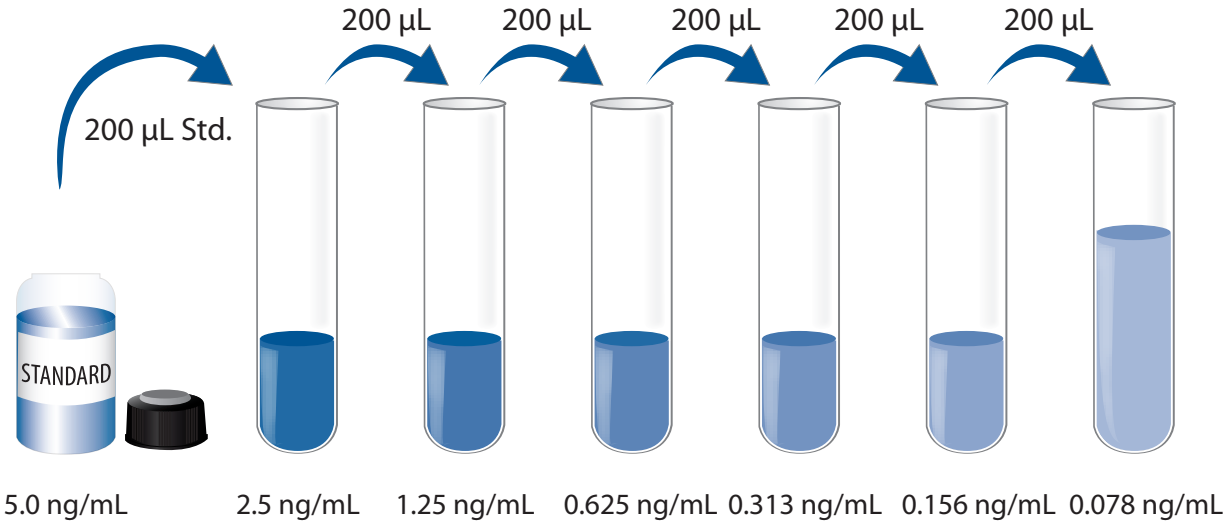
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5-26 (diluted 1:4) - Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Mouse PF4 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse PF4 Standard with Calibrator Diluent RD5-26 (diluted 1:4). 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 RD5-26 (diluted 1:4) into each tube. Use the standard stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse PF4 Standard (5.0 ng/mL) serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) 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 samples, control, and standards 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 RD1-40 to each well.
4. Add 50 µL of standard, control, or sample* per well. Mix by gently tapping the plate frame for 1 minute. 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 PF4 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

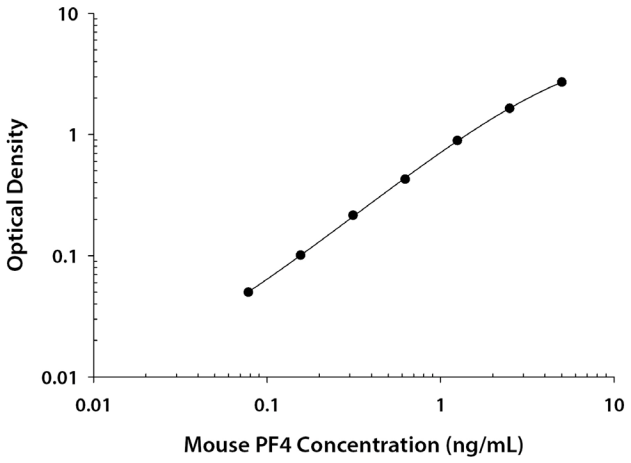
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 PF4 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	0.025 0.029	0.027	—
0.078	0.075 0.078	0.077	0.050
0.156	0.127 0.128	0.128	0.101
0.313	0.239 0.246	0.243	0.216
0.625	0.436 0.472	0.454	0.427
1.25	0.913 0.926	0.920	0.893
2.5	1.605 1.735	1.670	1.643
5.0	2.663 2.797	2.730	2.703

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 components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	0.23	0.41	1.51	0.25	0.45	1.46
Standard deviation	0.013	0.029	0.046	0.019	0.035	0.110
CV (%)	5.7	7.1	3.0	7.6	7.8	7.5

RECOVERY

The recovery of mouse PF4 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	102	90-120%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse PF4 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	
				EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	95	95	99	97
	Range (%)	91-102	91-101	94-103	92-101
1:4	Average % of Expected	97	96	101	95
	Range (%)	87-102	93-102	95-105	90-103
1:8	Average % of Expected	90	99	100	95
	Range (%)	85-100	93-103	95-105	86-106
1:16	Average % of Expected	89	94	95	96
	Range (%)	83-105	92-97	81-107	88-102

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

SENSITIVITY

Nineteen assays were evaluated and the minimum detectable dose (MDD) of mouse PF4 ranged from 0.003-0.010 ng/mL. The mean MDD was 0.006 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.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant mouse PF4 produced at R&D Systems®.

SAMPLE VALUES

Serum/Platelet-poor Plasma - Samples were evaluated for the presence of PF4 in this assay.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum (n=20)	7.01	3.7-9.1	1.42
Platelet-poor EDTA plasma (n=20)	1.83	0.26-4.70	1.49
Platelet-poor heparin plasma (n=20)	1.84	0.57-8.28	1.67

Cell Culture Supernates:

IC-21 mouse macrophage cells (5 x 10^4 cells/mL) were cultured in RPMI 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. An aliquot of the cell culture supernate was removed, assayed for mouse PF4, and measured 1.91 ng/mL.

P388D1 mouse lymphoma cells (1 x 10^4 cells/flask) were cultured in RPMI 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. An aliquot of the cell culture supernate was removed, assayed for mouse PF4, and measured 2.78 ng/mL.

J774A.1 mouse reticulum cell sarcoma macrophage cells (3.5 x 10^6 cells/flask) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 10 µg/mL streptomycin sulfate, and 100 ng/mL recombinant mouse GM-CSF, and incubated for 3 days. An aliquot of the cell culture supernate was removed, assayed for mouse PF4, and measured 36.7 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse PF4.

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 recombinant mouse PF4 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Coagulation Factor X
CXCL9/MIG
CXCL10/IP-10/CRG-2
IL-3
Thrombomodulin
$VEGF_{120}$
$VEGF_{164}$

Recombinant human:

CXCL4/PF4
CXCL8/IL-8
LRP-1
Thrombin

Up to 2% (w/v) heparin was added to a mid- or high-range mouse PF4 control and assayed for interference. No significant interference was observed.

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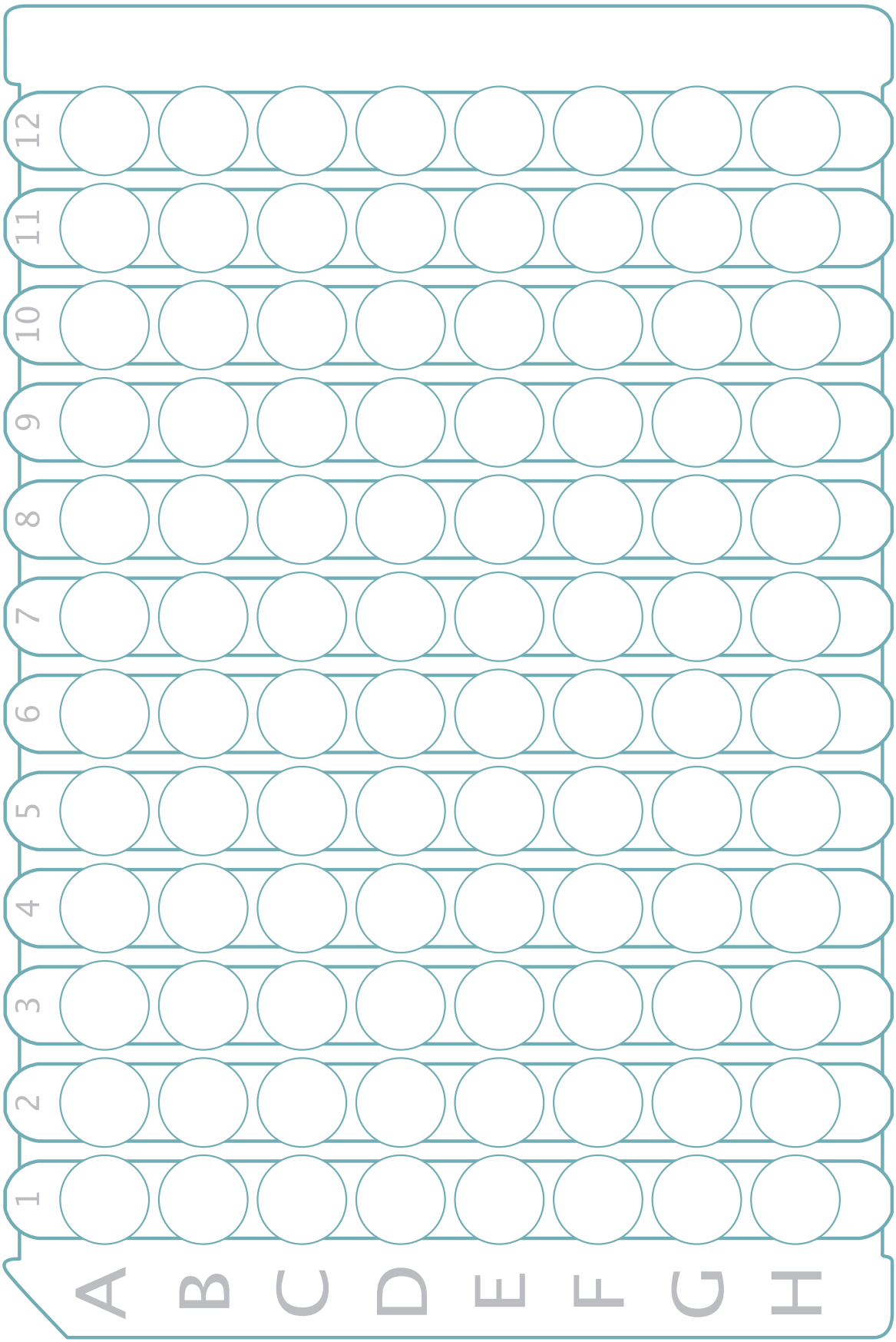
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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