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R&D SYSTEMS

Quantikine™ QuicKit™ ELISA

Human SP-D Immunoassay

Catalog Number QK1920
SK1920
PK1920

For the quantitative determination of human Surfactant Protein D (SP-D) concentrations in cell culture supernates, serum, plasma, and bronchoalveolar fluid (BALF).

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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Manufactured and Distributed by:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475 612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

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, bronchoalveolar fluid (BALF), 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) is associated with increased circulating SP-D (7, 8, 11, 12). In contrast, BALF 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 the 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 challenges 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). SP-D provides a mechanism for the clearance of small antigenic insults without the need for a damaging inflammatory response (3, 5, 6).

INTRODUCTION *CONTINUED*

The Quantikine™ QuicKit™ Human SP-D Immunoassay is a one-step, 80-minute solid phase ELISA designed to measure human SP-D levels in cell culture supernates, serum, plasma, and BALF. It contains recombinant human SP-D and antibodies raised against the recombinant protein. Results obtained for naturally occurring human SP-D showed linear curves that were parallel to the standard curves obtained using the recombinant QuicKit standards. These results indicate that this kit can be used to determine relative mass values for natural SP-D.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An anti-tag antibody has been pre-coated onto a microplate. Standards and samples are pipetted into the wells followed by an antibody cocktail. The antibody cocktail consists of an affinity tag labeled monoclonal capture antibody and an enzyme-linked monoclonal detection antibody, specific for human SP-D. After washing away any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of SP-D bound. 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 from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the 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™ QuicKit™ 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.
- Ensure reagent addition to plate wells is uninterrupted.
- To ensure accurate results, proper adhesion of the plate sealer during the incubation step 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.

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.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # QK1920	CATALOG # SK1920	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
QuicKit™ Coated Microplate	899063	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with an anti-tag monoclonal antibody.	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	899575	2 vials	12 vials	Recombinant human SP-D in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for the reconstitution volume.*	Use a freshly reconstituted standard for each assay. Discard after use.
Human SP-D Capture Ab Concentrate	899573	1 vial	6 vials	Lyophilized tagged monoclonal antibody specific for human SP-D.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-57	895207	1 vial	6 vials	11 mL of a buffered protein base with preservatives.	
Human SP-D Detection Ab Concentrate	899574	1 vial	6 vials	400 µL of a monoclonal antibody specific for human SP-D conjugated to horseradish peroxidase with preservatives.	
Calibrator Diluent RD5I	895134	1 vial	6 vials	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
TMB ELISA Substrate	642736	1 vial	6 vials	12 mL of a TMB ELISA substrate.	
ELISA Stop Solution	642827	1 vial	6 vials	12 mL of an acid solution.	
Plate Sealers	N/A	4 strips	8 strips	Adhesive strips.	

* Provided this is within the expiration date of the kit.

QK1920 contains sufficient materials to run an ELISA on one 96 well plate.
SK1920 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, # PK1920). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak has enough reagents to assay 50 microplates (96 wells/plate). Although the package inserts are the same as those for the single kit inserts, there are minor differences related to the number of reagents and their container sizes.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
  Note: *Additional wash buffer is available for purchase* (R&D Systems®, # WA126).

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
QuicKit™ Coated Microplate	899063	50 plates
Human SP-D Standard*	899575	25 vials
Human SP-D Capture Ab Concentrate	899573	50 vials
Human SP-D Detection Ab Concentrate	899574	50 vials
Assay Diluent RD1-57	895207	50 vials
Calibrator Diluent RD5I	895134	50 vials
Wash Buffer Concentrate	895126	9 bottles
TMB ELISA Substrate	642736	50 vials
ELISA Stop Solution	642827	50 vials
Plate Sealers	N/A	100 sheets

**If additional standard vials are needed, contact Technical Service at techsupport@bio-techne.com*

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
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- **Polypropylene** test tubes for dilution of standards
- Human SP-D Controls (optional; Catalog # QC320)

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.*

BALF: Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell culture supernates and BALF samples require a 2-fold dilution due to matrix effect. A suggested 2-fold dilution is 75 µL of sample + 75 µL of Calibrator Diluent RD5I.

Serum and plasma samples require a 3-fold dilution due to matrix effect. A suggested 3-fold dilution is 50 µL of sample + 100 µL of Calibrator Diluent RD5I.

Multiple dilutions are recommended for unknown samples.

Avoid repeated freeze/thaw cycles, which may cause erroneous results.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

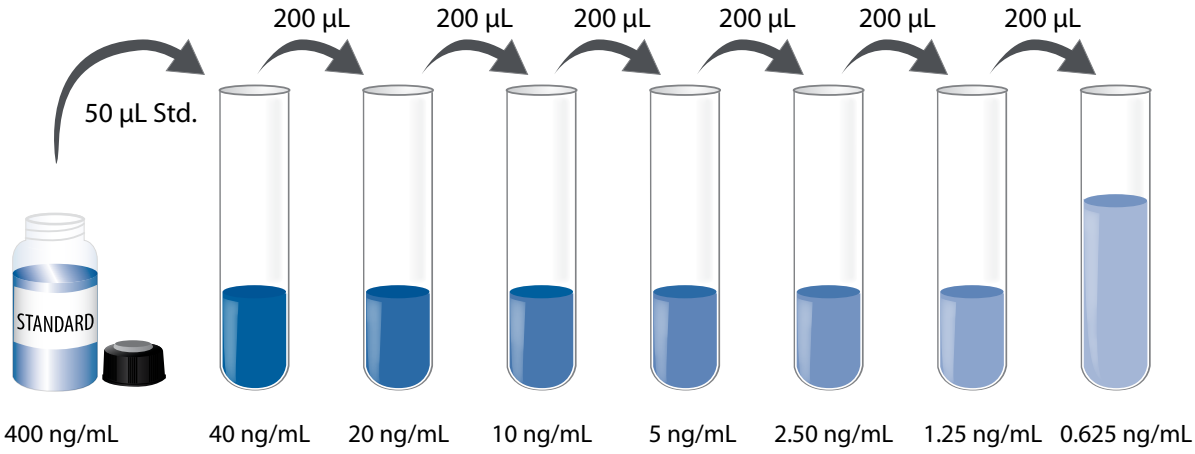
Human SP-D Capture Ab Concentrate - **Refer to the vial label for reconstitution volume.** Reconstitute the Human SP-D Capture Ab Concentrate with Assay Diluent RD1-57. This reconstitution produces a 20X Capture Antibody stock. Allow the capture antibody to sit for a minimum of 5 minutes with gentle agitation prior to diluting. Once reconstituted, the 20X capture antibody stock can be stored for 4 weeks at 2-8 °C.

Antibody Cocktail - Dilute the reconstituted Capture Ab stock and the Detection Ab Concentrate 20-fold in Assay Diluent RD1-57. For a full plate, add 300 µL of reconstituted Human SP-D Capture Ab stock and 300 µL of Human SP-D Detection Ab Concentrate to 5.4 mL of Assay Diluent RD1-57 to get 6 mL of Human SP-D Antibody Cocktail.

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 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Human SP-D Standard - **Refer to vial label for reconstitution volume.** Reconstitute the Human SP-D Standard with distilled or deionized water. This reconstitution produces a stock solution of 400 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD5I into the 40 ng/mL tube. Pipette 200 µL into the remaining tubes. 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. Calibrator Diluent RD5I 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, controls, and samples be assayed in duplicate.

1. Prepare all reagents, samples, and working standards 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 standard, control, or sample per well. A plate layout is provided to record standards and samples assayed.
4. Add 50 µL Antibody Cocktail to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12″ orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process twice for a total of three 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 TMB ELISA Substrate solution to each well. Incubate for 20 minutes at room temperature **on the benchtop. Protect from light.**
7. Add 100 µL of ELISA 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.
8. 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

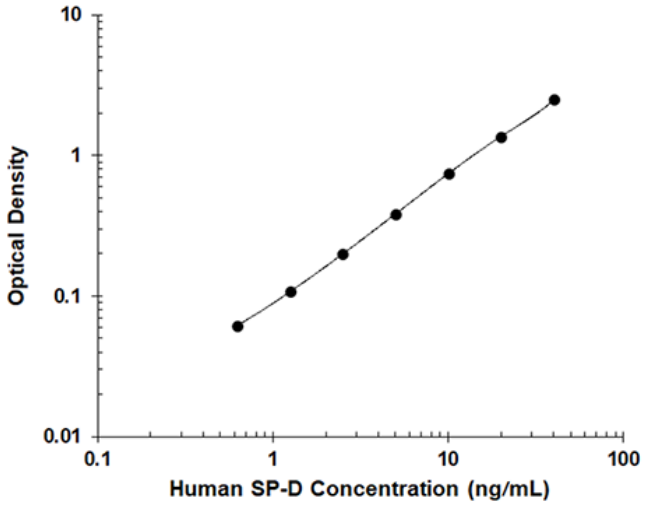
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human SP-D 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	0.012 0.012	0.012	—
0.625	0.059 0.063	0.061	0.049
1.25	0.105 0.110	0.108	0.096
2.50	0.197 0.202	0.200	0.188
5.0	0.378 0.389	0.384	0.372
10	0.741 0.747	0.744	0.732
20	1.368 1.368	1.368	1.356
40	2.500 2.539	2.520	2.508

PRECISION

Intra-Assay Precision (Precision within an assay)
Two samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Two samples of known concentration were tested in ten separate assays to assess inter-assay precision. Assays were performed by at least four technicians.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	1	2	1	2
n	20	20	10	10
Mean (ng/mL)	4.65	23.8	4.37	22.0
Standard deviation	0.210	1.27	0.277	1.62
CV (%)	4.5	5.3	6.3	7.4

RECOVERY

The recovery of human SP-D spiked to three different levels in samples throughout the range of the assay in cell culture media was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	110	106-114%
Serum (n=2)	90	77-100%
EDTA plasma (n=2)	90	81-99%
Heparin plasma (n=2)	86	79-95%
BALF (n=2)	96	93-99%

SENSITIVITY

Ten assays were evaluated and the minimum detectable dose (MDD) of human SP-D ranged from 0.020 - 0.163 ng/mL. The mean MDD was 0.064 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 spiked with high concentrations of human SP-D were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n= 2)	EDTA plasma (n= 2)	Heparin plasma (n= 2)	BALF (n= 2)
1:2	Average % of Expected	96	104	100	106	104
	Range (%)	93-99	96-112	94-105	101-112	102-106
1:4	Average % of Expected	96	107	103	113	101
	Range (%)	93-99	97-118	97-108	107-120	99-104
1:8	Average % of Expected	98	113	111	122	99
	Range (%)	98-99	101-126	106-117	113-130	97-100
1:16	Average % of Expected	98	115	115	123	104
	Range (%)	96-99	100-131	105-125	110-135	97-111

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma/BALF - 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 of Dectectable (ng/mL)	% Detectable	Range (ng/mL)
Serum (n=10)	9.68	90	ND-25.9
EDTA plasma (n=10)	8.12	90	ND-21.2
Heparin plasma (n=10)	8.82	90	ND-23.3
BALF (n=4)	5.20	75	ND-9.72

ND=Non-detectable

Cell Culture Supernates - A549 cells were grown in Kaighn's F12 + 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin sulfate until 80% confluent before treating with Nintedanib (Tocris™, Catalog # 7049). Cells were incubated with 5 µM Nintedanib for 48 hours; or incubated with 5 µM Nintedanib for 48 hours followed by 20 µM Nigericin sodium salt (Tocris, Catalog # 4312) for an additional 24 hours; or incubated with 5 µM Nintedanib for up to 72 hours before being treated with 20 µM Nigericin sodium salt or 500 ng/mL Ionomycin calcium salt (Tocris, Catalog # 1704) for an additional hour. After the incubation periods, cell supernatants were removed, diluted 2-fold, and assayed for levels of SP-D. All diluted samples measured less than the lowest human SP-D standard, 0.625 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human SP-D.

The factors listed below were prepared at 400 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 400 ng/mL in a low level recombinant human SP-D control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

DMBT-1
MFAP4
SP-A1

Recombinant mouse:

SP-D

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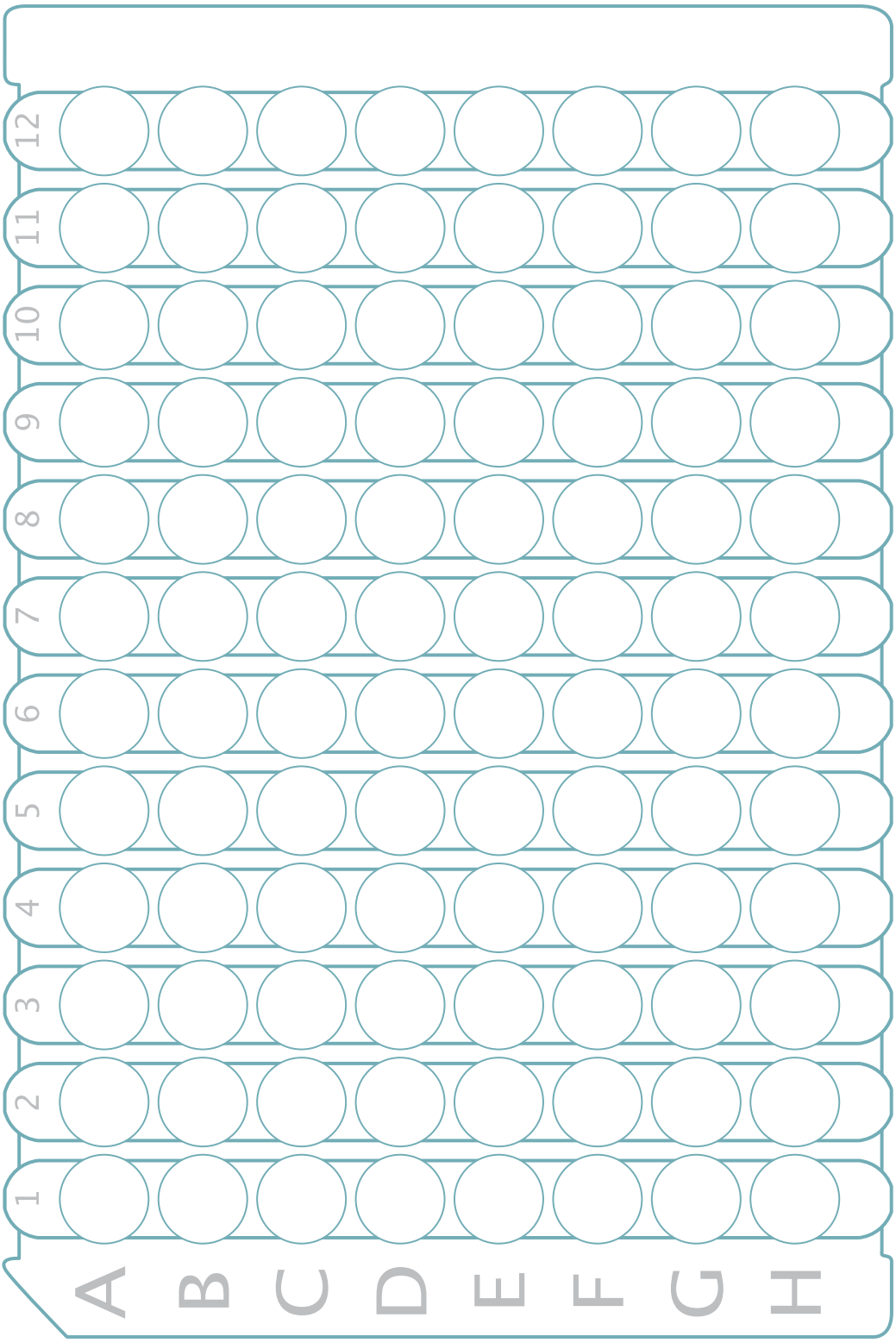
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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