Quantikine® ELISA

Mouse P-Selectin/CD62P Immunoassay

Catalog Number MPS00

For the quantitative determination of mouse P-Selectin 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

Mouse P-Selectin (also known as CD62P, GMP-140 and LECAM-3) is a member of a small family of leukocyte adhesion molecules that also includes E-Selectin (CD62E) and L-Selectin (CD62L). All selectins are Ca^{++}-dependent receptors that bind specifically to the carbohydrates of leukocyte cell surface glycoproteins (1-4). P-Selectin is stored in the α-granules of platelets and Weibel-Palade bodies of endothelial cells. Upon cell activation by cytokines, endotoxin or thrombotic events, P-Selectin is rapidly and transiently transported to the cell surface (5-7). Mature P-Selectin is a type I transmembrane glycoprotein that contains a 116 amino acid (aa) N-terminal, C-type lectin domain, a 42 aa EGF-like motif, eight 60-70 aa short concensus repeats (SCRs, also known as Sushi domains), a 24 aa transmembrane segment, and a short 35 aa cytoplasmic region. The number of SCRs differs between different species, with nine in human and six in bovine (7-9). Both the lectin and EGF domains are required for optimal binding to its counter-receptor (10). The primary structure of the mouse P-Selectin EGF-like domain is 92% identical to that in rat or human P-Selectin. The lectin domain of mouse P-Selectin is 92% and 76% identical to its rat and human counterparts, respectively (7, 11, 12). There is only 35% aa sequence identity between the mature mouse P-Selectin and E-Selectin proteins (7, 13).

The interaction between P-Selectin and its ligand(s)/counter-receptor(s) mediates the initial rolling and subsequent stable adhesion of leukocytes on both endothelial cells and platelets. This results in leukocyte activation by chemokines or through P-Selectin glycoprotein ligand-1 (PSGL-1) ligation, leading to either leukocyte extravasation at sites of inflammation, or leukocyte involvement in thrombus formation at sites of vascular injury (1-3, 14-16). P-Selectin binds to both highly clustered O-linked sialyl Lewis-x (sLe^x) tetrasaccharide, and sulfated tyrosine (16-18). These features are present on the major P-Selectin ligand, PSGL-1, a transmembrane sialomucin of 120 kDa that is constitutively expressed on hematopoietic cells (18). In addition, heparin sulfate, veriscan (a chondroitin-sulfate proteoglycan), platelet GP-Ibα and other poorly characterized sialylated, fucosylated, O-linked glycoproteins can also serve as ligands for P-Selectin (5, 19-22).

A 100-105 kDa soluble form of P-Selectin has been reported and is assumed to arise from proteolytic cleavage (23, 24). Cell surface P-Selectin is rapidly upregulated following cell activation (7), and shedding via proteolysis is reported to occur after the sixth SCR (9). Although the function of the shedding process is not known, it has been suggested to curtail leukocyte extravasation, and/or halt unactivated platelet recruitment to sites of thrombus formation (24). Soluble P-Selectin is known to be a marker for increased procoagulant activity (25), and to serve as a selective antagonist of the classical complement-activation pathway (23).

The Quantikine® Mouse P-Selectin/CD62P Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse P-Selectin levels in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant mouse P-Selectin and antibodies raised against the recombinant factor. Results obtained for natural mouse P-Selectin 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 of natural mouse P-Selectin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse P-Selectin has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any P-Selectin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse P-Selectin 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 P-Selectin 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.
- It is recommended that the samples be pipetted within 10 minutes.
- 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 P-Selectin Microplate	892178	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse P-Selectin.	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 P-Selectin Conjugate	892179	12 mL of a polyclonal antibody specific for mouse P-Selectin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse P-Selectin Standard	892180	Recombinant mouse P-Selectin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse P-Selectin Control	892181	Recombinant mouse P-Selectin 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 RD5-26 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.

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 - Collect plasma on ice using EDTA as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the separated 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: *Heparin and citrate plasma have not been validated for use in this assay.*

P-Selectin is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of P-Selectin, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommmended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

SAMPLE PREPARATION

Use polypropylene tubes.

Serum and plasma samples generally require at least a 50-fold dilution prior to assay. A suggested 50-fold dilution is 10 µL of sample + 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 P-Selectin Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

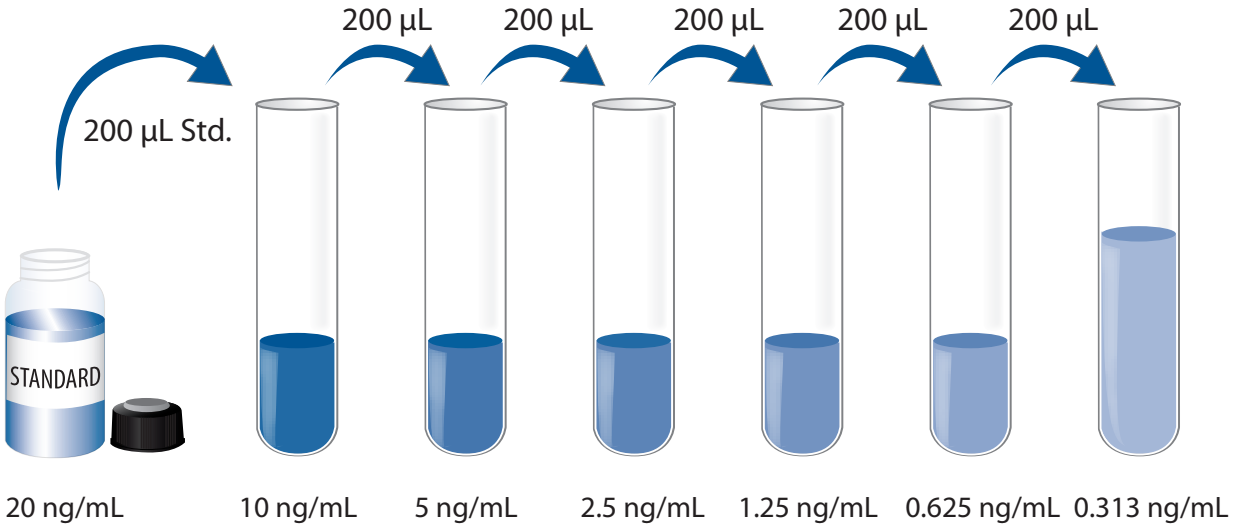
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 P-Selectin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse P-Selectin Standard with Calibrator Diluent RD5-26 (diluted 1:4). Do not substitute other diluents. This reconstitution produces a stock solution of 20 ng/mL. Mix the standard to ensure complete reconstitution and 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 P-Selectin Standard (20 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 standards, control, and samples be assayed in duplicate.

1. Prepare reagents, standard dilutions, 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. 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 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 Mouse P-Selectin 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

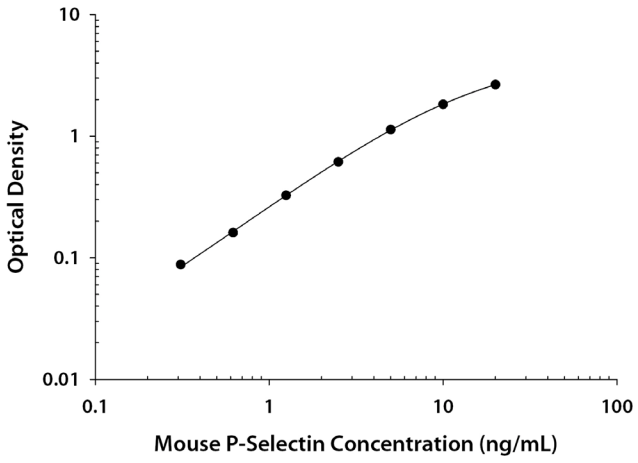
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 P-Selectin 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	0.008 0.008	0.008	—
0.313	0.091 0.102	0.096	0.088
0.625	0.167 0.171	0.169	0.161
1.25	0.327 0.342	0.334	0.326
2.5	0.605 0.639	0.622	0.614
5	1.102 1.178	1.140	1.132
10	1.749 1.919	1.834	1.826
20	2.620 2.697	2.658	2.650

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. 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	32	36	30
Mean (ng/mL)	1.27	2.92	12.9	1.27	2.90	12.6
Standard deviation	0.10	0.23	1.13	0.08	0.24	1.13
CV (%)	7.9	7.9	8.8	6.3	8.3	9.0

RECOVERY

The recovery of mouse P-Selectin spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernate (n=4)	100	89-110%
Serum* (n=10)	98	81-115%
EDTA plasma* (n=6)	94	80-112%

*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 mouse P-Selectin were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=5)	Serum* (n=6)	EDTA plasma* (n=6)
1:2	Average % of Expected	101	99	102
	Range (%)	97-106	90-107	97-106
1:4	Average % of Expected	97	94	104
	Range (%)	94-103	89-103	98-109
1:8	Average % of Expected	99	98	107
	Range (%)	97-104	91-107	104-112
1:16	Average % of Expected	95	96	107
	Range (%)	90-99	87-102	90-117

*Samples were diluted prior to assay.

SENSITIVITY

Four assays were evaluated and the minimum detectable dose (MDD) of mouse P-Selectin ranged from 0.007-0.018 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.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse P-Selectin/Fc chimera produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse P-Selectin in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)
Serum (n=20)	186	151-216
Platelet-poor EDTA plasma (n=11)	100	74-140

Cell Culture Supernates:
Lungs from two mice were cultured for 5 days in 40 mL of RPMI supplemented with 10% fetal bovine serum and stimulated with 10 µg/mL ConA. An aliquot of the cell culture supernate was removed, assayed for mouse P-Selectin, and measured 18.6 ng/mL.

Hearts from two mice were cultured for 6 days in 40 mL of RPMI supplemented with 10% fetal bovine serum. An aliquot of the cell culture supernate was removed, assayed for mouse P-Selectin, and measured 0.69 ng/mL.

Mouse splenocytes (1 x 10^6 cells/mL) were cultured for 4 days in RPMI supplemented with 10% fetal bovine serum, 50 µM β-mercaptoethanol, and 10 ng/mL of recombinant human IL-2. An aliquot of the cell culture supernate was removed, assayed for mouse P-Selectin, and measured 0.85 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse P-Selectin.

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 mouse P-Selectin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

E-Selectin
ICAM-1
ICAM-2
L-Selectin
VCAM-1

Recombinant human P-Selectin cross-reacts approximately 1.7% in this assay.

Normal rat serum and plasma samples read approximately 0.6 ng/mL. Porcine serum samples were also evaluated and measured less than the lowest standard, 0.31 ng/mL.

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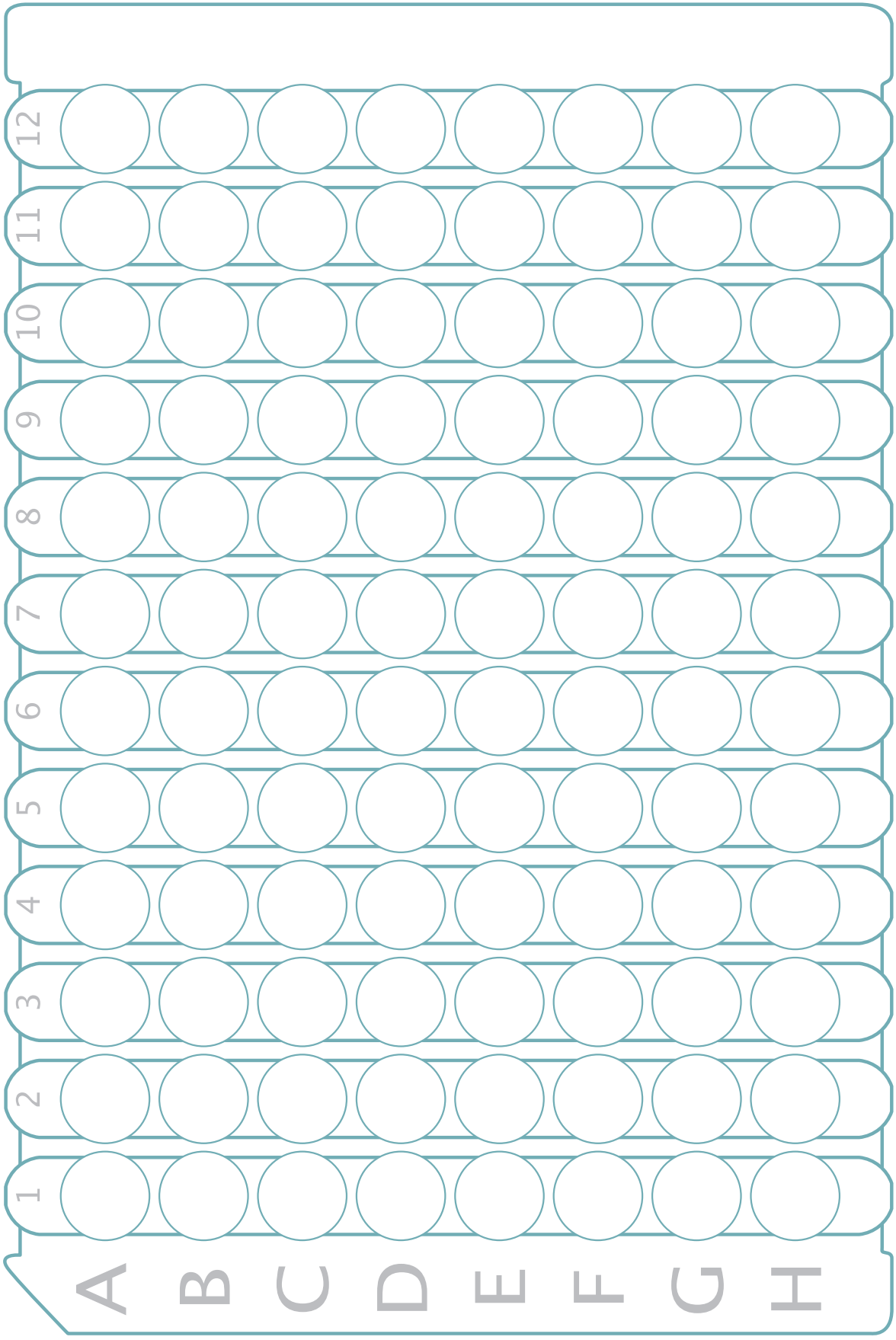
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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