Parameter™

Thromboxane B_2 Assay

Catalog Number KGE011

For the quantitative determination of Thromboxane B_2 (TXB_2) concentrations in cell culture supernates, serum, plasma, 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

Prostaglandins, thromboxanes, and leukotrienes belong to the prostanoid class of fatty acid derivatives of arachidonic acid. Arachidonic acid is liberated from membrane phospholipids by the action of phospholipases and then metabolized to Prostaglandin H_2 (PGH_2) by the cyclooxygenases COX-1 and COX-2. PGH_2 is converted into Thromboxane A_2 (TXA_2) by thromboxane synthase. TXA_2 production can be blocked by corticosteroids that inhibit the phospholipases or by non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit the cyclooxygenases (1). Thromboxane synthase can be blocked by a variety of compounds, some of which also function as antagonists of thromboxane receptors (2). TXA_2 is rapidly hydrolyzed to TXB_2 and then to additional derivatives including 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 (3-5). These molecules are significantly more stable than TXA_2, and therefore, TXB_2 is typically used as a surrogate marker for the estimation of TXA_2 (6).

TXA_2 is produced primarily by activated platelets and is a potent vasoconstrictor and promoter of thrombosis (1, 7, 8). It also increases cardiac contractility and protects against vascular hyporesponsiveness during systemic inflammation (9, 10). In its effects on the vasculature, TXA_2 functions as a counterbalance to the vasorelaxant and anti-thrombotic Prostacyclin (PGI_2) (11). TXA_2 production is induced in vascular endothelial cells (EC) following vascular injury (11). Consequently, measurements of serum thromboxanes can be artificially elevated due to TXA_2 release by EC damaged during blood collection and by platelets activated during blood handling (3, 12). Urinary thromboxane levels are not subject to these handling-induced errors (5).

TXA_2 exerts its biological effects through interactions with the 7-transmembrane G protein-coupled receptor TP (10, 11, 13-15). Two alternate splice forms of this receptor are expressed (TPα and TPβ) that have distinct C-terminal cytoplasmic regions (16). Both isoforms are expressed in platelets and in EC. Although both isoforms exhibit similar ligand binding characteristics, they have opposite effects on adenylyl cyclase activity (17, 18). TPβ signaling inhibits EC migration and FGF-2-induced angiogenesis, whereas TPα signaling does not alter EC migration (19, 20). Both receptor isoforms mediate EC apoptosis and the destabilization of vascular endothelial cell networks (18). TPα heterodimerizes with IP, the structurally related receptor for PGI_2, and both components of the dimer retain full affinity for their respective ligands (21). This receptor complex is internalized following ligand binding and follows an intracellular trafficking pattern determined by which receptor is ligated (22). In platelets, stimulation of IP leads to serine phosphorylation in the C-terminal tail of TPα and subsequent TPα desensitization (23). This phosphorylation site is absent in TPβ, and TPβ does not exhibit IP-induced desensitization (23).

Elevated levels of urinary and circulating TXA_2 and its metabolites are produced in a variety of pathologies that result in vascular injury or platelet activation. These include renal allograft rejection, capillary leak syndrome, cyclosporin-induced renal damage, type I and type II diabetes, pulmonary hypertension, angina pectoris, myocardial infarction, rheumatoid arthritis, IgE-induced anaphylaxis, and gingivitis (2, 24-34). Thromboxanes are also upregulated in oral epithelial cells and EC during gingivitis and accumulate in the crevicular fluid (35). Both isoforms of TP as well as TXA synthase and COX-2 are upregulated in prostate cancer and contribute to increased tumor aggressiveness (36, 37).

The Parameter TXB_2 Immunoassay is a 3.5 hour competitive enzyme immunoassay designed to measure TXB_2 in cell culture supernates, serum, plasma, and urine.

PRINCIPLE OF THE ASSAY

This assay is based on the forward sequential competitive binding technique in which TXB_2 present in a sample competes with horseradish peroxidase (HRP)-labeled TXB_2 for a limited number of binding sites on a mouse monoclonal antibody. TXB_2 in the sample is allowed to bind to the antibody in the first incubation. During the second incubation, HRP-labeled TXB_2 binds to the remaining antibody sites. Following a wash to remove unbound materials, 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 TXB_2 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 soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in this assay, 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.
- 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 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/RECONSTITUTED MATERIAL
Goat anti-mouse IgG Microplate	892575	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.*
TXB_2 Conjugate	893789	6 mL of TXB_2 conjugated to horseradish peroxidase with red dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
TXB_2 Standard	893791	200 ng of TXB_2 in buffer with preservatives; lyophilized.	
Primary Antibody Solution	893790	6 mL of a mouse monoclonal antibody for TXB_2 in buffer with blue dye and preservatives.	
Calibrator Diluent RD5-57	895944	2 vials (21 mL/vial) of a buffered protein base with preservatives.	
Wash Buffer 10X Concentrate	895222	100 mL of a 10-fold concentrated solution of buffered surfactant.	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895926	11 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.
- 1000 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.
- Indomethacin (Tocris, Catalog # I708 or equivalent).
- TXB_2 Controls (optional; available from R&D Systems).

PRECAUTIONS

Care should be taken when handling the TXB_2 Standard because of the known and unknown effects of thromboxanes.

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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.

Note: *Media containing serum may contain detectable levels of TXB_2. A media blank should be run with media containing serum.*

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 heparin or EDTA 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.*
Do not use lipemic, grossly hemolyzed, or turbid samples.
Thoroughly mix thawed samples before assay.

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.

***To inhibit thromboxane synthesis by COX-2, indomethacin should be added to serum and plasma collection tubes immediately following draw (to a final concentration of approximately 10 µg/mL).**

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

SAMPLE PREPARATION

All samples require at least a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5-57.

For urine samples, a 10-fold dilution is recommended. A suggested 10-fold dilution is 50 µL of sample + 450 µL of Calibrator Diluent RD5-57.

REAGENT PREPARATION

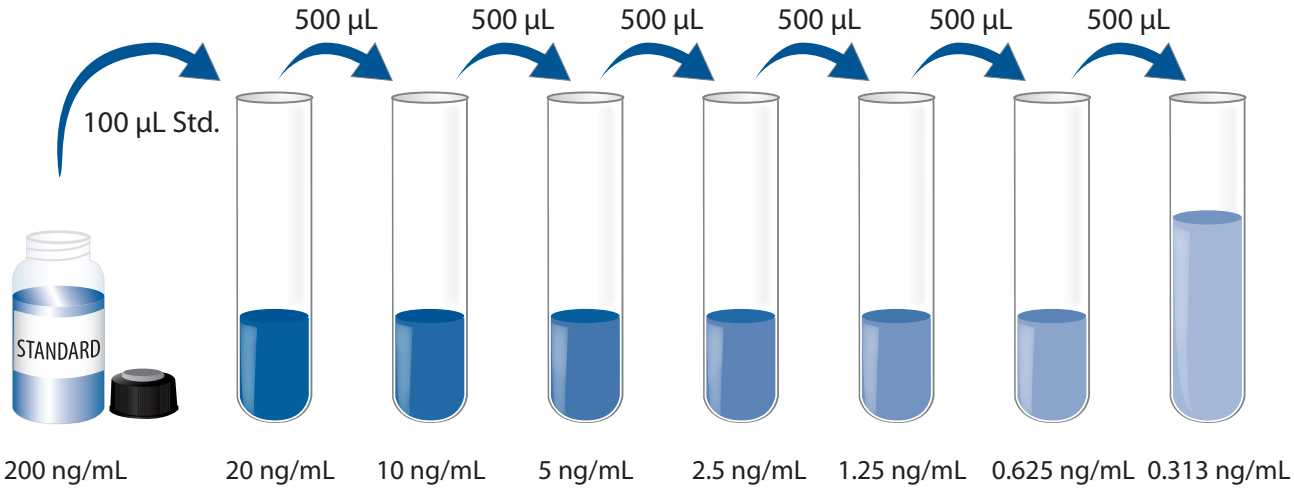
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 100 mL of Wash Buffer 10X Concentrate to 900 mL deionized or distilled water to prepare 1000 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.

TXB_2 Standard - Reconstitute the TXB_2 Standard with 1.0 mL of Calibrator Diluent RD5-57. This reconstitution produces a stock solution of 200 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-57 into the 20 ng/mL tube. Pipette 500 µL of Calibrator Diluent RD5-57 into the remaining tubes. Use the 200 ng/mL stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 20 ng/mL standard serves as the high standard, and Calibrator Diluent RD5-57 serves as the zero standard (B_0).



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.

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 150 µL of Calibrator Diluent RD5-57 to the non-specific binding (NSB) wells.
4. Add 100 µL of Calibrator Diluent RD5-57 to the zero standard (B_0) wells.
5. Add 100 µL of Standard, control, or sample* to the remaining wells.
6. Add 50 µL of Primary Antibody Solution to all wells **except** the NSB wells. All wells except the NSB wells will be blue in color.
7. Cover with the adhesive strip provided, and incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 rpm ± 50 rpm. A plate layout is provided to record standards and samples assayed.
8. **Do not aspirate or wash the plate.** Add 50 µL of TXB_2 Conjugate to all wells. All wells except the NSB wells will be violet in color (NSB wells will be pink).
9. Cover with a new adhesive strip, and incubate for 1 hour at room temperature on the shaker.
10. 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 for 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.
11. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
12. Add 100 µL of Stop Solution to each well. If the color in the wells does not appear uniform, gently tap the plate to ensure thorough mixing.
13. 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 the 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.

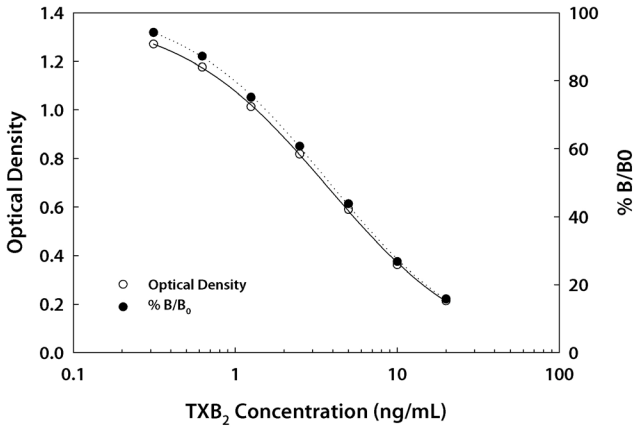
If desired, % B/B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

Calculate the concentration of TXB_2 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.



(ng/mL)	O.D.	Average	Corrected	% B/B_0
NSB	0.005 0.007	0.006	—	—
0 (B_0)	1.346 1.363	1.355	1.349	—
0.313	1.268 1.285	1.277	1.271	94.2
0.625	1.177 1.187	1.182	1.176	87.2
1.25	1.001 1.037	1.019	1.013	75.1
2.5	0.815 0.833	0.824	0.818	60.7
5	0.588 0.604	0.596	0.590	43.8
10	0.361 0.374	0.368	0.362	26.8
20	0.216 0.223	0.220	0.214	15.8

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	3.87	6.95	13.2	3.86	7.05	14.4
Standard deviation	0.23	0.38	0.51	0.34	0.45	0.74
CV (%)	5.9	5.5	3.9	8.9	6.4	5.1

RECOVERY

The recovery of TXB_2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media* (n=4)	98	88-110%
Serum* (n=4)	98	86-111%
EDTA plasma* (n=4)	94	86-105%
Heparin plasma* (n=4)	97	87-107%
Urine* (n=4)	98	84-111%

*Samples were diluted prior to assay. See Sample Preparation section.

SENSITIVITY

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of TXB_2 ranged from 0.114-0.310 ng/mL. The mean MDD was 0.223 ng/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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of TXB_2 were serially diluted with Calibrator Diluent RD5-57 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)	Urine* (n=4)
1:2	Average % of Expected	98	97	93	96	98
	Range (%)	90-104	95-100	87-97	90-98	94-106
1:4	Average % of Expected	98	102	97	98	100
	Range (%)	94-105	96-108	90-100	88-102	95-105
1:8	Average % of Expected	100	97	104	97	102
	Range (%)	84-110	92-103	91-114	81-113	100-104
1:16	Average % of Expected	103	107	100	95	105
	Range (%)	94-119	100-116	93-108	82-106	98-112

*Samples were diluted prior to assay. See Sample Preparation section.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of TXB_2 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)	3.08	1.11-11.3	2.26
Urine (n=26)	22.1	3.50-72.5	14.3

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
EDTA plasma (n=36)	2.27	97	ND-10.1
Heparin plasma (n=36)	2.22	92	ND-9.26

ND=Non-detectable

Cell Culture Supernates - Human peripheral blood lymphocytes (PBLs) were cultured in DMEM supplemented with 5% fetal calf serum, 50 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. Aliquots of the cell culture supernates were removed and assayed for levels of TXB_2.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Stimulated	38.7	69.0
Unstimulated	4.34	5.35

Note: *Sample values have been corrected for TXB_2 present in the media.*

SPECIFICITY

The factors listed below were prepared at 200 ng/mL or 50 ng/mL in Calibrator Diluent RD5-57 and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL or 50 ng/mL in a mid-range TXB_2 control were assayed for interference. Cross-reactivity is listed below.

Condition	% Cross-reactivity
2,3-dinor-TXB_3	13.5%
11-dehydro-TXB_2	6.5%
$PGF_{2\alpha}$	6.5%
PGE_2	5.9%
$PGF_{1\alpha}$	4.7%
6-keto-$PGF_{1\alpha}$	4.5%
PGE_1	3.4%
PGE_3	3.3%
2,3-dinor-TXB_2	1.7%
PGA_2	0.7%
Arachidonyl Ethanolamide	0.3%
Arachidonic Acid	0.2%
PGB_2	0.2%

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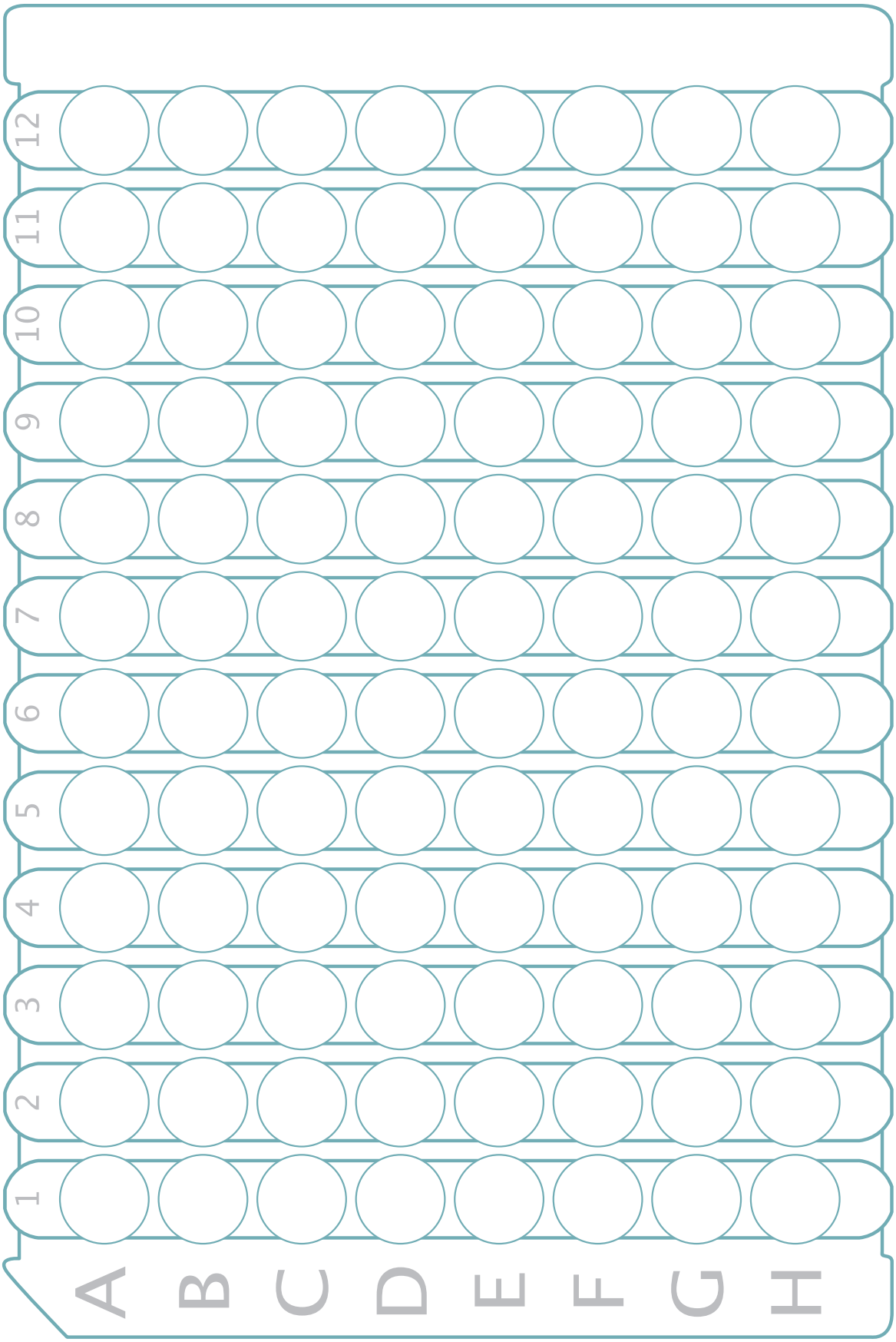
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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