Parameter™

Substance P Assay

Catalog Number KGE007
SKGE007
PKGE007

For the quantitative determination of Substance P in cell culture supernates, serum, plasma, saliva, and urine.

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

Substance P (SP, Neurokinin-1) is member of the tachykinin peptide family. It was first discovered in 1931 as a hypotensive and spasmogenic factor in extracts of equine brain and intestine (1, 2). The isolation, characterization, and sequencing of Substance P took place in the early 1970s (3-5). Human Substance P is processed from preprotachykinin α, β, γ, and δ isoforms that also generate other tachykinin family members Neuropeptide K, Neurokinin A, Neurokinin B, and Neuropeptide γ (6, 7). Signal sequence and other processing results in the active 1348 Da, 11 amino acid (aa) peptide: RPKPQQFFGLM. Studies of synthetic peptides have shown that Phe at position 7 and amidation at the C-terminus are crucial for function, and that a basic or neutral aa at position 5 and an aromatic aa at position 8 are selective for the NK1 receptor (8). The sequence of Substance P is identical across mammalian species. Alligator and avian species have a single conservative amino acid substitution at position 3. Homologs such as physalaemin are present in the skin of amphibians as defense proteins. Non-neuronal Substance P is present in the endocrine cells of the intestinal mucosa and may be the source of Substance P found in the blood. Tumor epithelial cells may express and store Substance P and other tachykinins. Neuronal tissue is the main source of Substance P, both in the central nervous system (many areas of the brain and spinal cord) and in primary sensory neurons (C-type fibers) in the gut, respiratory tract, blood vessels, urinary system, skin, and lymphoid organs (6).

Substance P binds primarily to the neurokinin 1 receptor (NK1 receptor, Substance P receptor, tachykinin receptor 1), and with low affinity to the NK2 and NK3 receptors (6, 7). The NK1 receptor is a 407 amino acid, seven-transmembrane G-protein-coupled receptor that activates a phosphatidylinositol-calcium second messenger system. The NK-1 receptor has potential N-glycosylation sites at the N-terminus, an intermolecular disulfide bond (C105 to C180), and an S-palmitoylation site at C322. NK2 and NK3 receptors have similar structure but limited sequence identity.

Substance P is secreted from primary sensory afferent neurons after stimulation through protease-activated receptor 2 (PAR2). Calcitonin gene-related peptide (CGRP) is often co-secreted and induces inflammation in concert with SP (9, 10). SP is traditionally considered a neurotransmitter that engages NK1 receptors on neurons in the dorsal horn, transmitting pain signals through the central nervous system (9). However, when genes for either preprotachykinin A or NK1 receptor are disrupted, mice show normal pain thresholds but blunted responses to higher intensity stimuli (11, 12). Response to Substance P depends on the target cell and includes inducing hypotension, tachycardia, release of inflammatory mediators, and other responses. Direct antimicrobial effects of Substance P have also been shown (13).

The Parameter Substance P Immunoassay is a 3.5 hour competitive enzyme immunoassay designed to measure Substance P in cell culture supernates, serum, plasma, saliva, and urine. It contains a synthetically derived human Substance P peptide and has been shown to accurately quantitate this peptide. Results obtained using samples containing natural Substance P showed linear curves that were parallel to the standard curves obtained using the Parameter kit standards. These results indicate that this kit can be used to determine relative mass values for natural Substance P.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which Substance P present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled Substance P for sites on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of Substance P in the sample.

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 this assay, the possibility of interference cannot be excluded.
- Neurokinins (NKs) are a family of highly conserved structurally related neuropeptides. Substance P and Neurokinin A are both encoded by the preprotachykinin A gene. The antibody utilized in this assay also binds to Neurokinin A. See the Specificity section on page 10.
- **Samples containing mouse or rat IgG may interfere with this assay.**

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.
- Pre-rinse the pipette tip when pipetting.
- Pipette standards and samples to the bottom of the wells. Add all other reagents to the side of the wells to avoid contamination.
- 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. Gently tap the side of the plate to ensure thorough mixing.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at ≤ -20 °C in a manual defrost freezer. Do not use past kit expiration date.

PART	PART #	CATALOG # KGE007	CATALOG # SKGE007	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Goat Anti-mouse Microplate	892575	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse polyclonal antibody.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
Substance P Conjugate	893078	1 vial	6 vials	6 mL/vial of Substance P conjugated to horseradish peroxidase with red dye and preservatives.	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Substance P Standard	893080	1 vial	6 vials	50 ng/vial of Substance P in buffer with preservatives; lyophilized.	
Primary Antibody Solution	893079	1 vial	6 vials	6 mL/vial of a mouse monoclonal antibody for Substance P in buffer with blue dye and preservatives.	
Calibrator Diluent RD5-45	895905	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	4 adhesive strips.	

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

KGE007 contains sufficient materials to run an ELISA on one 96 well plate.
SKGE007 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PKGE007). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 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.
- Aprotinin (Sigma, Catalog # A1153).
- Collection device for saliva samples that has no protein binding or filtering capabilities such as a Salivette® or equivalent.
- Test tubes for dilution of standards and samples.
- Substance P Controls (optional; available from R&D Systems).

PRECAUTIONS

Substance P is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

Care should be taken when handling the Standard because of the known and unknown effects of Substance P.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please 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.

Samples containing mouse or rat IgG may interfere with this assay.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and add aprotinin within 5 minutes of collection. 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 samples on ice using heparin as an anticoagulant. Add aprotinin within 5 minutes of collection. 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.

Aprotinin should be added to serum and plasma samples within 5 minutes of collection to a final concentration of 0.014 TIU/mL.

Note: *EDTA is not recommended for use in this assay.*
Citrate plasma has not been validated for use in this assay.

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell culture supernates, serum, plasma, and urine samples require a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-45.

Saliva samples require a 20-fold dilution. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-45.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

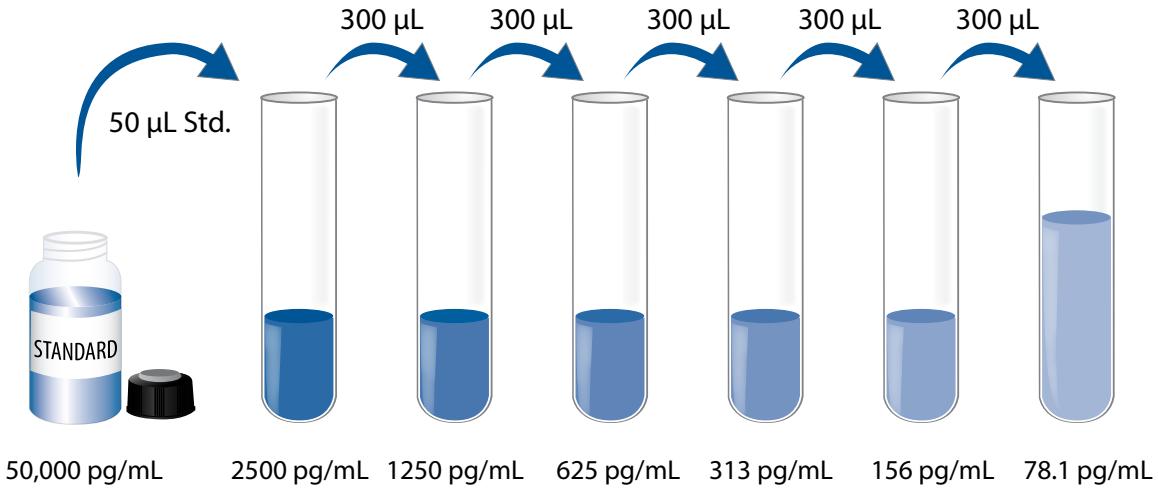
Note: *High concentrations of Substance P are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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

Substance P Standard - Reconstitute the Substance P Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/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.

Pipette 950 µL of Calibrator Diluent RD5-45 into the 2500 pg/mL tube. Pipette 300 µL of Calibrator Diluent RD5-45 into the remaining tubes. Use the 50,000 pg/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 2500 pg/mL standard serves as the high standard and the Calibrator Diluent RD5-45 serves as the zero standard (B_0) (0 pg/mL). **Use diluted standards within 60 minutes of preparation.**



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *High concentrations of Substance P are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 Calibrator Diluent RD5-45 to the non-specific binding (NSB) wells.
4. Add 50 µL of Calibrator Diluent RD5-45 to the zero standard (B_0) wells.
5. Add 50 µL of Standard, control, or sample* to the remaining wells. A plate layout is provided to record the standards and samples assayed.
6. Add 50 µL of Primary Antibody Solution to each well **(excluding the NSB wells)**. All wells except the NSB wells will now be blue in color.
7. Add 50 µL of the Substance P Conjugate to each well. All wells except the NSB wells will now be violet in color. Cover with the adhesive strip provided.
8. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
9. 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.
10. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
11. 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.
12. 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 require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

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

Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. Do not include the B_0 in the standard curve.

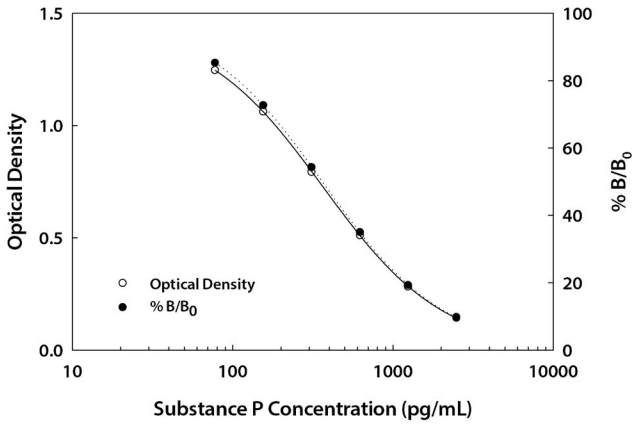
If desired, % B/B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

Calculate the concentration of Substance P corresponding to the mean absorbance from the standard curve.

Since 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	% B/B_0
NSB	0.012 0.012	0.012	—	—
0 (B_0)	1.477 1.476	1.475	1.463	—
78.1	1.269 1.245	1.257	1.245	85.2
156	1.089 1.055	1.072	1.060	72.6
312	0.809 0.799	0.804	0.792	54.2
625	0.526 0.518	0.522	0.510	34.9
1250	0.297 0.288	0.293	0.281	19.2
2500	0.156 0.152	0.154	0.142	9.7

PRECISION

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

Inter-assay Precision (Precision between assays)
Four 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	4	1	2	3	4
n	20	20	20	20	20	20	20	20
Mean (pg/mL)	146	258	616	1973	140	262	684	1923
Standard deviation	12.3	15.1	21.5	142	18.1	26.8	103	180
CV (%)	8.4	5.9	3.5	7.2	12.9	10.2	15.1	9.4

RECOVERY

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

Sample Type	Average % Recovery	Range %
Cell culture media (n=4)	94	82-110%
Serum (n=4)	101	85-117%
Heparin plasma (n=4)	100	85-116%
Urine (n=4)	89	76-107%
Saliva (n=4)	102	90-110%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Substance P were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture media (n=4)	Serum (n=4)	Heparin plasma (n=4)	Saliva (n=4)	Urine (n=4)
1:2	Average % of Expected	90	100	99	103	96
	Range (%)	85-93	95-105	94-103	101-103	93-98
1:4	Average % of Expected	85	107	106	94	96
	Range (%)	81-90	97-111	100-112	85-100	92-99
1:8	Average % of Expected	99	121	120	111	104
	Range (%)	91-103	112-129	112-125	90-125	90-117

SENSITIVITY

Twenty-one assays were evaluated and the minimum detectable dose (MDD) of Substance P ranged from 16.8-43.8 pg/mL. The mean MDD was 31.5 pg/mL.

The MDD was determined by subtracting two standard deviations from the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of Substance P in this assay. No medical histories were available for the donors used in this study. Samples were treated and diluted prior to assay as directed in Sample Preparation section.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=22)	628	100	402-1576
Heparin plasma (n=23)	508	100	322-1286
Urine (n=10)	214	80	ND-371
Saliva (n=11)	161	9	ND-161

ND=Non-detectable

Cell Culture Supernates - Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 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. Aliquots of the cell culture supernate were removed and assayed for levels of Substance P. No detectable levels were observed.

SPECIFICITY

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-45 and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range Substance P control were assayed for interference. No interference was observed. Cross-reactivity is listed below.

Compound	% Cross-reactivity
Hemokinin-1 (Sourced from Tocris)	100%
Substance P (4-11)*	88.4%
Substance P (2-11)*	81.2%
Neurokinin A*	71.4%
Physalaemin*	68.1%
Substance P (8-11)*	1.4%
Neurokinin B*	1.1%
Substance P (1-7)*	< 0.1%
*Factors were sourced from Sigma/Aldrich	

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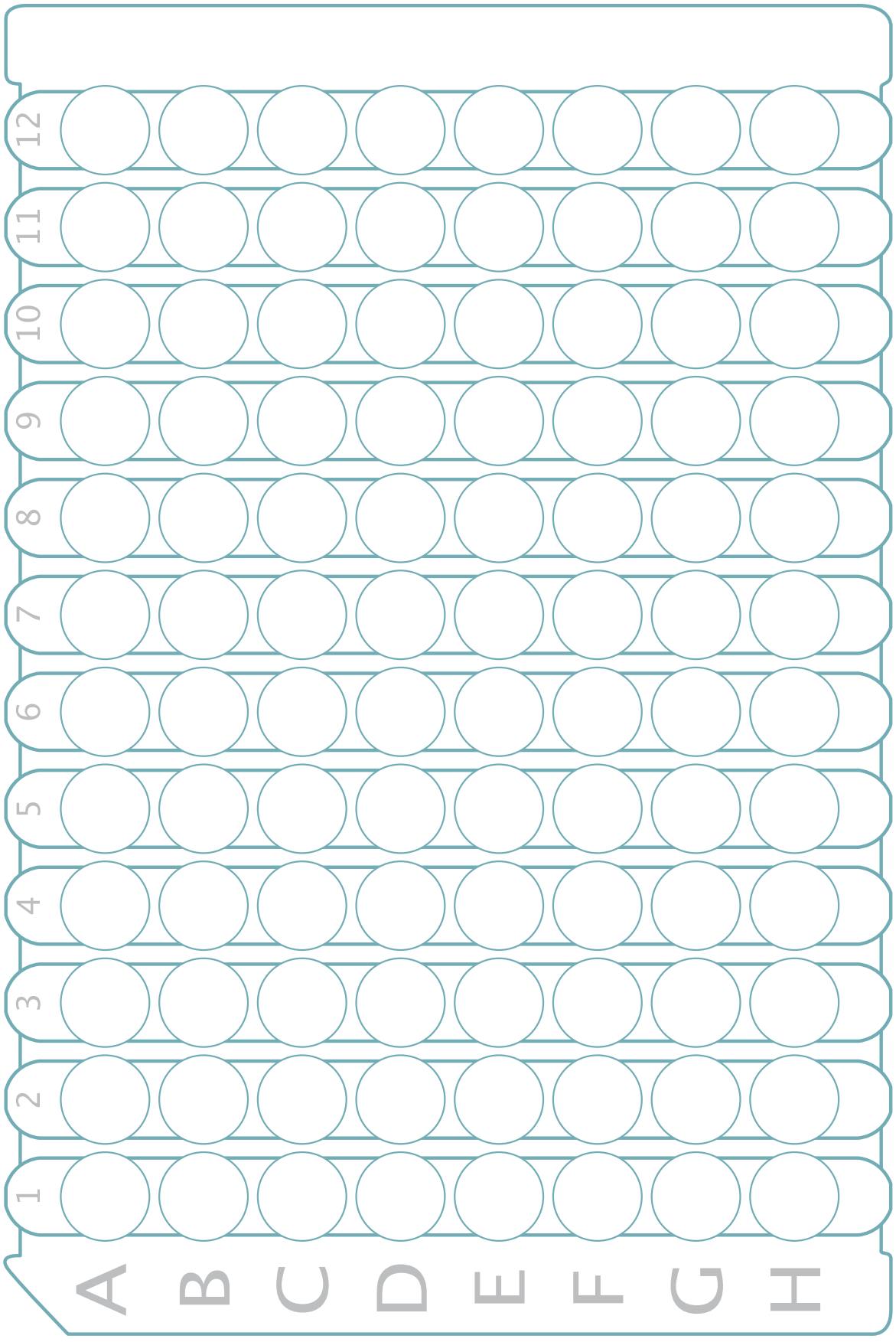
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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