Quantikine™ ELISA

Human SP-D Immunoassay

Catalog Number DSFPD0

For the quantitative determination of human Surfactant Protein D (SP-D) 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

SP-D (surfactant protein-D; also known as SFTPD and PSP-D) is a 43 kDa member of the collectin family of innate immune modulators (1-7). Its principal components consist of a collagen-like region and a C-terminal carbohydrate recognition domain (CRD), a structure that places it in a subset of pattern recognition proteins termed defense collagens (1-7). Mature human SP-D shares 75% and 78% amino acid (aa) sequence identity with mouse and porcine SP-D, respectively. It is constitutively secreted by alveolar lining cells and epithelium associated with tubular structures and induced in cardiac smooth muscle and endothelial cells (2, 8-10). Human SP-D is found in serum, plasma, broncho-alveolar lavage (BAL) fluid, and amniotic fluid (1, 2, 8). Injury due to lung conditions such as idiopathic pulmonary fibrosis, interstitial pneumonia, pulmonary alveolar proteinosis, severe acute respiratory syndrome (SARS), and chronic obstructive pulmonary disease (COPD) are associated with increased circulating SP-D (7, 8, 11, 12). In contrast, BAL fluid may show decreased SP-D in cystic fibrosis and SARS patients and chronic smokers, correlating with impaired host defense (3, 7, 13). Diurnal variation in plasma SP-D has been reported with a peak at 10:00 a.m. and a valley at 10:00 p.m. (14).

SP-D forms a glycosylated, disulfide-linked 150 kDa trimer with an α-helical coiled-coil structure and a "head" of three symmetrical CRDs (1, 2, 15, 16). Each CRD recognizes the hydroxides of one monosaccharide (2, 17). Trimerization allows for the discrimination of monosaccharide patterns specific to microbial pathogens (15, 17). Typically, SP-D forms a higher-order 620 kDa, X-shaped dodecamer through disulfide bonds associated with the N-terminus (1-4, 16). This allows for even finer discrimination of self vs. non-self carbohydrate patterns and facilitates binding and phagocytosis of microbes expressing complex antigens (3, 7, 17, 18). A human polymorphism, Met11Thr, interferes with the formation of oligomers, potentially affecting the ability of affected individuals to interact with microorganisms (3, 18-21). In addition, the cysteines involved in dodecamer formation can be S-nitrosylated by reactive nitrogen-oxygen intermediates, blocking dodecamer formation and aggregation, and inducing macrophage chemoattraction (22, 23). This potentially upregulates the inflammatory role of SP-D (22, 23).

SP-D binds both secreted and transmembrane proteins that transduce its function. It binds human neutrophil defensins, modulating influenza anti-viral defense (24). It binds MD-2/LY96, a secreted protein that cooperates with Toll-like receptors (TLRs) in the response of macrophages to bacterial lipopolysaccharides (LPS) or cell wall components (25). It also binds macrophage CD14 and TLRs directly, blocking binding of LPS and down-regulating TNF-α secretion (2-4, 6, 26, 27). SP-D binding of both SIRPα and the calreticulin/CD91 complex on macrophages allows for a graded response to environmental challenge via the following mechanism: when the ratio of antigen/pathogen to available CRDs is low, antigen can be bound without occupying all available CRDs. The free CRDs will bind to SIRPα, generating a signal that downmodulates the inflammatory response. When virtually all CRDs are occupied by ligand, however, free CRDs are not available for SIRPα binding. Instead, the dodecamer is thought to rearrange, exposing the N-termini of all four linked trimers which bind to the calreticulin/CD91 complex and initiate inflammation (28, 29). Thus, SP-D provides a mechanism for the clearance of small antigenic insults without the need for a damaging inflammatory response (3, 5, 6).

The Quantikine™ Human SP-D Immunoassay is a 5.5 hour solid-phase ELISA designed to measure human SP-D in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human SP-D and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human SP-D 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 for natural human SP-D.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human SP-D has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any SP-D present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human SP-D is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of SP-D bound in the initial step. The color development is stopped and the intensity of the color is measured.

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 these from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate 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.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate 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
Human SP-D Microplate	893381	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human SP-D.	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.*
Human SP-D Standard	893383	Recombinant human SP-D in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and freeze at ≤ -20 °C in a manual defrost freezer for up to 1 month.* Avoid repeated freeze-thaw cycles.
Human SP-D Conjugate	893382	21 mL of a monoclonal antibody specific for human SP-D conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-57	895207	11 mL of a buffer with blue dye and preservative.	
Calibrator Diluent RD5-54	895598	21 mL of a buffered protein base with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD6-27	895339	21 mL of a buffered animal serum with preservatives. *For serum and plasma samples.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. *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	895032	6 mL of 2 N sulfuric 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

PRECAUTIONS

Calibrator Diluent RD6-27 contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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 SDS 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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 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 15 minutes at 1000 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.*

REAGENT PREPARATION

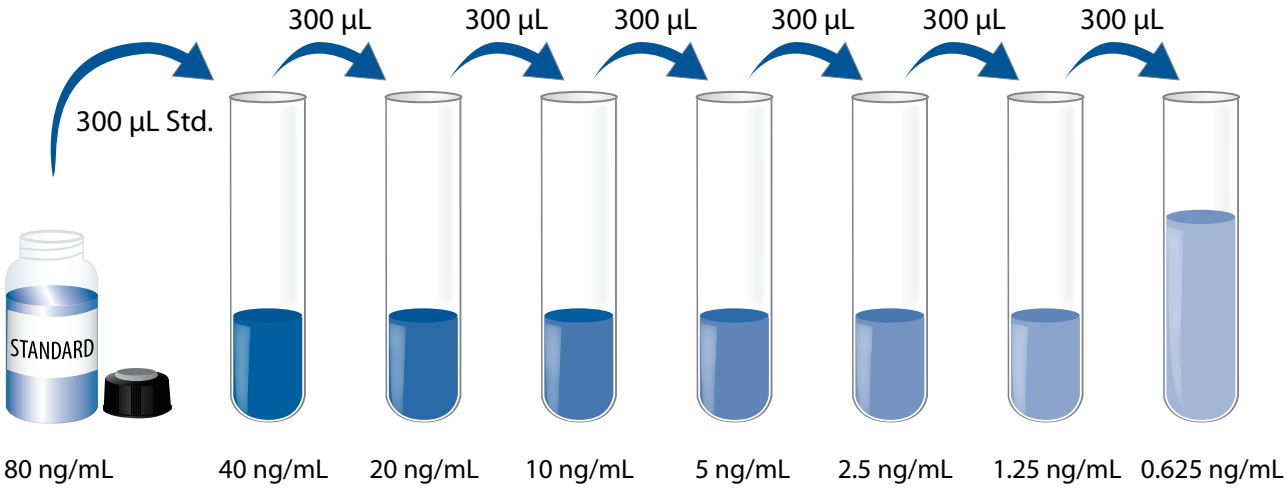
Bring all reagents to room temperature before use.

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 480 of 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. 200 µL of the resultant mixture is required per well.

Human SP-D Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human SP-D Standard with deionized or distilled water. This reconstitution produces a stock solution of 80 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene test tubes. Pipette 300 µL of Calibrator Diluent RD5-54 (*for cell culture supernate samples*) or Calibrator Diluent RD6-27 (*for serum and plasma samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 40 ng/mL standard serves as the high standard. The appropriate calibrator diluent 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 and samples be assayed in duplicate.

1. Prepare all reagents, working standards, 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 100 µL of Assay Diluent RD1-57 to each well.
4. Add 50 µL of standard or sample per well. Cover with the adhesive strip provided. Incubate for 3 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 200 µL of Human SP-D 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 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the 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.

CALCULATION OF RESULTS

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

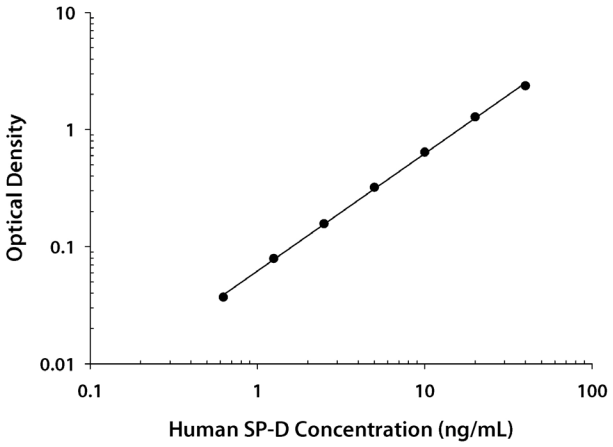
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human SP-D concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

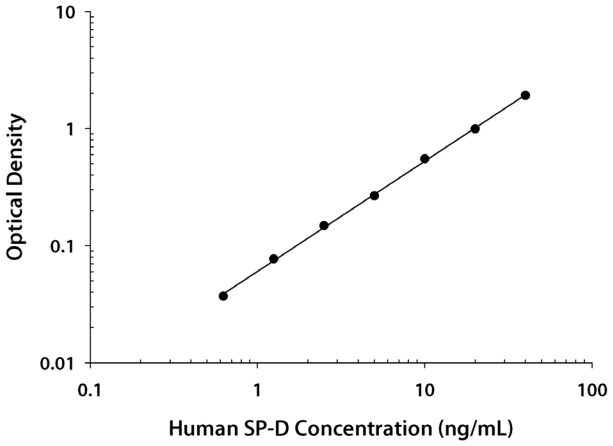
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.019 0.020	0.020	—
0.625	0.057 0.057	0.057	0.037
1.25	0.096 0.102	0.099	0.079
2.5	0.170 0.184	0.177	0.157
5.0	0.325 0.356	0.341	0.321
10	0.651 0.670	0.661	0.641
20	1.214 1.389	1.302	1.282
40	2.364 2.407	2.386	2.366

SERUM/PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.020 0.020	0.020	—
0.625	0.057 0.057	0.057	0.037
1.25	0.096 0.097	0.097	0.077
2.5	0.165 0.170	0.168	0.148
5.0	0.285 0.287	0.286	0.266
10	0.571 0.571	0.571	0.551
20	1.007 1.016	1.012	0.992
40	1.910 1.956	1.933	1.913

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. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	6.07	11.7	23.4	5.63	11.2	22.8
Standard deviation	0.495	0.588	1.46	0.495	0.999	1.78
CV (%)	8.2	5.0	6.2	8.8	8.9	7.8

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	5.99	11.3	24.3	6.63	13.1	26.9
Standard deviation	0.371	0.921	1.63	0.617	1.14	2.50
CV (%)	6.2	8.2	6.7	9.3	8.7	9.3

RECOVERY

The recovery of human SP-D spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range (%)
Cell culture media (n=4)	102	95-109
Serum (n=4)	106	89-118
EDTA plasma (n=4)	110	99-120
Heparin plasma (n=4)	104	85-118

SENSITIVITY

Eighty assays were evaluated and the minimum detectable dose (MDD) of human SP-D ranged from 0.02-0.37 ng/mL. The mean MDD was 0.11 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 human SP-D were serially diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	104	101	106	103
	Range (%)	100-112	95-104	102-110	97-111
1:4	Average % of Expected	104	98	103	101
	Range (%)	96-120	94-103	98-105	89-117
1:8	Average % of Expected	103	98	109	103
	Range (%)	91-114	88-105	93-116	93-114
1:16	Average % of Expected	98	90	106	97
	Range (%)	89-102	83-95	95-114	83-120

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human SP-D in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=36)	7.08	0.935-18.9	4.73
EDTA plasma (n=36)	6.83	0.955-23.0	4.84
Heparin plasma (n=36)	6.89	0.945-20.4	4.57

Cell Culture Supernates - Human peripheral blood leukocytes were cultured in DMEM supplemented with 5% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for human SP-D. All samples measured below the lowest standard, 0.625 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human SP-D.

The factors listed below were prepared in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors were assayed for interference in a mid-range recombinant human SP-D control. No significant cross-reactivity or interference was observed.

Recombinant human (prepared at 50 ng/mL):

4-1BB Ligand
APRIL
BCMA
CD30 Ligand
EDA
EDA-A2
Fas Ligand
GITR Ligand
LIGHT
Lymphotactin α1/β2
Lymphotactin α2/β1
OX40 Ligand
TACI
TNF-α
TWEAK

Recombinant human (prepared at 400 ng/mL):

C1q
C1qTNF9
Calreticulin
LRP-1 Cluster II
LRP-1 Cluster IV
MD-2
SIRPα/CD172a
TLR1
TLR2
TLR3
TLR4/MD-2 Complex

Recombinant human (prepared at 5000 ng/mL):

CD14

Recombinant mouse (prepared at 50 ng/mL):

4-1BB Ligand
BAFF
BCMA
CD27 Ligand
CD30 Ligand
Fas Ligand
LIGHT
OX40 Ligand
TACI
TNF-α
TWEAK

Recombinant mouse (prepared at 400 ng/mL):

TLR1
TLR2
TLR3

Other recombinants (prepared at 50 ng/mL):

bovine TNF-α
canine TNF-α
cotton rat TNF-α
equine TNF-α
feline TNF-α
porcine TNF-α
rat TNF-α
rhesus macaque TNF-α

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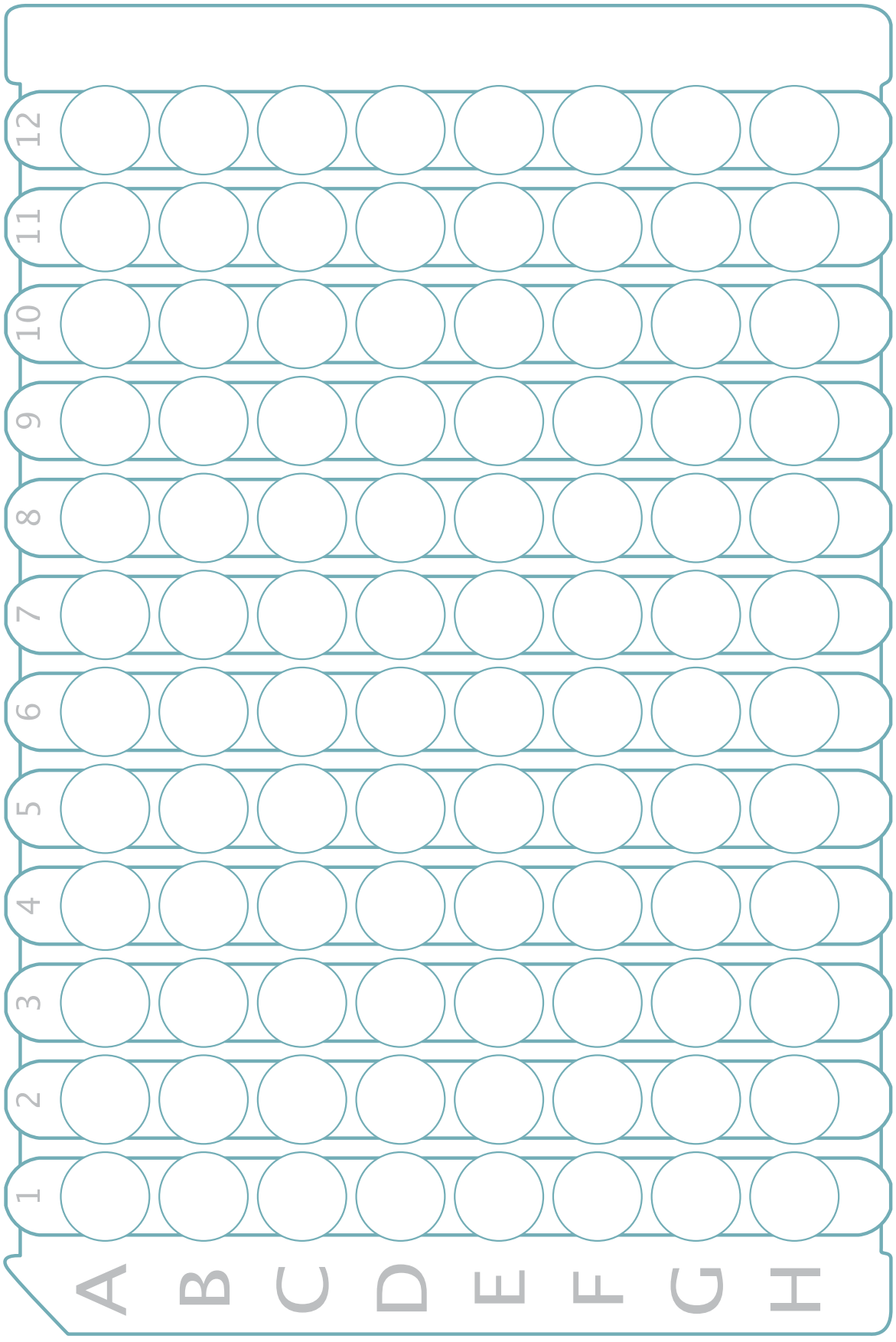
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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