Quantikine® ELISA

Human Angiogenin Immunoassay

Catalog Number DAN00

For the quantitative determination of human Angiogenin (ANG) concentrations in cell culture supernates, serum, and plasma.

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

Human Angiogenin (ANG) is a single chain, 14 kDa, non-glycosylated polypeptide that is classified as a member of the RISBASE family of ribonucleases (1-3). RISBASE family molecules exhibit both ribonuclease activity and special biological actions. ANG is 123 amino acid (aa) residues in length and contains three intra-chain disulfide bonds. When optimally aligned with pancreatic ribonuclease, ANG shows 35% sequence identity. This is reflected in its modest RNA catalytic activity (2-4). It has been suggested that angiogenin binding may result in significant RNase activity (5, 6). Relative to human ANG, mouse ANG is 75% identical at the aa level (2) and human ANG is reported to be active in the murine system (7). Cells known to express ANG include vascular endothelial cells (8), smooth muscle cells (8), fibroblasts (8), colonic columnar epithelium (9), lymphocytes (9), and primary adenocarcinoma cells as well as select tumor cell lines (9, 10). The receptor for angiogenin is unknown, however, it would appear that actin, either as a receptor or as a binding molecule, is essential for the expression of angiogenin's effects (11-13).

Functionally, ANG is most commonly associated with the angiogenic process. It has been suggested that ANG first binds to actin, followed by dissociation of the actin-ANG complex and subsequent activation of tissue plasminogen activator. This generates plasmin which is known to degrade basement membrane laminin and fibronectin (14, 15). Destruction of the basement membrane is considered a prerequisite for endothelial cell migration during neovascularization (16, 17). Although ANG would appear to act principally extravascularly or perivascularly, circulating ANG has been detected in normal serum in ng/mL concentrations (18, 19). Under pathological conditions, elevated levels of ANG have been detected in patients with pancreatic cancer (18) and arterial occlusive disease (19).

The Quantikine® Human Angiogenin Immunoassay is a 2.5 hour solid-phase ELISA designed to measure human ANG in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human ANG and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human ANG 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 ANG.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human ANG has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ANG present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human ANG 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 ANG bound. 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 ANG Microplate	890102	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human ANG.	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 ANG Standard	890104	Recombinant human ANG in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at 2-8 °C.* Do not freeze.
Human ANG Conjugate	890103	21 mL of a polyclonal antibody specific for human ANG 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 RD5C Concentrate	895046	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
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.
- 100 mL and 500 mL graduated cylinders.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human ANG Controls (optional; R&D Systems®, Catalog # QC21).

PRECAUTIONS

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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 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.

Note: *Human serum used in the preparation of cell culture media may contain high levels of ANG. Because of the low species cross-reactivity of this kit, human ANG levels in culture media containing 10% bovine or fetal bovine serum can be assayed without interference.*

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.

SAMPLE PREPARATION

Use polypropylene tubes. Do not use glass.

Serum and plasma samples require a 200-fold dilution. A suggested 200-fold dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD5C (diluted 1:5)*. Complete the 200-fold dilution by adding 25 µL of the diluted sample to 475 µL Calibrator Diluent RD5C (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

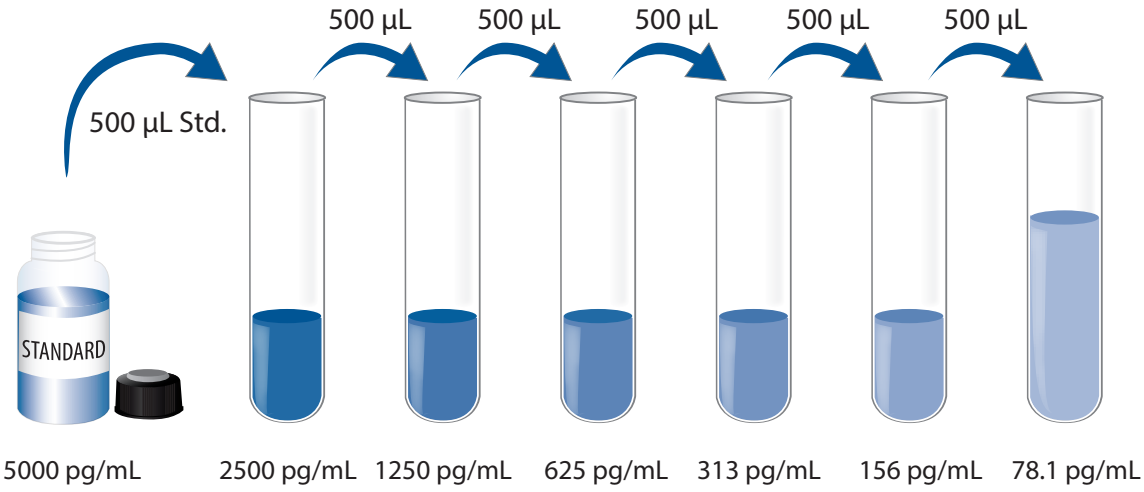
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5C (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5C Concentrate to 80 mL of deionized or distilled water to yield 100 mL of Calibrator Diluent RD5C (diluted 1:5).

Human ANG Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human ANG Standard with Calibrator Diluent RD5C (diluted 1:5). This reconstitution produces a stock solution of 5000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD5C (diluted 1:5) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human ANG Standard (5000 pg/mL) serves as the high standard. Calibrator Diluent RD5C (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 samples, controls, and standards 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 RD1W to each well.
4. Add 200 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice for a total of three 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 ANG Conjugate to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **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

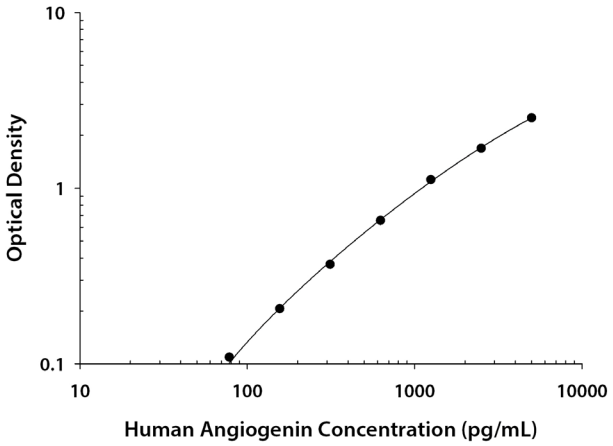
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 ANG 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	0.019 0.020	0.020	—
78.1	0.128 0.130	0.129	0.109
156	0.223 0.228	0.226	0.206
313	0.387 0.388	0.388	0.368
625	0.670 0.678	0.674	0.654
1250	1.123 1.152	1.138	1.118
2500	1.687 1.723	1.705	1.685
5000	2.521 2.546	2.534	2.514

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)	356	717	1739	664	1911	3671
Standard deviation	10	24	50	47	166	309
CV (%)	2.8	3.3	2.9	7.1	8.7	8.4

RECOVERY

The recovery of human ANG 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	97-117%
Serum* (n=10)	103	84-126%
EDTA plasma* (n=10)	101	79-121%
Heparin plasma* (n=10)	102	80-137%
Citrate plasma* (n=10)	102	84-126%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

The minimum detectable dose (MDD) of human ANG is typically less than 6.0 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 *E. coli*-expressed recombinant human Angiogenin produced at R&D Systems®.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human ANG in each matrix were diluted with calibrator diluent and assayed. Results from typical sample dilutions are shown.

Mouse Samples	Dilution	Observed (pg/mL)	Expected (pg/mL)	$\frac{\text{Observed}}{\text{Expected}} \times 100$
Cell culture media	Neat	4546	———	———
	1:2	2184	2273	96
	1:4	1131	1136	100
	1:8	560	568	99
	1:16	265	284	93
Serum*	Neat	447,000	———	———
	1:2	207,000	224,000	92
	1:4	104,000	112,000	93
	1:8	53,000	56,000	95
	1:16	24,000	28,000	86
EDTA plasma*	Neat	652,000	———	———
	1:2	317,000	326,000	97
	1:4	155,000	163,000	95
	1:8	82,000	82,000	100
	1:16	37,000	41,000	90
Heparin plasma*	Neat	310,000	———	———
	1:2	141,000	155,000	91
	1:4	68,000	78,000	87
	1:8	34,000	39,000	87
	1:16	15,000	20,000	75
Citrate plasma*	Neat	328,000	———	———
	1:2	148,000	164,000	90
	1:4	79,000	82,000	96
	1:8	37,000	41,000	90
	1:16	17,000	20,000	85

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human ANG 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=40)	360,000	196,000-437,000	57,000
EDTA plasma (n=40)	321,000	160,000-476,000	75,000
Citrate plasma (n=40)	260,000	160,000-370,000	49,000
Heparin plasma (n=40)	287,000	156,000-452,000	67,000

Cell Culture Supernates:
Human peripheral blood mononuclear cells ($1x10^6$ cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and stimulated with 10 µg/mL PHA and recombinant human IL-2. Aliquots of the cell culture supernate were removed on days 1, 3, and 5 and assayed for levels of human ANG.

Day 1 (pg/mL)	Day 3 (pg/mL)	Day 5 (pg/mL)
0	149	409

Cell culture supernates from confluent HT-29 human colon adenocarcinoma cells, were sampled after 24 hours and measured in this assay. The levels of human ANG secreted into the media averaged 567 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human ANG.

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

Recombinant human:

EGF
G-CSF
GM-CSF
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-6
IL-7
IL-8
LIF
MIP-1α
MIP-1β
TGF-α
TGF-β1
TNF-α
TNF-β

Recombinant mouse:

EGF
GM-CSF
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
MIP-1α
MIP-1β

Natural proteins:

bovine panc. RNase A
bovine panc. RNase B
human plac. RNase Inhib.

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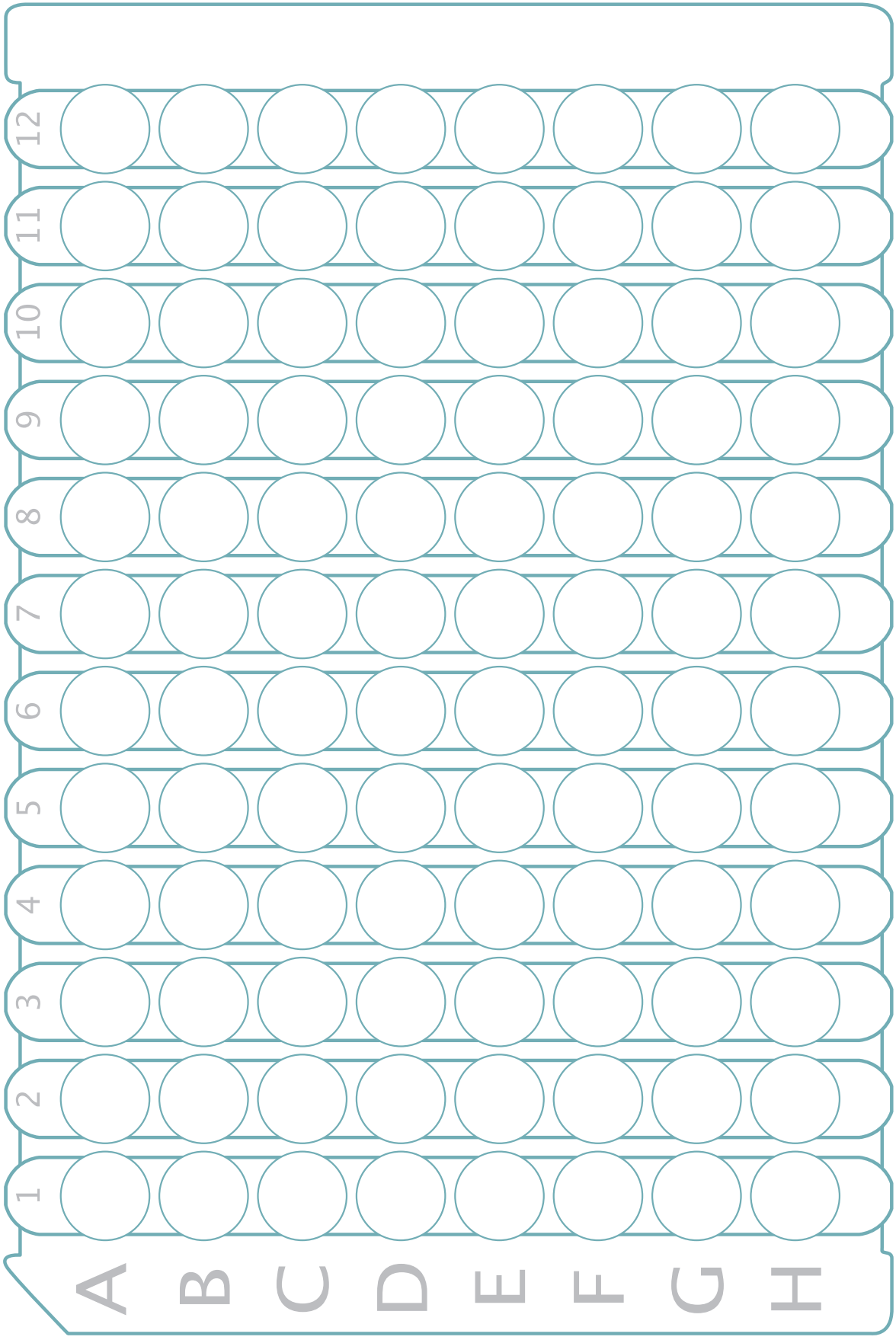
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES