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R&D SYSTEMS

Quantikine® ELISA

Human Axl Immunoassay

Catalog Number DAXL00

For the quantitative determination of human Axl concentrations in cell culture supernates, cell lysates, cerebrospinal fluid , 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

Axl (Ufo, Ark), Dtk (Sky, Tyro3, Rse, Brt), and Mer (human and mouse homologues of chicken c-Eyk) constitute a subfamily of the receptor tyrosine kinases (1, 2). The extracellular domains of these proteins contain two Ig-like motifs and two fibronectin type III motifs. This characteristic topology is also found in neural cell adhesion molecules and in receptor tyrosine phosphatases. The human Axl cDNA encodes an 887 amino acid (aa) precursor that includes an 18 aa signal sequence, a 426 aa extracellular domain, a 21 aa transmembrane segment, and a 422 aa cytoplasmic domain. The extracellular domains of human and mouse Axl share 81% aa sequence identity. A short alternately spliced form of human Axl is distinguished by a 9 aa deletion in the extracellular juxtamembrane region. These receptors bind the vitamin K-dependent protein growth arrest specific gene 6 (Gas6) which is structurally related to the anticoagulation factor protein S. Binding of Gas6 induces receptor autophosphorylation and downstream signaling pathways that can lead to cell proliferation, migration, or the prevention of apoptosis (3). This family of tyrosine kinase receptors is involved in hematopoiesis, embryonic development, tumorigenesis, and regulation of testicular functions.

The over expression of Axl has been identified in several cancers (4, 5): For example, the serum concentrations of Axl were significantly increased in Hepatocellular Carcinoma compared to healthy controls (6-8) and expression levels of Axl in renal cell carcinomas correlate with tumor progression and survival (9). Additionally, it is reported that there are increased serum or plasma levels of Axl in severe cases of Preeclampsia, Systemic Lupus Erythematosus, Sepsis, and in Chronic Obstructive Pulmonary Disease (10-13).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Axl has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Axl present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Axl 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 Axl 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 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 Axl Microplate	898993	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Axl.	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 Axl Standard	898995	2 vials of recombinant human Axl in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new Standard for each assay. Discard after use.
Human Axl Conjugate	898994	21 mL of a polyclonal antibody specific for human Axl conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	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.
- Test tubes for dilution of standards and samples.
- Human Axl Controls (optional; R&D Systems®, Catalog # QC250).

If using cell lysate samples, the following are also required:

- Cell Lysis Buffer 17 (R&D Systems®, Catalog # 895943).
- PBS

PRECAUTIONS

Axl 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. 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.

Cell Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

Cerebrospinal Fluid - Freeze sample within one hour of collection to avoid aggregation. Store samples at ≤ -70 °C.

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 or heparin 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.

SAMPLE PREPARATION

Cerebrospinal fluid samples require a 20-fold dilution due to high endogenous levels. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5P (diluted 1:5)*.

Serum and plasma samples require a 50-fold dilution due to high endogenous levels. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD5P (diluted 1:5)*.

Urine samples require a 40-fold dilution due to high endogenous levels. A suggested 40-fold dilution is 10 µL of sample + 390 µL of Calibrator Diluent RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

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

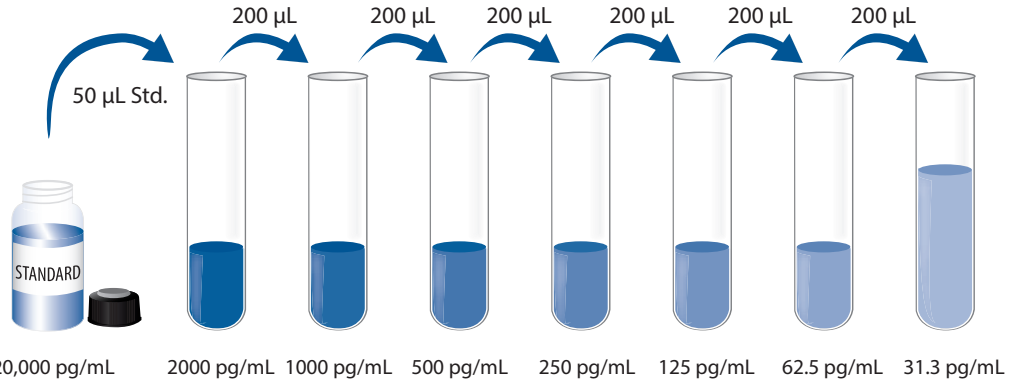
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5P (diluted 1:5) - Add 10 mL of Calibrator Diluent RD5P to 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5P (diluted 1:5).

Human Axl Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Axl Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5P (diluted 1:5) into the 2000 pg/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 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) 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.

Note: *Axl 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 50 µL of Assay Diluent RD1W 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 for a record of 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 Axl 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

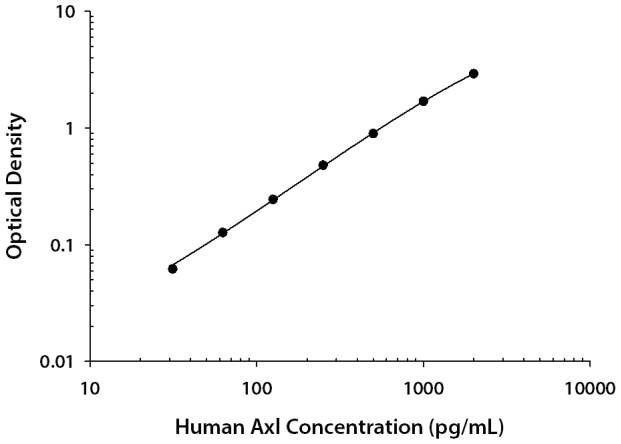
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 Axl 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 prior to assay, 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	0.007 0.008	0.008	—
31.3	0.069 0.071	0.070	0.062
62.5	0.134 0.135	0.135	0.127
125	0.251 0.254	0.253	0.245
250	0.479 0.497	0.488	0.480
500	0.888 0.919	0.904	0.896
1000	1.686 1.720	1.703	1.695
2000	2.889 2.966	2.928	2.920

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	249	565	1115	258	582	1131
Standard deviation	4.21	10.0	18.6	11.3	19.4	42.4
CV (%)	1.7	1.8	1.7	4.4	3.3	3.7

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	89	81-98%
Cell lysis buffer (n=1)	90	88-93%
Saliva (n=4)	94	82-101%

SENSITIVITY

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of human Axl ranged from 0.589-4.60 pg/mL. The mean MDD was 1.13 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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Axl were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Cell lysates* (n=4)	Cerebrospinal fluid* (n=4)	Serum* (n=4)
1:2	Average % of Expected	98	102	98	99
	Range (%)	95-102	93-108	95-104	98-101
1:4	Average % of Expected	97	102	96	101
	Range (%)	95-100	95-108	93-99	100-102
1:8	Average % of Expected	98	104	97	102
	Range (%)	97-98	99-108	95-99	100-104
1:16	Average % of Expected	96	106	95	98
	Range (%)	94-99	———	93-97	95-103

		EDTA plasma* (n=4)	Heparin plasma* (n=4)	Saliva (n=4)	Urine* (n=4)
1:2	Average % of Expected	103	101	101	95
	Range (%)	101-105	98-103	97-108	92-99
1:4	Average % of Expected	105	105	101	96
	Range (%)	103-106	102-109	99-105	94-98
1:8	Average % of Expected	105	105	105	97
	Range (%)	102-106	100-107	101-112	96-98
1:16	Average % of Expected	102	102	106	95
	Range (%)	96-106	96-106	101-115	94-97

*Samples were diluted prior to assay.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Axl manufactured at R&D Systems®.

SAMPLE VALUES

Cerebrospinal Fluid/Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Axl 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)
Cerebrospinal fluid (n=10)	18,248	5940-32,640	9182
Serum (n=30)	36,515	21,737-55,200	7435
EDTA plasma (n=30)	36,356	23,442-53,100	7262
Heparin plasma (n=30)	34,442	22,095-55,400	6917
Urine (n=10)	28,956	6560-56,680	18,115

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Saliva (n=12)	82.1	92	ND-188

ND=Non-detectable

Cell Culture Supernates:
U-87 MG human glioblastoma/astrocytoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 1 mM sodium pyruvate, until confluent. An aliquot of the cell culture supernate was removed, assayed for human Axl, and measured 16,440 pg/mL.

HepG2 human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for human Axl, and measured 117 pg/mL.

A172 human glioblastoma carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for human Axl, and measured 29,720 pg/mL.

Cell Lysates - A172 human glioblastoma carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. Cells were then washed with PBS and solubilized in Lysis Buffer 17 using 3-5 times the pellet volume and put on ice for 15 minutes. Tubes were centrifuged at 14,000 x g for 5 minutes to remove insoluble material. The remaining whole cell extract was removed, aliquoted into a clean test tube, and stored at ≤ 20 °C. Whole cell extract protein concentration was quantified using a total protein assay. 2.5 µg of the cell lysate was removed, assayed for human Axl, and measured 249 pg/mL.

Note: *Quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is 2-50 µg/well.*

SPECIFICITY

This assay recognizes natural and recombinant human Axl.

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

Recombinant human:

Dtk
EGFR
Gas6
GRB2
Mer
Protein S

Recombinant mouse:

Axl

Recombinant mouse Gas6 (Short Length) and recombinant mouse Gas6 (Full Length) do not cross-react in this assay but do interfere at concentrations > 10 ng/mL.

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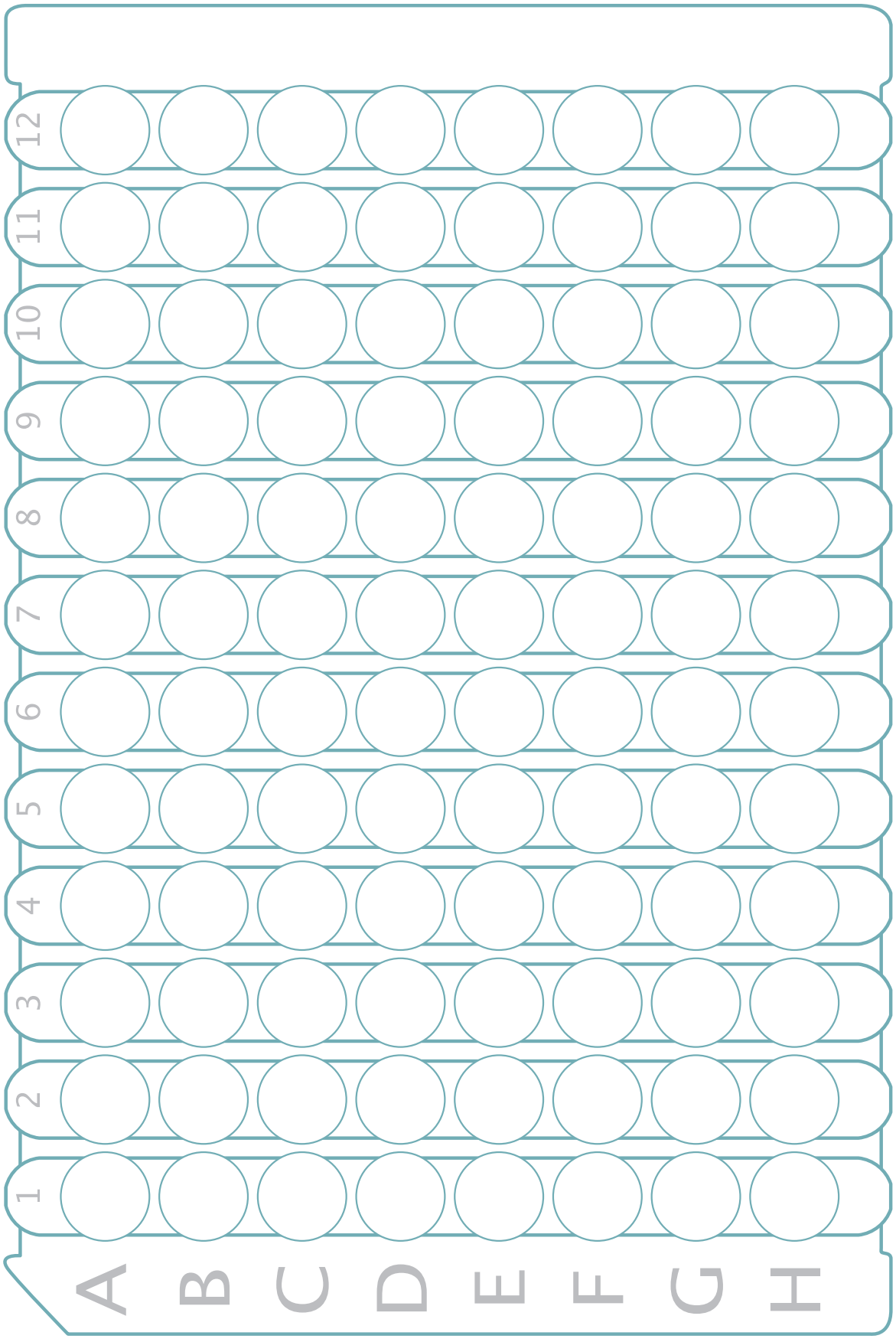
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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