Quantikine® ELISA

Human Carbonic Anhydrase IX Immunoassay

Catalog Number DCA900

For the quantitative determination of human Carbonic Anhydrase IX (CA9) 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.

TABLE OF CONTENTS

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INTRODUCTION

Carbonic Anhydrase IX (CA9 or CA IX, also known as membrane antigen MN) is a member of the carbonic anhydrase protein family. It is a transmembrane protein that consists of an N-terminal signal peptide, an extracellular proteoglycan-related domain and catalytic domain, a transmembrane segment, and a C-terminal intracellular tail (1). The major physiologic function for CA9 is to regulate pH by catalyzing the reversible hydration of carbon dioxide to carbonic acid, which subsequently decomposes to HCO_3^- and H_3O^+ (2). Primarily expressed in the gut, CA9 is ectopically up-regulated in many types of tumors, such as renal cell carcinoma, non-small cell lung cancer, breast cancer, and cervical cancer (3-6). One of the mechanisms underlying the over-expression of CA9 in tumor tissues is hypoxia. Under hypoxic conditions, the CA9 gene is induced by its upstream regulator, the hypoxia-inducible factor (HIF)-1 transcription factor. CA9 facilitates the tumor cells to create an acidified local environment to promote growth and metastasis (7). Another mechanism governing the enhanced expression of CA9 in tumor tissues is the loss of the tumor suppressor gene, von Hippel-Lindau (VHL). Some experimental evidence has shown that mutations in the VHL gene can lead to over-expression of CA9 in cancer cell lines (8). CA9 has also been found to have connections with some signal transduction pathways. The tyrosine moiety of the intracellular tail of CA9 can be phosphorylated in an epidermal growth factor-dependent manner and the phosphorylated CA9 can interact with PI-3-kinase (9). Additionally, CA9 has been demonstrated to mediate cell adhesion (10). The expression of CA9 in tumor tissues is often correlated with aggressive phenotypes. Furthermore, CA9 as a therapeutic target has also been proposed (8).

The ectodomain of CA9 can be released into the extracellular milieu. This soluble form of CA9 has been detected in cell culture supernates as well as biological fluids, such as serum and urine (11). The metalloprotease, TNFα-converting enzyme (TACE/ADAM17), is likely to regulate this process (12).

The Quantikine® Human Carbonic Anhydrase IX Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human CA9 in cell culture supernates, serum, plasma, and urine. It contains NS0-expressed recombinant human CA9 ectodomain and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human CA9 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 natural human CA9.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human CA9 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CA9 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human CA9 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 CA9 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, dilute the 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.
- 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 Carbonic Anhydrase IX Microplate	893173	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human CA9.	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 Carbonic Anhydrase IX Conjugate	893174	21 mL of a polyclonal antibody specific for human CA9 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Carbonic Anhydrase IX Standard	893175	Recombinant human CA9 in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-21	895215	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-12	895214	21 mL of a buffered animal serum 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.
- Test tubes for dilution of standards.
- Human Carbonic Anhydrase IX Controls (optional; R&D Systems®, Catalog # QC24).

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.

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 EDTA, heparin, or citrate 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.

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.

REAGENT PREPARATION

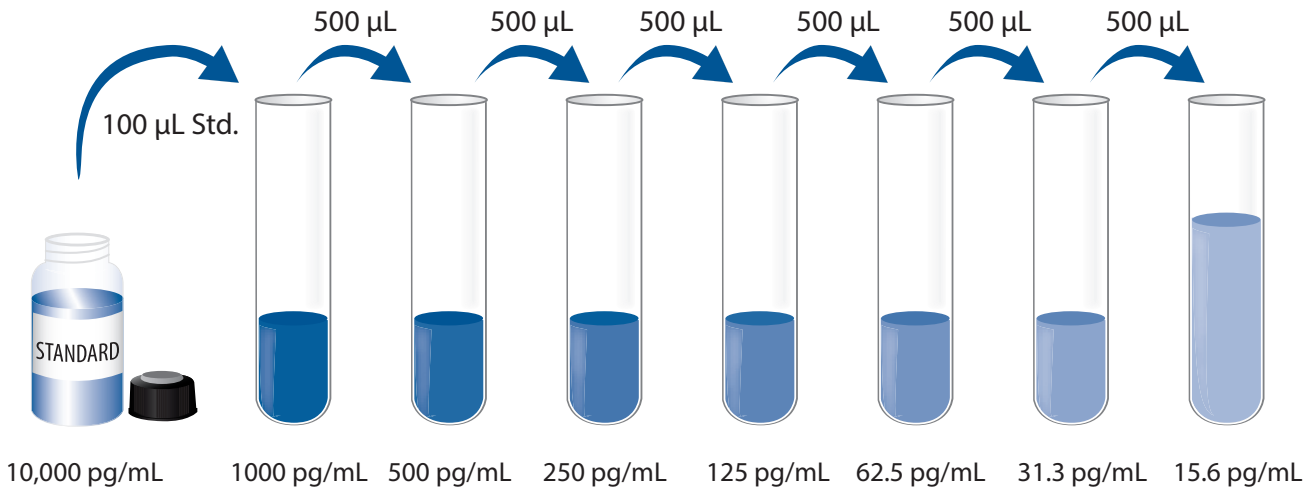
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 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. 200 µL of the resultant mixture is required per well.

Human Carbonic Anhydrase IX Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Carbonic Anhydrase IX Standard with Calibrator Diluent RD6-12. This reconstitution produces a stock solution of 10,000 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 900 µL of Calibrator Diluent RD6-12 into the 1000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD6-12 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, controls, and samples 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 50 µL of Assay Diluent RD1-21 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 Carbonic Anhydrase IX 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.

CALCULATION OF RESULTS

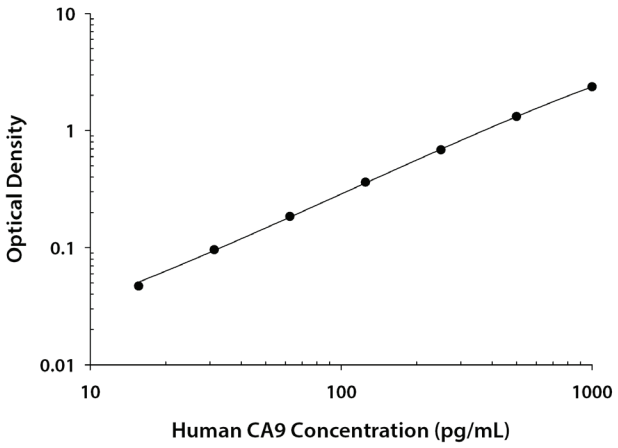
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human CA9 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	0.034 0.035	0.035	—
15.6	0.081 0.083	0.082	0.047
31.3	0.128 0.134	0.131	0.096
62.5	0.218 0.221	0.220	0.185
125	0.389 0.404	0.397	0.362
250	0.716 0.722	0.719	0.684
500	1.332 1.375	1.354	1.319
1000	2.385 2.417	2.401	2.366

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. 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	40	40	40
Mean (pg/mL)	166	338	673	166	333	647
Standard deviation	6.36	7.19	20.5	10.5	21.6	39.7
CV (%)	3.8	2.1	3.0	6.3	6.5	6.1

RECOVERY

The recovery of human CA9 spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	110	103-114%
Serum (n=4)	97	87-106%
EDTA plasma (n=4)	99	90-110%
Heparin plasma (n=4)	97	88-112%
Citrate plasma (n=4)	96	89-111%
Urine (n=4)	101	89-114%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human CA9 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)	Citrate plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	94	99	95	94	96	97
	Range (%)	92-95	92-104	92-98	88-98	91-100	92-107
1:4	Average % of Expected	94	102	97	96	97	96
	Range (%)	91-97	94-106	94-98	92-103	93-102	92-109
1:8	Average % of Expected	91	99	94	93	97	95
	Range (%)	89-93	95-101	90-97	89-96	88-102	90-106
1:16	Average % of Expected	91	94	93	90	96	94
	Range (%)	90-92	92-102	85-99	89-93	88-101	89-107

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) of human CA9 ranged from 0.665-4.39 pg/mL. The mean MDD was 2.28 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 NS0-expressed recombinant human CA9 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human CA9 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=35)	57.0	100	17.7-216
EDTA plasma (n=35)	55.2	100	16.1-205
Heparin plasma (n=35)	55.7	97	ND-194
Citrate plasma (n=35)	47.8	94	ND-178

ND=Non-detectable

Cell Culture Supernates:
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine 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 of human CA9. No detectable levels were observed.

HT-29 human colon adinocarinoma cells (0.25 x 10^5 cells/mL) were cultured in McCoy's 5a media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 7 days. An aliquot of the cell culture supernate was removed, assayed for human CA9, and measured 12,110 pg/mL.

COLO 205 human colorectal adenocarcinoma cells (0.75 x 10^5 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. An aliquot of the cell culture supernate was removed, assayed for human CA9, and measured 964 pg/mL.

Urine - Ten urine samples were evaluated for the presence of human CA9 in this assay. No detectable levels were observed.

SPECIFICITY

This assay recognizes natural and recombinant human CA9.

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 CA9 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CA1
CA2
CA3
CA4
CA5 A
CA5 B
CA6
CA7
CA8
CA10
CA11
CA12
CA13
CA14

Recombinant mouse:

CA9

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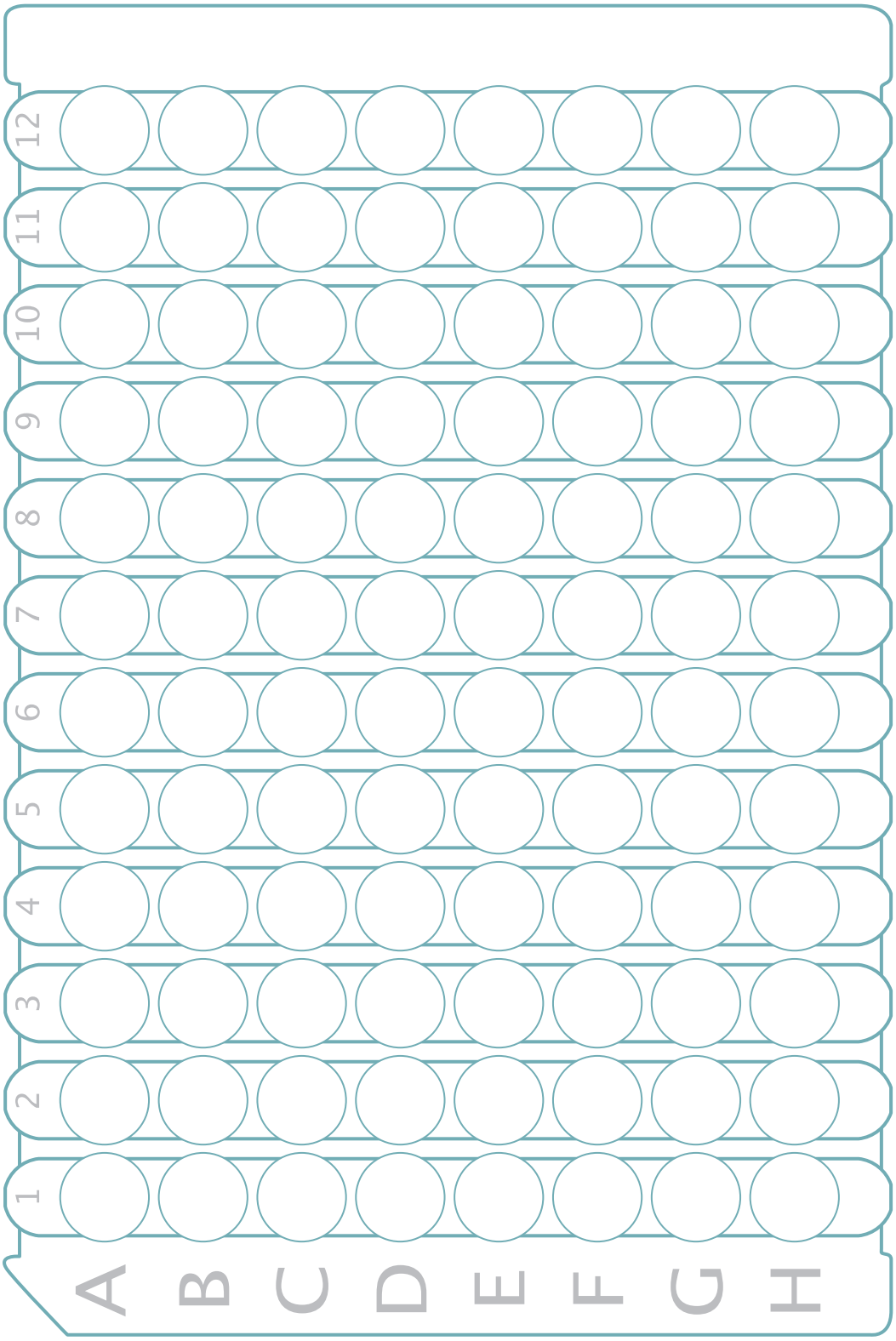
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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