Quantikine® ELISA

Human Amphiregulin Immunoassay

Catalog Number DAR00

For the quantitative determination of human Amphiregulin concentrations in cell culture supernates, cell lysates, 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

Amphiregulin (AR or AREG), also known as Schwannoma-derived growth factor (SDGF), is a widely-expressed member of the epidermal growth factor (EGF) family of growth factors. This family also includes TGF-α, HB-EGF, Betacellulin (BTC), Epiregulin, and the Neuregulins 1 through 4. All EGF family members are synthesized as type I transmembrane precursors and contain one or several EGF-like domains in their extracellular region (1-4). Human Amphiregulin is synthesized as an approximately 50 kDa transmembrane glycoprotein that contains an 80 amino acid (aa) N-terminal propeptide, a heparin-binding domain, one EGF-like domain, a 23 aa transmembrane segment, and a 31 aa cytoplasmic domain (5). Mature human Amphiregulin shares 78% and 76% aa sequence identity with mouse and rat Amphiregulin, respectively. Several Amphiregulin fragments ranging from 9 kDa to 43 kDa are produced by proteolytic cleavage of the transmembrane proprotein (6-9). Shedding of bioactive Amphiregulin is mediated by ADAM17/TACE and ADAM10 (10-12). Soluble Amphiregulin is released in response to ATP stimulation of dendritic cells and in response to CXCL12 stimulation of prostate epithelial cells (12, 13). The intracellular distribution of proAmphiregulin, cell surface shedding, and internalization of the residual fragments are altered by cellular infection by Neisseria (9, 14).

Amphiregulin exerts autocrine and paracrine activities through EGF R and ErbB2 (15-19). Its interaction with heparan sulfate proteoglycans enhances receptor activation and biological effect (17, 20). Amphiregulin functions as a mitogen for epithelial cells, keratinocytes, vascular smooth muscle cells, and fibroblasts (5, 13, 16-19, 21, 22). It plays an important role in branching morphogenesis of the lung and mammary gland (16, 17, 21), PGE_2-induced regeneration of the intestinal epithelium (19), and suppression of Fas-mediated liver damage (6).

Amphiregulin is elevated in sputum during asthmatic attacks and in rheumatoid but not osteoarthritis synovial fluid (22, 23). Transmembrane and soluble forms are upregulated by activated basophils, mast cells, and Th2 cells (24-26). Amphiregulin promotes inflammatory cytokine production and the clearance of nematode infections (22, 26). In cancer, Amphiregulin is upregulated by tumor-associated dendritic cells (13, 27). It promotes tumor progression by increasing proliferation, invasion, and angiogenesis and also contributes to tumor cell cisplatin resistance (2, 27, 28). In humans, Amphiregulin additionally promotes oocyte maturation and cumulus cell expansion and is elevated in the follicular fluid following exposure to HCG (29). It is upregulated in the rodent reproductive tract around the time of embryo implantation in the uterus and at mid-pregnancy (30, 31).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Amphiregulin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Amphiregulin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Amphiregulin 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 Amphiregulin 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
Amphiregulin Microplate	894412	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against Amphiregulin.	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.*
Amphiregulin Standard	894414	2 vials (10 ng/vial) of recombinant human Amphiregulin in a buffered protein base with preservatives; lyophilized.	Use a new Standard for each assay. Discard after use.
Amphiregulin Conjugate	894413	21 mL of a polyclonal antibody against Amphiregulin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-14	895180	12 mL of a buffered protein base with preservatives. *May contain a precipitate. Mix well before and during use.*	
Calibrator Diluent RD5-18	895335	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.
- Human Amphiregulin Controls (optional; available from R&D Systems).

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

- Cell Lysis Buffer 2 (R&D Systems, Catalog # 895347).
- PBS

PRECAUTIONS

Amphiregulin 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.

Cell Lysates - Prior to assay, cells must be lysed according to the directions in the Cell Lysis Procedure.

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.

CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Wash cells three times in cold PBS.
2. Resuspend cells at 1 x 10^7 cells/mL of Cell Lysis Buffer 2.
3. Incubate with gentle agitation for up to 60 minutes at room temperature.
4. Centrifuge at 8000 x g for 5 minutes to remove cell debris.
5. Assay immediately or aliquot the lysis supernates and store at ≤ -20 °C until ready for use.

REAGENT PREPARATION

The Conjugate must remain at 2-8 °C. Bring all remaining reagents to room temperature before use.

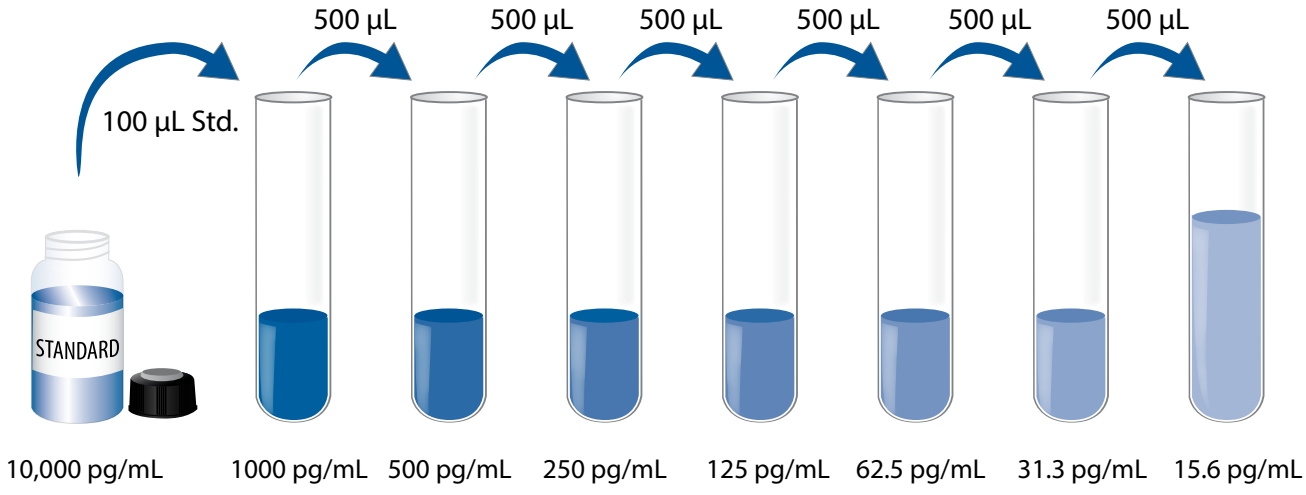
Note: *Amphiregulin is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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.

Amphiregulin Standard - Reconstitute the Amphiregulin Standard with 1.0 mL of deionized or distilled water. 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 5 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-18 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 RD5-18 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

The Conjugate must remain at 2-8 °C. Bring all remaining reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *Amphiregulin 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 RD1-14 to each well. *Assay diluent RD1-14 may contain a precipitate. Mix well before and during use.*
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 **cold** Amphiregulin Conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at 2-8 °C on the benchtop.**
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

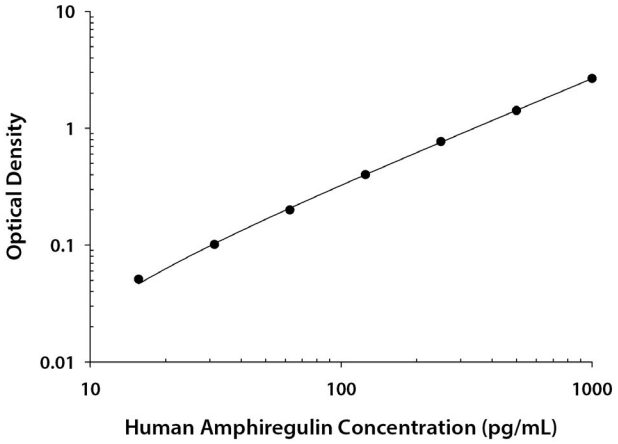
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Amphiregulin 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	0.012 0.013	0.013	—
15.6	0.062 0.065	0.064	0.051
31.3	0.112 0.115	0.114	0.101
62.5	0.211 0.215	0.213	0.200
125	0.409 0.416	0.413	0.400
250	0.780 0.783	0.782	0.769
500	1.427 1.432	1.430	1.417
1000	2.643 2.700	2.672	2.659

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)	102	314	631	99.4	308	614
Standard deviation	5.63	10.5	22.4	9.85	20.5	28.8
CV (%)	5.5	3.3	3.5	9.9	6.7	4.7

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	95	88-101%
Serum (n=4)	95	85-103%
EDTA plasma (n=4)	93	82-105%
Heparin plasma (n=4)	94	82-103%
Saliva (n=4)	104	98-113%
Urine (n=4)	88	75-99%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Amphiregulin 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=3)	Serum (n=3)	EDTA plasma (n=3)	Heparin plasma (n=3)	Saliva (n=4)	Urine (n=4)
1:2	Average % of Expected	102	100	102	102	105	99	102
	Range (%)	100-104	97-103	102-103	102-103	103-109	94-106	96-106
1:4	Average % of Expected	104	101	107	102	111	102	110
	Range (%)	100-106	95-108	104-110	95-106	103-116	98-109	103-114
1:8	Average % of Expected	104	101	109	110	115	106	112
	Range (%)	101-106	99-104	103-113	102-117	107-120	99-112	105-115
1:16	Average % of Expected	102	———	104	100	108	99	105
	Range (%)	96-109	———	101-107	94-106	97-118	92-107	96-109

*Samples were diluted prior to assay.

SENSITIVITY

Forty-one assays were evaluated and the minimum detectable dose (MDD) of human Amphiregulin ranged from 0.500-3.56 pg/mL. The mean MDD was 1.43 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 *E. coli*-expressed recombinant human Amphiregulin (aa 101-198 of accession # Q5U026) manufactured at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Thirty-five serum, EDTA plasma, and heparin plasma samples from apparently healthy volunteers were evaluated for the presence of human Amphiregulin in this assay. Amphiregulin was detectable in one volunteer and measured 39.2 pg/mL, 64.5 pg/mL, and 31.4 pg/mL respectively. No medical histories were available for the donors used in this study.

Thirteen urine samples were evaluated for the presence of Amphiregulin in this assay. All samples were non-detectable.

Sample Type	Mean (pg/mL)	% Detectable	Range (pg/mL)
Saliva (n=9)	26.9	67	ND-56.1

ND=Non-detectable

Cell Culture Supernates/Cell Lysates:
T47D human breast cancer cells were cultured for 3 days in hybridoma serum-free media containing porcine transferrin, EX-CYTE® VLE, 10% Primatone® HS, streptomycin and supplemented with 5% fetal bovine serum.

MDA-MB-453 human breast cancer cells were cultured until confluent in RPMI and supplemented with 10% fetal bovine serum and 2 mM L-glutamine until confluent.

Aliquots of cell culture supernates and cell lysates were removed and assayed for human Amphiregulin.

Cell Line	Cell Culture Supernates (pg/mL)	Cell Lysates (pg/mL)
T47D	982	315
MDA-MB-453	31.2	196

SPECIFICITY

This assay recognizes natural and recombinant human Amphiregulin. Recombinant human Amphiregulin (aa 101-198) and recombinant human Amphiregulin (aa 107-184) were tested in this assay. Both variants were detectable.

The factors listed below were prepared at 10 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 10 ng/mL in a mid-range recombinant human Amphiregulin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Betacellulin
EGF
EGF R
Epiregulin
ErbB2/Her2
HB-EGF
NRG1/HRG1
NRG1-α1/HRG1-α1 EGF domain
NRG1-β1/HRG1-β1
NRG1-β1/HRG1-β1 EGF domain
NRG1 Isoform GGF2
NRG1 Isoform SMDF
NRG2
TGF-α

Recombinant mouse:

Amphiregulin

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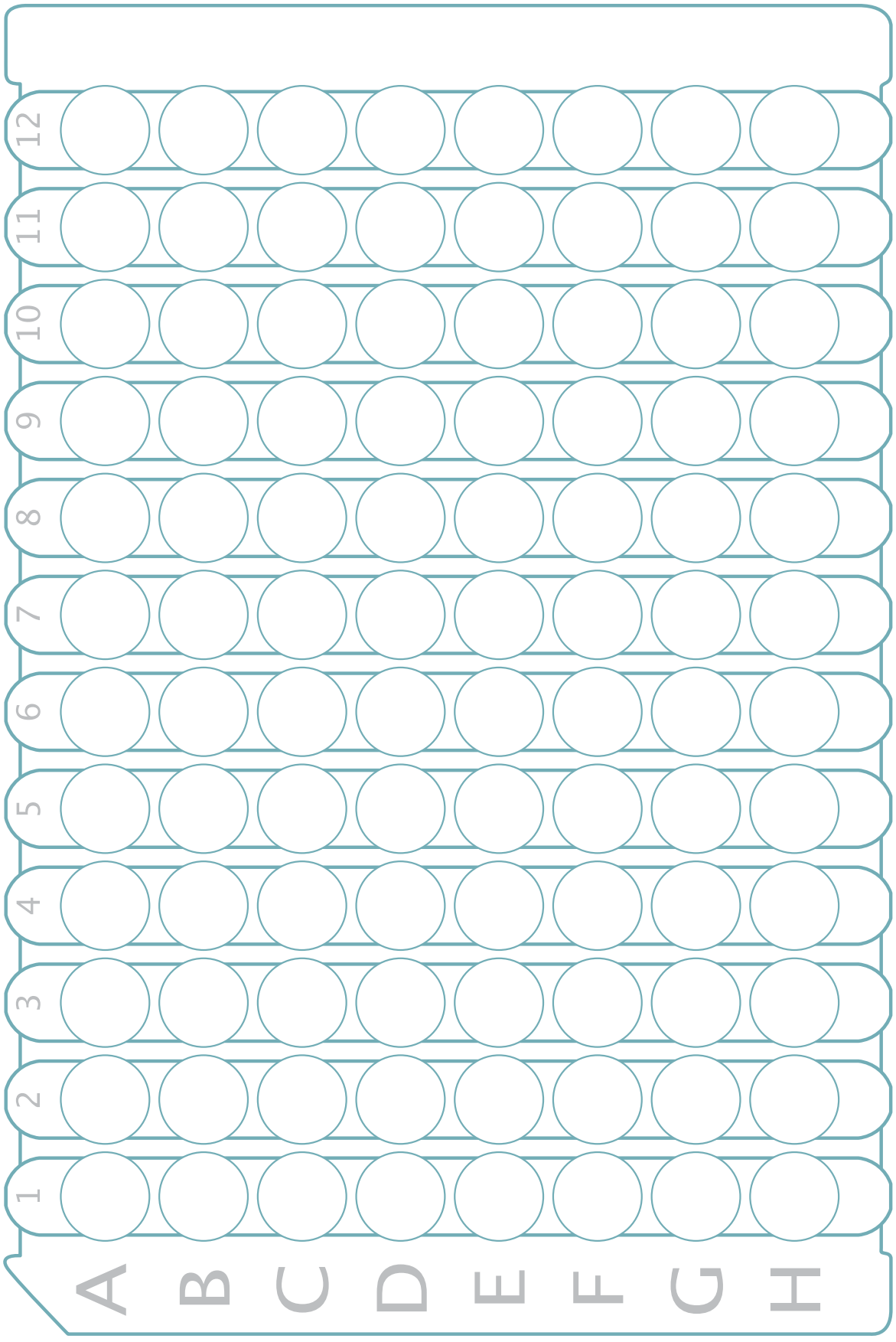
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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