Quantikine® ELISA

Human Cyr61/CCN1 Immunoassay

Catalog Number DCYR10

For the quantitative determination of human Cysteine-rich angiogenic inducer 61 (Cyr61) concentrations in cell culture supernates, serum, plasma, saliva, 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.

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INTRODUCTION

Cyr61, also known as CCN1 or IGFBP-10 (IGF-binding protein 10), is a multifunctional 40-45 kDa matricellular glycoprotein in the CCN (Cysteine-rich 61, Connective tissue growth factor, and Nephroblastoma overexpressed) family that also includes CTGF/CCN2, NOV/CCN3, WISP1 (Wnt1-inducible signaling protein 1; CCN4), WISP2/CCN5 and WISP3/CCN6 (1-3). Like other CCN family members, Cyr61 is a modular protein consisting of IGFBP, VWF, TSP, and cysteine knot domains (2, 3). Mature human Cyr61 shares 93% amino acid sequence identity with mouse and rat Cyr61. It is widely expressed during development and in adult tissues (1-4).

Cyr61 associates with the extracellular matrix (ECM) and with many cell surface molecules including integrins αVβ3, αVβ5, αMβ2, and α6β1, Syndecan-4, SOST/sclerostin, and heparan sulfate proteoglycans (2-8). It mediates the adhesion and migration of multiple cell types, and can promote vascular endothelial cell tubule formation and osteoblast differentiation (5-11). Its expression can be upregulated by mechanical and genotoxic stress, hypoxia, and cytokines such as TNF-α (1, 4,12). Cyr61 exhibits both tumorigenic and tumor suppressor properties, depending in part on whether it is acting on the tumor microenvironment or the tumor itself. It may be up regulated and promote tumorigenesis, angiogenesis, and metastasis in breast, renal, gastric, squamous cell, pancreatic, and colorectal carcinomas as well as in glioma (13-20). It is shown to promote migration of breast cancer cells by inducing expression of MMP-1 in stromal fibroblasts adjacent to the tumor (20). In contrast, it can suppress tumor growth in endometrial, hepatic, gastric, and non-small cell lung cancers and melanoma, and may be down regulated in these cancers (4, 21-24). Cyr61 is up regulated in injured skin and bone where it induces the expression of growth factors, cytokines, proteases, and integrins involved in wound repair (25, 26). It inhibits fibrosis in scars by inducing fibroblast senescence at the end of wound healing (27). It participates in TNF-α-induced apoptosis by contributing to induction of reactive oxygen species (28, 29).

Plasmin cleavage of ECM-bound Cyr61 releases a 28 kDa N-terminal fragment which retains the ability to promote endothelial cell migration and fibroblast mitogenesis (30, 31). Metalloproteinases such as MMP-14 can also mediate Cyr61 shedding (32). Released Cyr61 is found in serum or plasma, where it can increase the adhesion and migration of circulating $CD34^+$ hematopoietic precursor cells (33). Increased serum Cyr61 may correlate with metastasis in prostate cancer, while decreased placental and serum Cyr61 may correlate with failure of uterine vascular remodeling during preeclampsia (34, 35). It is found in bovine milk, and may be increased in the early stages of involution (36).

The Quantikine Human Cyr61/CCN1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Cyr61 in cell culture supernates, serum, plasma, saliva, and human milk. It contains CHO cell-expressed recombinant human Cyr61 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Cyr61 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 Cyr61.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Cyr61 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Cyr61 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for Cyr61 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 Cyr61 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
Cyr61 Microplate	894369	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against Cyr61.	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.*
Cyr61 Standard	894371	3 vials (25 ng/vial) of recombinant human Cyr61 in a buffer with preservatives; lyophilized.	Discard after use. Use a fresh standard for each assay.
Cyr61 Conjugate	894370	21 mL of a polyclonal antibody against Cyr61 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-36	895272	11 mL of a buffer with blue dye and 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 Cyr61 Controls (optional; available from R&D Systems).

PRECAUTIONS

Cyr61 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 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 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: *Heparin plasma is not recommended for use in this assay.*
Citrate plasma has not been validated for use in this assay.
Hemolyzed or icteric samples are not recommended 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.

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

SAMPLE PREPARATION

Human milk samples require at least a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD6-63.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

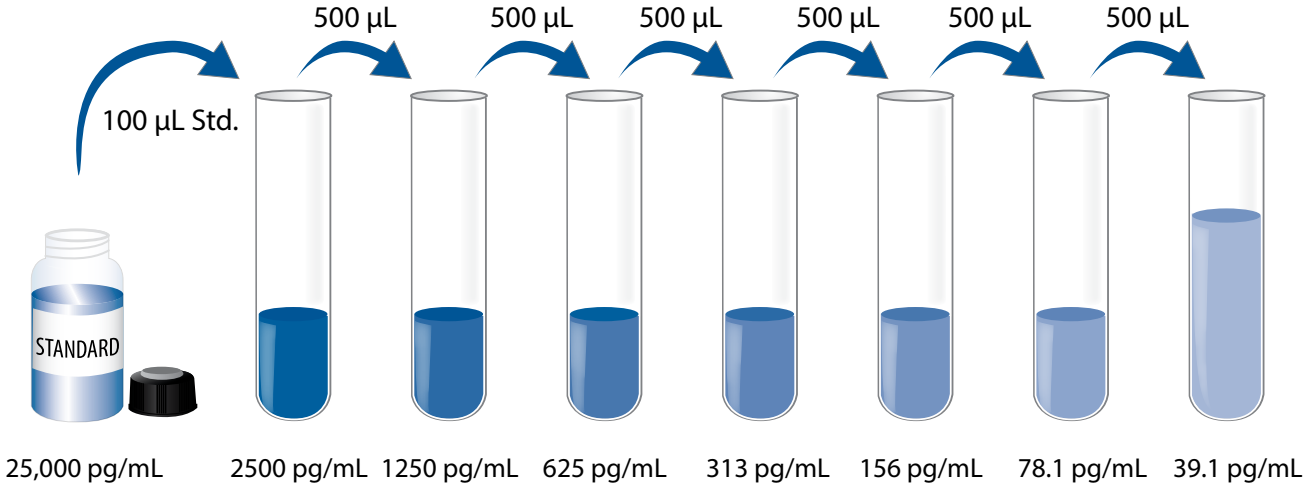
Note: *High concentrations of Cyr61 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into 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.

Cyr61 Standard - Reconstitute the Cyr61 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 25,000 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.

Use Polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD6-63 into the 2500 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 2500 pg/mL standard serves as the high standard. Calibrator Diluent RD6-63 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 samples, controls, and standards be assayed in duplicate.

Note: *High concentrations of Cyr61 are 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 RD1-36 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 Cyr61 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

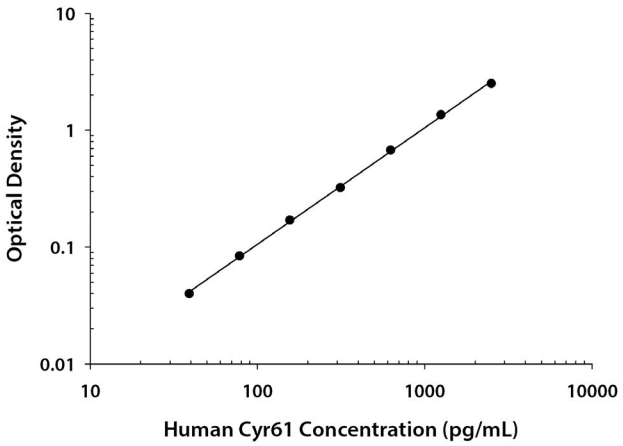
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Cyr61 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	0.008 0.010	0.009	—
39.1	0.047 0.051	0.049	0.040
78.1	0.092 0.094	0.093	0.084
156	0.178 0.180	0.179	0.170
313	0.322 0.339	0.331	0.322
625	0.675 0.692	0.684	0.675
1250	1.362 1.365	1.364	1.355
2500	2.495 2.538	2.517	2.508

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	256	721	1442	249	712	1445
Standard deviation	5.94	14.2	33.5	15.9	35.0	73.3
CV (%)	2.3	2.0	2.3	6.4	4.9	5.1

RECOVERY

The recovery of Cyr61 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	107	98-117%
Serum (n=4)	101	95-113%
EDTA plasma (n=4)	98	91-104%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Cyr61 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Human milk* (n=4)
1:2	Average % of Expected	97	100	94	98
	Range (%)	93-101	96-105	87-97	95-103
1:4	Average % of Expected	94	107	99	93
	Range (%)	88-97	106-108	96-103	90-99
1:8	Average % of Expected	91	109	102	91
	Range (%)	83-98	108-110	96-106	86-98
1:16	Average % of Expected	88	107	102	85
	Range (%)	80-94	104-109	94-108	80-95

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-eight assays were evaluated and the minimum detectable dose (MDD) of Cyr61 ranged from 0.786-3.80 pg/mL. The mean MDD was 1.54 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 CHO cell-expressed recombinant human Cyr61 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of Cyr61 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=35)	248	166-506	64.0
EDTA plasma (n=35)	200	124-370	42.7
Saliva (n=10)	140	41.0-570	158
Human milk (n=9)	13,483	1411-44,800	14,228

Cell Culture Supernates:

HeLa human cervical epithelial carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for levels of natural Cyr61, and measured 2594 pg/mL.

SK-HEP-1 human liver adenocarcinoma cells were cultured in MEM supplemented with 10% fetal bovine serum and sodium pyruvate. An aliquot of the cell culture supernate was removed, assayed for levels of natural Cyr61, and measured 20,865 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant and natural human Cyr61.

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 Cyr61 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CTGF
IGF-I
IGF-II
IL-6
Integrin α6
Integrin αMβ2
Integrin αVβ3
Integrin αVβ5
CCL2/MCP-1
CCL22/MDC
CCL25/TECK
CCL27/CTACK
NOV/CCN3
Syndecan-4
TGF-β1
TGF-β1.2
TGF-β2
TGF-β3
WISP-1/CCN4
WISP-3/CCN6

Recombinant mouse:

Cyr61
CCL12/MCP-5

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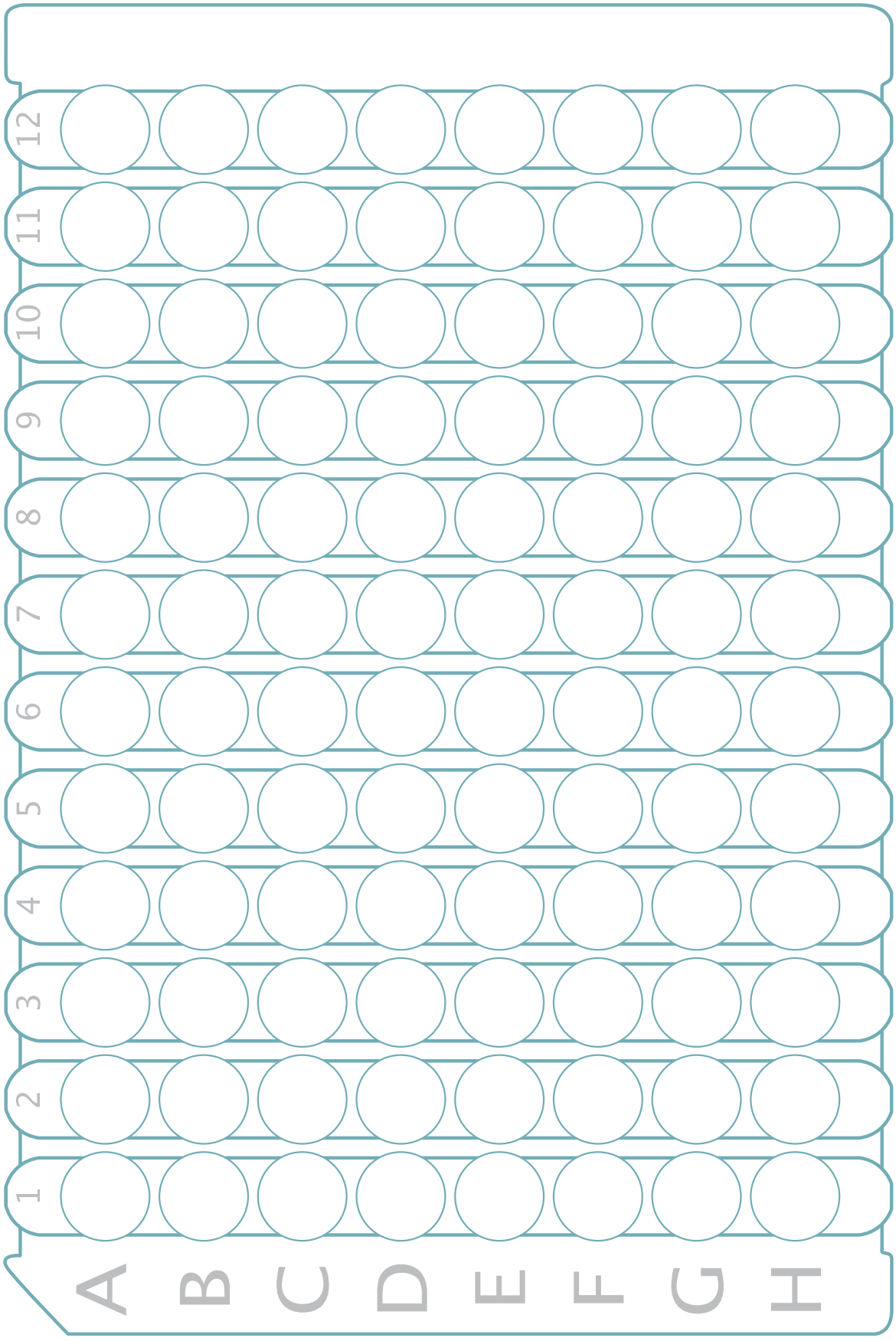
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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