Quantikine™ ELISA

Mouse SP-D Immunoassay

Catalog Number MSFPD0

For the quantitative determination of mouse Surfactant Protein-D (SP-D) concentrations in cell culture supernates, tissue lysates, 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

Surfactant Protein-D (SP-D), also known as SFTPD or PSP-D, is a 43 kDa member of the collectin family of innate immune modulators. 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 (1-3). Mature mouse SP-D shares 76% and 92% amino acid sequence identity with human and rat SP-D, respectively. It is constitutively secreted by alveolar lining cells and epithelium associated with tubular structures, and is inducible in cardiac smooth muscle and endothelial cells (4-6). SP-D is found in serum and plasma as well as broncho-alveolar lavage (BAL) fluid and amniotic fluid (4). 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 (4 ,7 ,8). In contrast, BAL fluid may show decreased SP-D in cystic fibrosis, SARS patients, and chronic smokers, correlating with impaired host defense (1, 2, 9). Diurnal variation in plasma SP-D has been reported, with a peak at 10 a.m. and a nadir at 10 p.m. (10).

SP-D forms a glycosylated, disulfide-linked 150 kDa trimer with an α-helical coiled-coil structure and a "head" of three symmetrical CRDs (11, 12). Each CRD recognizes the hydroxides of one monosaccharide (11, 13). Trimerization allows for the discrimination of monosaccharide patterns specific to microbial pathogens (11, 13). Typically, SP-D forms a higher-order 620 kDa, X-shaped dodecamer through disulfide bonds associated with the N-terminus (12). This allows for even finer discrimination of self *vs.* nonself carbohydrate patterns and facilitates binding and phagocytosis of microbes expressing complex antigens (13, 14). In humans, a polymorphism (Met11Thr) interferes with the formation of oligomers, potentially affecting the ability of affected individuals to respond to microorganisms (14-17). In addition, the cysteines involved in dodecamer formation can be S-nitrosylated by reactive nitrogen-oxygen intermediates, thereby blocking dodecamer formation and aggregation and inducing macrophage chemoattraction (18, 19). This potentially enhances the inflammatory role of SP-D (18, 19). SP-D can be cleaved and inactivated by MMP-9 mediated proteolysis (20).

SP-D functions through both secreted and transmembrane proteins. It binds human neutrophil defensins, modulating influenza antiviral defense (21). 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 (22). It also binds macrophage CD14 and TLRs directly, blocking binding of LPS and down-regulating TNF-α secretion (23, 24). SP-D binding of both SIRPα and the calreticulin/CD91 complex on macrophages (25, 26) 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 (25, 26). During high CRD ligand binding (low SIRPα binding), the dodecamer rearranges to expose N-termini that bind the calreticulin/CD91 complex, an event that initiates inflammation (1, 2). Thus, SP-D provides a mechanism for the clearance of small antigenic insults without the need for a damaging inflammatory response (1, 2). Also, direct and indirect binding of neutrophil defensins and macrophage CD14 and TLRs to SP-D can modulate response to viruses and bacterial lipopolysaccharides (23, 24).

INTRODUCTION *CONTINUED*

The Quantikine™ Mouse SP-D immunoassay is a 4.5 hour solid-phase ELISA designed to measure SP-D in mouse cell culture supernates, tissue lysates, serum, and plasma. It contains CHO cell-expressed recombinant mouse SP-D and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant mouse SP-D. Results obtained using natural mouse SP-D showed dose response curves that were parallel to the standard curves obtained using the Quantikine mouse kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse SP-D.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse SP-D has been pre-coated onto a microplate. Standards, control, 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 mouse 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. 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 SP-D 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 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.

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 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse SP-D Microplate	894709	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse 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.*
Mouse SP-D Conjugate	894710	12 mL of a monoclonal antibody specific for mouse SP-D conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse SP-D Standard	894711	Recombinant mouse SP-D in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse SP-D Control	894712	Recombinant mouse SP-D 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 RD1S	895137	11 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5-3	895436	21 mL of a 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
- 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples

OTHER SUPPLIES REQUIRED FOR TISSUE LYSATE SAMPLES

- Cell Lysis Buffer 2 (R&D Systems, Catalog # 895347)
- PBS

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.

Tissue Lysates - Tissue must be lysed prior to assay as directed in the Sample Values section.

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.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 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.*

SAMPLE PREPARATION

Serum and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-3.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

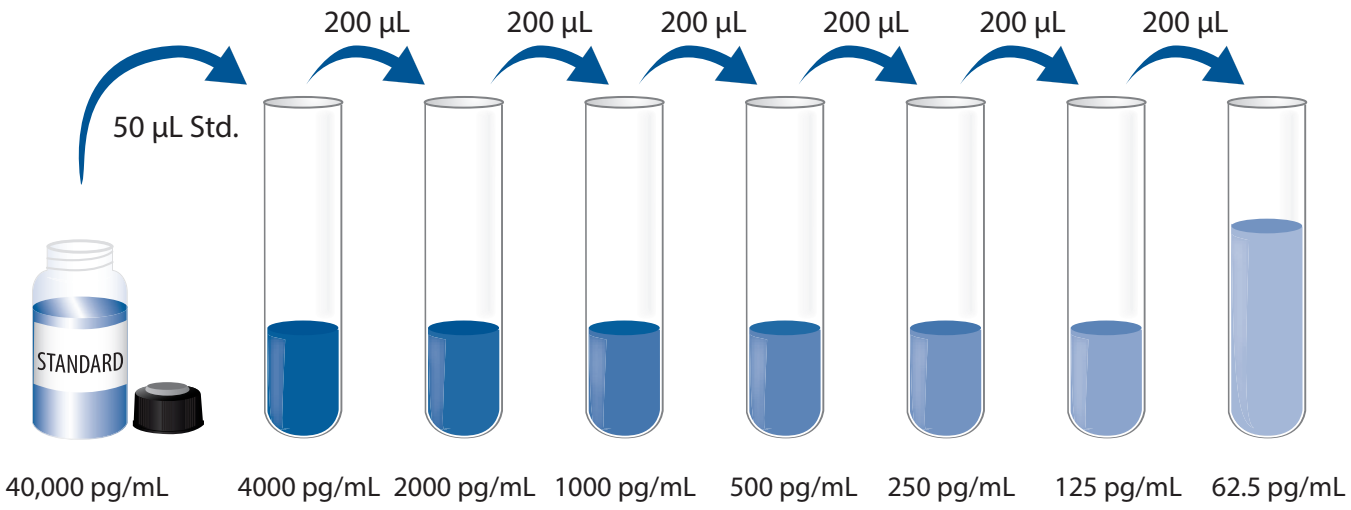
Mouse SP-D Control - Reconstitute the control with 1 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

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 480 mL 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. 100 µL of the resultant mixture is required per well.

Mouse SP-D Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse SP-D Standard with deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-3 into the 4000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-3 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 all reagents, standard dilutions, 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 RD1S 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 a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. 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 SP-D Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. 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

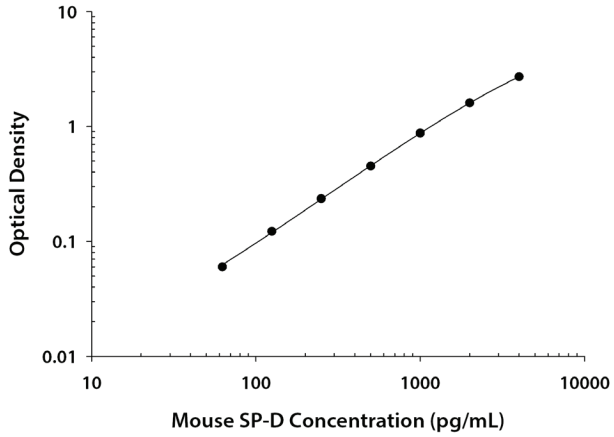
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 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.



(pg/mL)	O.D.	Average	Corrected
0	0.012 0.013	0.013	—
62.5	0.072 0.074	0.073	0.060
125	0.131 0.138	0.135	0.122
250	0.248 0.248	0.248	0.235
500	0.464 0.465	0.465	0.452
1000	0.885 0.888	0.887	0.874
2000	1.595 1.629	1.612	1.599
4000	2.698 2.738	2.718	2.705

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 kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	158	534	1270	169	531	1267
Standard deviation	5.75	13.8	43.0	14.0	34.4	120
CV (%)	3.6	2.6	3.4	8.3	6.5	9.5

RECOVERY

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

	Average % Recovery	Range
Cell culture media (n=4)	99	92-105%
Tissue lysates (n=4)	101	91-113%
Serum (n=4)	96	84-109%
EDTA plasma (n=4)	97	90-103%
Heparin plasma (n=4)	97	84-104%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse SP-D in each matrix were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Tissue lysates (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	98	98	106	107	107
	Range (%)	95-100	95-101	102-109	104-109	104-110
1:4	Average % of Expected	98	100	111	110	109
	Range (%)	95-102	96-103	105-114	106-114	103-112
1:8	Average % of Expected	98	101	114	111	111
	Range (%)	91-104	100-103	104-120	106-114	107-114
1:16	Average % of Expected	98	103	108	108	107
	Range (%)	95-99	99-106	100-117	103-113	102-112

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

SENSITIVITY

Twenty-nine assays were evaluated and the minimum detectable dose (MDD) of mouse SP-D ranged from 1.14-9.43 pg/mL. The mean MDD was 3.05 pg/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 CHO cell-expressed recombinant mouse SP-D produced at R&D Systems®.

SAMPLE VALUES

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

Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	4618	2232-9212	1969
EDTA plasma (n=6)	3889	2252-4938	964
Heparin plasma (n=6)	3075	1730-4626	1178

Cell Culture Supernates - Lungs from mice were rinsed with PBS then homogenized with a tissue homogenizer and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 1 µg/mL LPS for 3 days. Aliquots of the cell culture supernates were removed and assayed for mouse SP-D

Condition	Value (pg/mL)
Unstimulated	375
Stimulated	354

Tissue Lysates - Organs from mice were rinsed with PBS, cut into 1-2 mm pieces, and homogenized with a tissue homogenizer in PBS. An equal volume of Cell Lysis Buffer 2 was added and tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was then removed by centrifugation. Aliquots of the lysates were removed and assayed for mouse SP-D.

Tissue	Value (pg/mL)
Heart	82.3
Lung	73,620

SPECIFICITY

This assay recognizes natural and recombinant mouse SP-D.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range mouse SP-D control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:
CD14
TLR1
TLR2
TLR3
TLR4

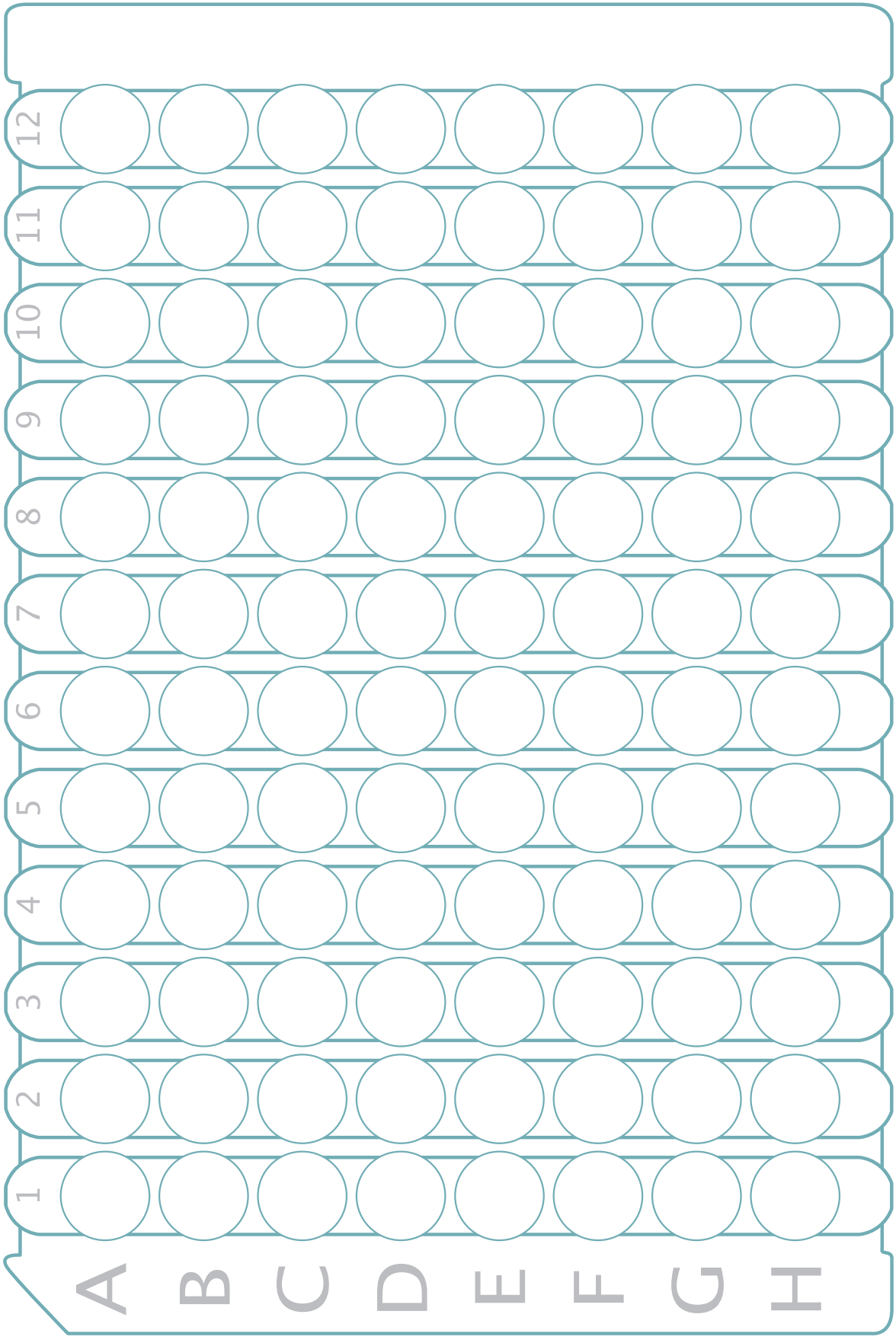
Recombinant human:
SP-D

REFERENCES

1. Pandit, H. *et al.* (2012) Front. Biosci. (Elite Ed.) **4**:651.
2. Jakel, A. *et al.* (2013) Front. Biosci. (Landmark Ed.) **18**:1129.
3. Motwani, M. *et al.* (1995) J. Immunol. **155**:5671.
4. Honda, Y. *et al.* (1995) Am. J. Respir. Crit. Care Med. **152**:1860.
5. Kim, J.K. *et al.* (2007) Am. J. Physiol. Lung Cell. Mol. Physiol. **292**:L879.
6. Snyder, G.D. *et al.* (2008) Am. J. Physiol. Heart Circ. Physiol. **294**:H2053.
7. Sin, D.D. *et al.* (2008) Ther. Adv. Respir. Dis. **2**:65.
8. Wu, Y.P. *et al.* (2009) Scand. J. Immunol. **69**:508.
9. Cooley, J. *et al.* (2008) J. Leukoc. Biol. **83**:946.
10. Hoegh, S.V. *et al.* (2009) Immunobiology **215**:314.
11. Hakansson, K. *et al.* (1999) Structure **7**:225.
12. Crouch, E. *et al.* (1994) J. Biol. Chem. **269**:17311.
13. Crouch, E.C. *et al.* (2006) Am. J. Respir. Cell Mol. Biol. **35**:84.
14. Leth-Larsen, R. *et al.* (2005) J. Immunol. **174**:1532.
15. Sorensen, G.L. *et al.* (2009) Mol. Immunol. **46**:3060.
16. Brandt, E.B. *et al.* (2008) J. Allergy Clin. Immunol. **121**:1140.
17. Haczku, A. (2008) J. Allergy Clin. Immunol. **122**:861.
18. Guo, C.J. *et al.* (2008) PLoS Biol. **6**:e266.
19. Matalon, S. *et al.* (2009) FASEB J. **23**:1415.
20. Bratcher, P.E. *et al.* (2012) PLoS ONE **7**:e41881.
21. Hartshorn, K.L. *et al.* (2006) J. Immunol. **176**:6962.
22. Nie, X. *et al.* (2008) Biochemistry **47**:12878.
23. Ohya, M. *et al.* (2006) Biochemistry **45**:8657.
24. Yamazoe, M. *et al.* (2008) J. Biol. Chem. **283**:35878.
25. Janssen, W.J. *et al.* (2008) Am. J. Respir. Crit. Care Med. **178**:158.
26. Gardai, S.J. *et al.* (2003) Cell **115**:13.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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