Quantikine® ELISA

Human APRIL/TNFSF13 Immunoassay

Catalog Number DAPR00

For the quantitative determination of human A Proliferation-Inducing Ligand (APRIL) concentrations in cell culture supernates, serum, platelet-poor plasma, and saliva.

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

APRIL (a proliferation-inducing ligand), also known as TNFSF13, TALL2, TRDL1, and CD256, is a member of the TNF ligand superfamily (1). It is synthesized as a 32 kDa proprotein which is cleaved by furin in the Golgi to release the active 17 kDa soluble molecule (2-4). Secreted human APRIL, which consists almost entirely of a single TNF homology domain, shares 85% amino acid sequence identity with mouse and rat APRIL (2, 3). Both APRIL and its close relative BAFF/TNFSF13B bind and signal through the TNF superfamily receptors TACI and BCMA, while BAFF additionally functions through BAFF R (2, 5, 6). APRIL binds to heparan sulfate proteoglycans (HSPGs) independently of its binding to TACI and BCMA (6, 7). The interaction with HSPGs induces APRIL oligomerization, and this augments TACI-, or BMCA-mediated effects (7-9). HSPGs are also critical for the tumor growth-promoting effects attributed to APRIL (6). APRIL can form bioactive heterotrimers with BAFF, and these circulate in the serum of patients with rheumatic immune disorders (10). TWE-PRIL is a bioactive hybrid protein produced by gene splicing. It consists of the intracellular domain, transmembrane segment, and stalk region of TWEAK/TNFSF12 fused to the TNF homology domain of APRIL (11). TWE-PRIL is expressed in monocytes and activated T cells and, in contrast to APRIL, is presented on the cell surface (11). APRIL enhances the proliferation and survival of plasma cells and also promotes T cell-dependent humoral responses (2, 12, 13). In the context of autoimmune disorders, however, APRIL can inhibit pathologic humoral responses as well as disease progression (14). Its expression by $CD4^+$ T cells inhibits the production of Th2 cytokines and allergic inflammation (15). APRIL is elevated in the serum during coronary artery disease (16), and it is also elevated in many cancers primarily due to expression by tumor-infiltrating neutrophils (4, 7, 17).

The Quantikine Human APRIL/TNFSF13 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human APRIL in cell culture supernates, serum, platelet-poor plasma, and saliva. It contains HEK293-expressed recombinant human APRIL and antibodies raised against the recombinant factor. Results obtained using natural human APRIL 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 naturally occurring human APRIL.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human APRIL has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any APRIL present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human APRIL 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 APRIL 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 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 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 APRIL Microplate	894831	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human APRIL.	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 APRIL Standard	894833	2 vials of recombinant human APRIL in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human APRIL Conjugate	894832	21 mL of a monoclonal antibody specific for human APRIL conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-68	895528	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-63	895994	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	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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human APRIL Controls (optional; R&D Systems, Catalog # QC199).

PRECAUTIONS

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

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. 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.

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.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly icteric and lipemic samples are not suitable for use in this assay.

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.

Saliva samples require a 2-fold dilution due to matrix effect. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD6-63.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

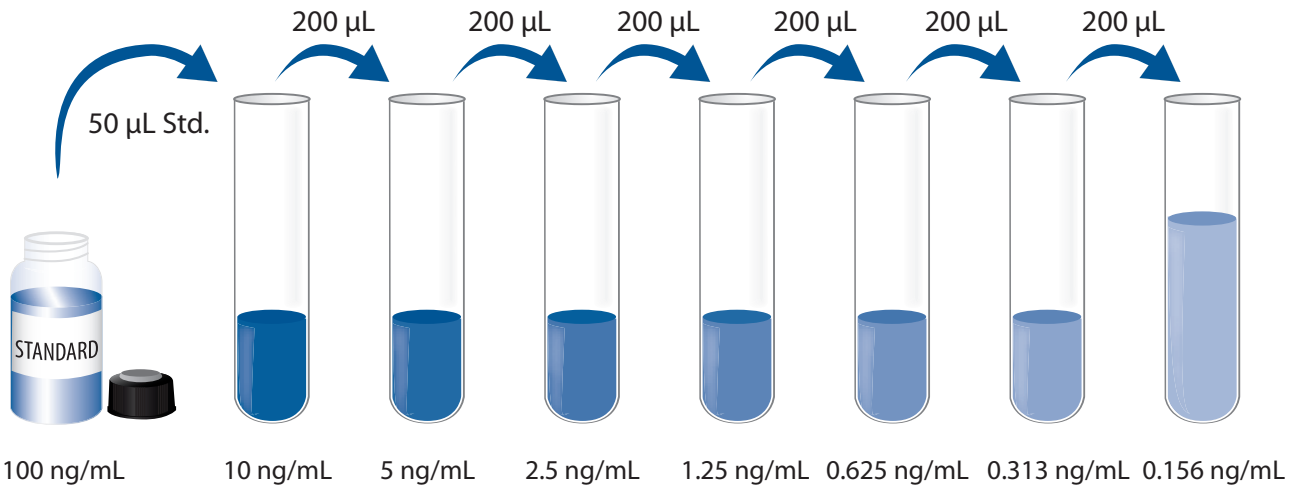
Note: *APRIL is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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.

Human APRIL Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human APRIL Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of **30 minutes** with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD6-63 into the 10 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 10 ng/mL standard serves as the high standard. Calibrator Diluent RD6-63 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 samples, controls, and standards be assayed in duplicate.

Note: *APRIL is 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 Assay Diluent RD1-68 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 200 µL of Human APRIL 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 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. 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.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

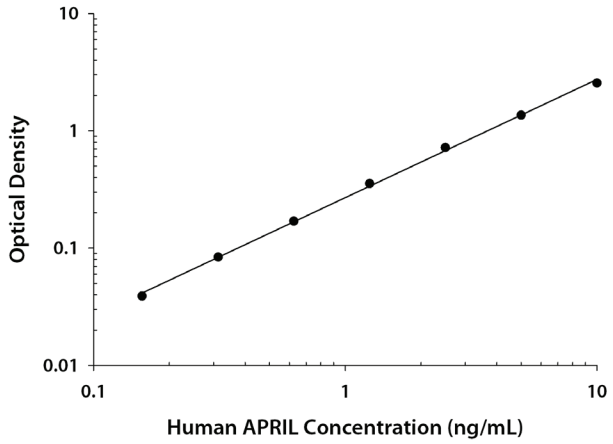
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 APRIL concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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	0.011 0.012	0.012	—
0.156	0.050 0.051	0.051	0.039
0.313	0.094 0.097	0.096	0.084
0.625	0.180 0.184	0.182	0.170
1.25	0.366 0.367	0.367	0.355
2.5	0.729 0.733	0.731	0.719
5	1.353 1.392	1.373	1.361
10	2.559 2.579	2.569	2.557

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 components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	1.42	2.84	6.24	1.48	3.06	6.09
Standard deviation	0.075	0.109	0.265	0.126	0.198	0.401
CV (%)	5.3	3.8	4.2	8.5	6.5	6.6

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	99	96-105%
Serum (n=4)	102	94-115%
Platelet-poor EDTA plasma (n=4)	102	92-111%
Platelet-poor heparin plasma (n=4)	104	100-109%

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of human APRIL ranged from 0.003-0.015 ng/mL. The mean MDD was 0.007 ng/mL.

The MDD was determined by adding two standard deviations to 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 human APRIL were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	Platelet-poor		Saliva* (n=4)
				EDTA plasma (n=4)	Heparin plasma (n=4)	
1:2	Average % of Expected	105	101	103	103	104
	Range (%)	99-112	98-103	100-105	97-106	97-109
1:4	Average % of Expected	107	97	103	103	106
	Range (%)	103-115	93-103	98-111	98-110	100-112
1:8	Average % of Expected	106	92	99	101	105
	Range (%)	99-115	86-98	95-109	97-107	94-111
1:16	Average % of Expected	100	91	91	97	105
	Range (%)	93-111	87-96	87-99	92-105	103-108

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

CALIBRATION

This immunoassay is calibrated against highly purified HEK293-expressed recombinant human APRIL produced at R&D Systems.

SAMPLE VALUES

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

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Platelet-poor EDTA plasma (n=35)	———	———	ND
Platelet-poor heparin plasma (n=35)	0.171	17	ND-0.227

ND=Non-detectable

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=35)	2.01	1.09-3.61	0.564
Saliva (n=10)	3.17	0.392-6.58	1.89

Cell Culture Supernates:

$CD14^+$ monocytes were cultured in DMEM and supplemented with 10% fetal bovine serum. Cells were cultured stimulated with 100 ng/mL of recombinant human IFN-γ for 40 hours. An aliquot of the cell culture supernate was removed, assayed for levels of human APRIL, and measured 0.454 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human APRIL.

The factors listed below were prepared at 100 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

4-1BB Ligand
April-2/BAFF-1 Heterodimer
BAFF
BAFF-2/APRIL-1 Heterodimer
EDA-A1
EDA-A2
Fas Ligand
GITR Ligand
LIGHT
OX40 Ligand
TACI
TL1A
TNF-α
TRAIL
TRANCE
TWEAK

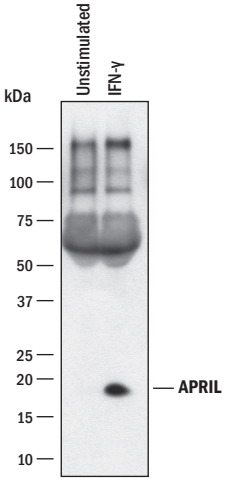
Recombinant mouse:

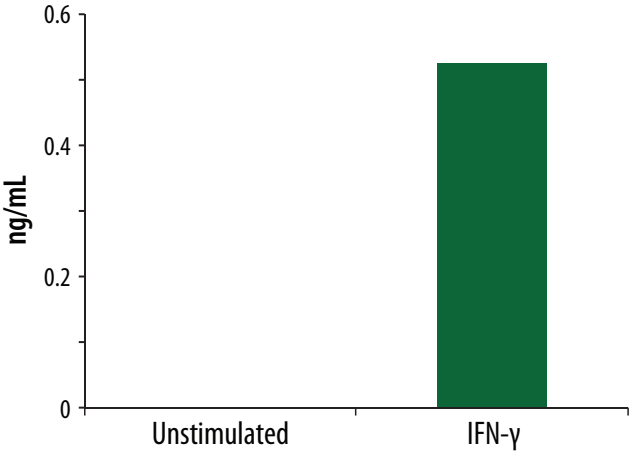
4-1BB Ligand
APRIL
BAFF
EDA-A1
Fas Ligand
GITR Ligand
LIGHT
OX40 Ligand
TL1A
TNF-α
TRAIL
TRANCE
TWEAK

Other recombinants:

bovine TNF-α
canine TNF-α
cotton rat TNF-α
equine TNF-α
feline TNF-α
guinea pig TNF-α
porcine TNF-α
rabbit TNF-α
rat TNF-α

Recombinant human BCMA does not cross-react in this assay but does interferes at concentrations > 0.5 ng/mL in this assay.





Conditioned media samples were analyzed by Western blot and Quantikine ELISA. Human $CD14^+$ cells were isolated from PBMC, and left unstimulated or treated with 100 ng/mL recombinant human IFN-γ, and the conditioned media harvested. For the Western blot, samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with a polyclonal antibody specific for human APRIL. The Western blot shows a direct correlation with the ELISA value for these samples.

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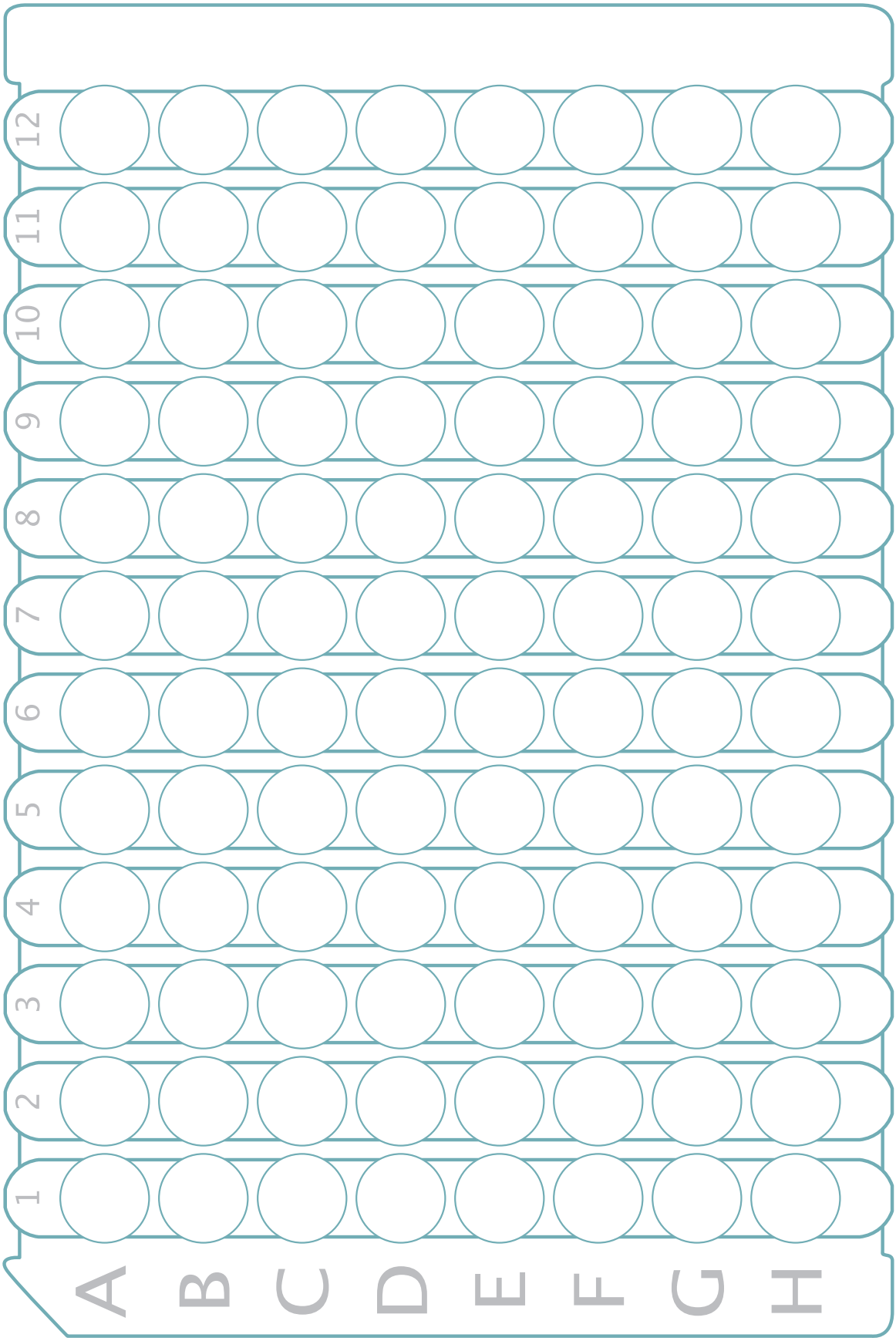
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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