Quantikine® ELISA

Human CD40 Immunoassay

Catalog Number DCCD40

For the quantitative determination of human CD40 concentrations in cell culture supernates, serum, plasma, saliva, urine, and human milk.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

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INTRODUCTION

CD40, also known as TNFRSF5, is a 45-50 kDa variably glycosylated type I transmembrane protein that belongs to the TNF receptor superfamily. It exhibits diverse activities in normal immune system development and function and participates in the control or progression of a variety of diseases (1, 2). Mature human CD40 consists of a 173 amino acid (aa) extracellular domain (ECD) with four TNFR repeats, a 22 aa transmembrane segment, and a 62 aa cytoplasmic domain (3). Within the ECD, human CD40 shares 58% and 56% aa sequence identity with mouse and rat CD40, respectively. Alternate splicing of human CD40 generates a secreted isoform and an isoform that lacks the third TNFR repeat (4). Soluble CD40 (27-28 kDa), which functions as an inhibitor of CD40 bioactivity, can be generated by alternate splicing or by proteolytic cleavage of transmembrane CD40 by TACE (4-6).

CD40 is expressed on B cells, dendritic cells, monocytes, macrophages, T cells, neutrophils, platelets, fibroblasts, smooth muscle cells, epithelial cells, endothelial cells, neurons, and in many hematopoietic and epithelial cancers (1, 7, 8). It is upregulated on these cell types during inflammation and notably on $CD4^+$ T cells in autoimmune mice (9-11). CD40 homotrimerization is important for its ability to initiate signaling through TRAF family proteins (12). CD40 signaling is induced by its binding to the 39 kDa transmembrane glycoprotein CD40 Ligand/CD154 (CD40L). CD40L itself is upregulated on T cells, B cells, dendritic cells, neutrophils, platelets, vascular endothelial cells, and smooth muscle cells during activation or inflammation (1, 7, 13, 14). CD40L can be expressed on the cell surface in heteromultimers of different isoforms (15). Soluble CD40L is generated by intracellular proteolytic cleavage and is secreted as a homotrimer of 15-18 kDa subunits (15, 16). Both the membrane bound and soluble forms of CD40L induce signaling through CD40 (17, 18).

Interactions between CD40 and CD40L are involved in multiple aspects of humoral immunity, cellular immunity, and inflammation. CD40L on activated $CD4^+$ T cells provides a costimulatory signal that augments B cell proliferation, germinal center formation, immunoglobulin class switching, and antibody secretion (1, 11, 18). CD40 ligation likewise promotes the activation of the many other cell types which express it and additionally promotes the development of medullary thymic epithelial, Th17, and $FoxP3^+$ Treg cells (14, 19-23). CD40-CD40L interactions regulate lymphocyte development and the balance between immune self-tolerance and autoimmunity (11, 24). CD40 signaling reinforces inflammatory responses but can also play a tissue protective role by enhancing epithelial cell survival during oxidative stress (7, 23, 25-28). CD40-CD40L interactions in disease can be beneficial (contributing to the immune response to cancer) or detrimental (contributing to the progression of atherosclerosis and graft versus host disease) (1, 7, 8). CD40-mediated responses are modulated by TLR, RANK, and TNF receptor activity through intracellular signaling crosstalk or direct interaction of CD40 with those receptors (2, 20, 21).

Circulating soluble CD40 is normally excreted in the urine but is elevated in the plasma of chronic renal failure patients (29). Plasma levels of soluble CD40 are also elevated in patients with liver disease, systemic sclerosis, Hashimoto's thyroiditis, hypertension-associated severe organ damage, or hematologic malignancies (30-34). It is elevated in the plasma of Alzheimer's patients, and these levels correlate with decreased cognitive performance (35, 36).

The Quantikine Human CD40 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human CD40 in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains NS0-expressed recombinant human CD40 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human CD40 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 CD40.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for human CD40 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CD40 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human CD40 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 CD40 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 CD40 Microplate	894014	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for human CD40.	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.*
Human CD40 Conjugate	894015	21 mL of polyclonal antibody specific for human CD40 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human CD40 Standard	894016	12.5 ng of recombinant human CD40 in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1-106	895960	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-15	895244	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 CD40 Controls (optional; R&D Systems, Catalog # QC60).

PRECAUTIONS

CD40 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 ProClin® 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.

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.*

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

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

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and centrifuge twice more for a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.

Urine samples require at least a 5-fold dilution. A suggested 5-fold dilution is 100 µL of sample + 400 µL of Calibrator Diluent RD6-15.

Human milk samples require at least a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD6-15.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

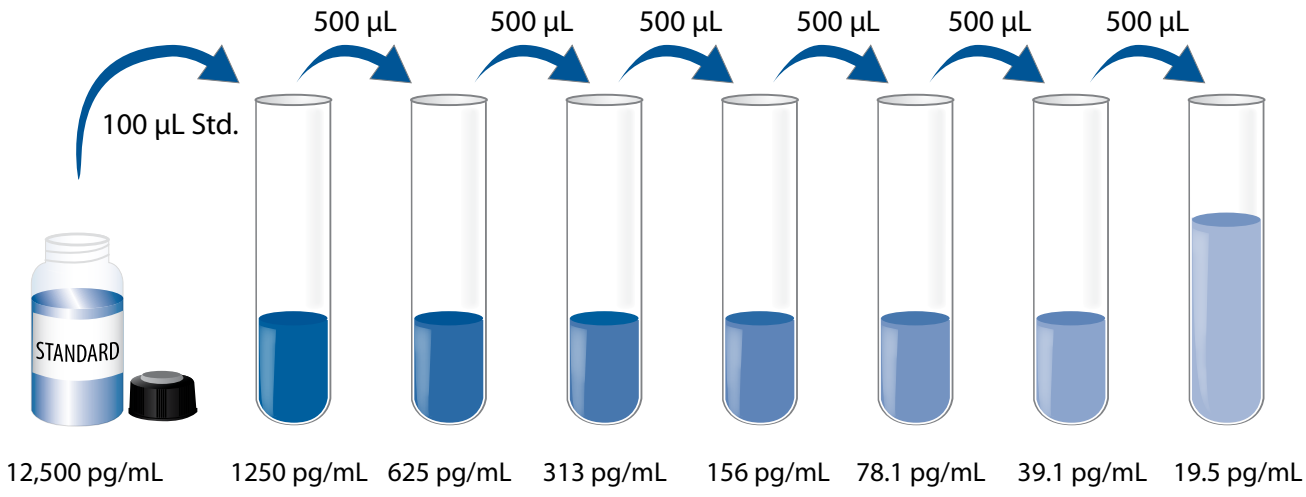
Note: *CD40 is detectable in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 CD40 Standard - Reconstitute the Human CD40 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 12,500 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD6-15 into the 1250 pg/mL tube. Pipette 500 µL of Calibrator Diluent RD6-15 into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1250 pg/mL standard serves as the high standard. Calibrator Diluent RD6-15 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, samples, and controls be assayed in duplicate.

Note: *CD40 is detectable 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 RD1-106 to each well.
4. Add 100 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 CD40 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 Sample Preparation section.

CALCULATION OF RESULTS

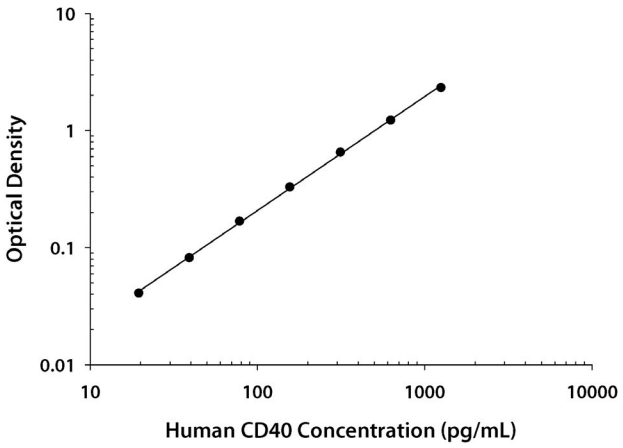
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 CD40 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the 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	0.027 0.027	0.027	—
19.5	0.068 0.068	0.068	0.041
39.1	0.108 0.109	0.109	0.082
78.1	0.193 0.196	0.195	0.168
156	0.353 0.361	0.357	0.330
313	0.674 0.686	0.680	0.653
625	1.226 1.282	1.254	1.227
1250	2.252 2.460	2.356	2.329

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 (pg/mL)	97.5	301	561	104	307	574
Standard deviation	4.65	14.7	24.2	9.53	19.6	29.9
CV (%)	4.8	4.9	4.3	9.2	6.4	5.2

RECOVERY

The recovery of human CD40 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	95-115%
Serum (n=4)	92	85-101%
EDTA plasma (n=4)	92	87-104%
Heparin plasma (n=4)	93	82-102%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human CD40 were serially diluted with Calibrator Diluent 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)	Saliva (n=4)	Urine* (n=4)	Human milk* (n=5)
1:2	Average % of Expected	107	104	104	104	92	104	111
	Range (%)	103-111	101-107	103-106	101-108	86-101	102-106	107-116
1:4	Average % of Expected	105	103	103	105	95	104	111
	Range (%)	97-115	100-107	99-108	99-109	85-101	98-109	104-122
1:8	Average % of Expected	102	98	99	99	94	105	121
	Range (%)	93-111	89-106	96-104	91-103	88-99	92-113	110-132
1:16	Average % of Expected	98	94	93	91	———	106	———
	Range (%)	90-110	88-100	87-106	85-97	———	90-113	———

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of human CD40 ranged from 0.54-5.56 pg/mL. The mean MDD was 1.63 pg/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.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human CD40 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human CD40 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=36)	583	409-870	102
EDTA plasma (n=36)	535	381-833	108
Heparin plasma (n=36)	516	347-762	104
Saliva* (n=17)	206	85-395	77.9
Urine (n=10)	3539	2101-5008	1055
Human milk (n=8)	301	105-664	101

*One sample assayed read above the top standard of 1250 pg/mL and was not included in this summary.

Cell Culture Supernates:

Human peripheral blood leukocytes (PBL) were cultured in DMEM and supplemented with 5% fetal calf serum, 5 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 for 1 or 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of natural human CD40.

Sample Type	Day 1 (pg/mL)	Day 5 (pg/mL)
PBL unstimulated	29.7	170
PBL stimulated w/PHA	ND	289

ND=Non-detectable

MDA-MB-231 human breast cancer cells were cultured in Leibovitz L-15 supplemented with 10% fetal bovine serum and 2 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for levels of natural human CD40, and measured 244 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human CD40.

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 recombinant human CD40 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

4-1BB
BAFF R
CD27
CD30
CD40 Ligand
Fas
GITR
Lymphotoxin βR
OX40
TNF RI
TNF RII
TRAIL R1
TRAIL R2
TRAIL R3
TRAIL R4
TWEAK R

Recombinant mouse:

4-1BB
BAFF R
CD27
CD30
CD40
CD40 Ligand
DcTRAIL R1
DcTRAIL R2
Fas
GITR
Lymphotoxin βR
OX40
TNF RI
TNF RII
TWEAK R

Recombinant rat:

Fas

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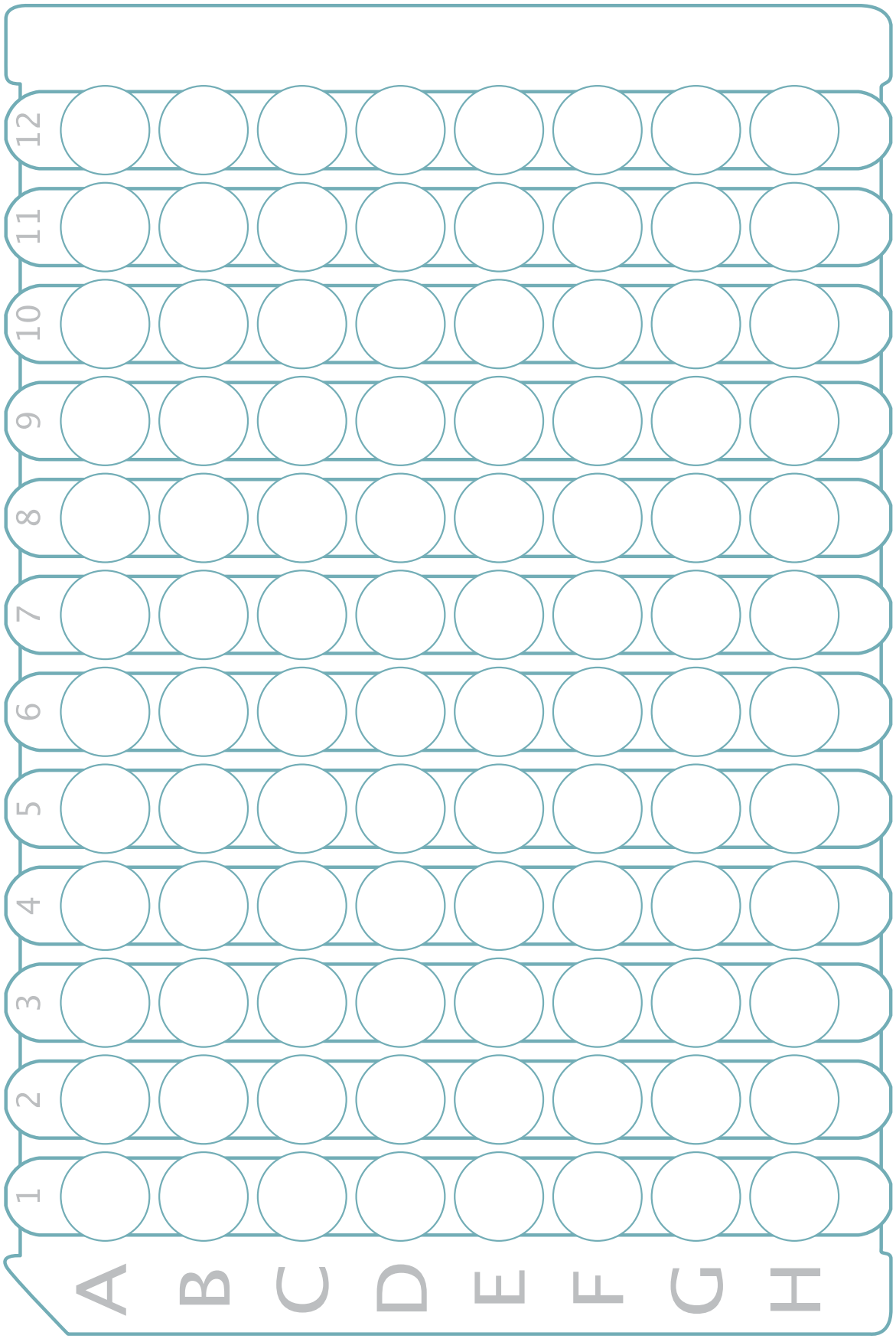
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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