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R&D SYSTEMS

Quantikine™ ELISA

Mouse/Rat Angiopoietin-2 Immunoassay

Catalog Number MANG20

For the quantitative determination of mouse and rat Angiopoietin-2 concentrations in cell culture supernates, tissue lysates, 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

Angiopoietin-2 is an approximately 70 kDa secreted glycoprotein that plays a complex role in angiogenesis, inflammation, and vascular development. Mature mouse Angiopoietin-2 contains a coiled-coil domain that mediates multimerization and a C-terminal Fibrinogen-like domain that mediates receptor binding (1, 2). It forms disulfide-linked dimers, trimers, tetramers, and pentamers (2-4). Mature mouse Angiopoietin-2 shares 86% and 96% amino acid (aa) sequence identity with human and rat Angiopoietin-2, respectively. Angiopoietin-2 is expressed in vascular endothelial (EC) and smooth muscle cells in highly angiogenic tissues (*e.g.* placenta, ovaries, uterus, and tumor vasculature) (5-9), lung epithelial cells, differentiating myotubes, and neural progenitor cells (10-12). It is upregulated by cellular stress (7, 10, 13, 14) and circulates at elevated levels in sepsis and acute lung injury (10, 15).

Both Angiopoietin-2 and the related Angiopoietin-1 are ligands for the receptor tyrosine kinase Tie-2 (5). Angiopoietin-2 acts a Tie-2 agonist, although it can also partially antagonize the activation of Tie-2 by Angiopoietin-1 (5, 7, 15-17). Angiopoietin-2 promotes EC survival, proliferation, and migration and also promotes sprouting angiogenesis (7, 17, 18). It induces the loss of pericytes from vessel walls and an increase of vascular permeability (14, 15, 19-21). Angiopoietin-2 is required for the development of lymphatic vessels as well as the postnatal remodeling of both lymphatic and vascular vessels (6, 19, 22). In cancer, Angiopoietin-2 production is induced by tumor-derived VEGF and augments VEGF-induced angiogenesis and tumor growth (8, 19, 23). In addition, Angiopoietin-2 modulates cell adhesion and promotes tumor cell migration through interactions with Integrins containing α3, α5, αV, β1, or β3 chains (24-26). It promotes leukocyte adhesion to the vascular endothelium and extravasation to inflammatory sites (10, 13, 19, 27). Aside from the circulatory system, Angiopoietin-2 promotes the differentiation of myoblasts, regulatory T cells, and neurons (11, 12, 28).

The Quantikine™ Mouse/Rat Angiopoietin-2 Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse and rat Angiopoietin-2 in cell culture supernates, tissue lysates, serum, and plasma. It contains CHO cell-expressed recombinant mouse Angiopoietin-2 and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant factor accurately. Results obtained using natural Angiopoietin-2 showed dose-response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit can be used to determine relative mass values for natural Angiopoietin-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse/rat Angiopoietin-2 has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any Angiopoietin-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat Angiopoietin-2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of Angiopoietin-2 bound in the initial step. The sample values are then read off the standard curve.

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 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.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

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
Mouse/Rat Angiopoietin-2 Microplate	894685	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse/rat Angiopoietin-2.	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.*
Mouse/Rat Angiopoietin-2 Standard	894687	Recombinant mouse Angiopoietin-2 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 ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Mouse/Rat Angiopoietin-2 Control	894688	2 vials of recombinant mouse Angiopoietin-2 in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	Use a fresh control for each assay. Discard after use.
Mouse/Rat Angiopoietin-2 Conjugate	894686	12 mL of a polyclonal antibody specific for mouse/rat Angiopoietin-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-40	895513	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Used diluted 1:4 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	895174	23 mL of diluted hydrochloric 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
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples

SUPPLIES REQUIRED FOR TISSUE LYSATE SAMPLES

- PBS
- Sample Diluent Concentrate 2 (2X) (R&D Systems™, Catalog # DYC002 or 1% NP-40 Alternative, 20 mM Tris (pH 8.0), 137 mM NaCl,10% glycerol, 2 mM EDTA, 1 mM activated Sodium Orthovanadate). Dilute 2-fold in deionized or distilled water immediately before use.

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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates - Tissues must be lysed prior to assay as described in the Sample Values section.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C.

Note: *Repeated freeze-thaw cycles will cause sample variation.*

Plasma - Collect plasma using EDTAor heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 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.*

SAMPLE PREPARATION

Mouse serum and plasma samples require a 40-fold dilution. A suggested 40-fold dilution is 10 µL of sample + 390 µL of Calibrator Diluent RD5-26 (diluted 1:4).*

Rat serum and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 15 µL of sample + 135 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

Mouse and rat tissue lysate samples require a 10-fold dilution. A suggested 10-fold dilution is 15 µL of lysate + 135 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse/Rat Angiopoietin-2 Control - Reconstitute the control with 1 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

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.

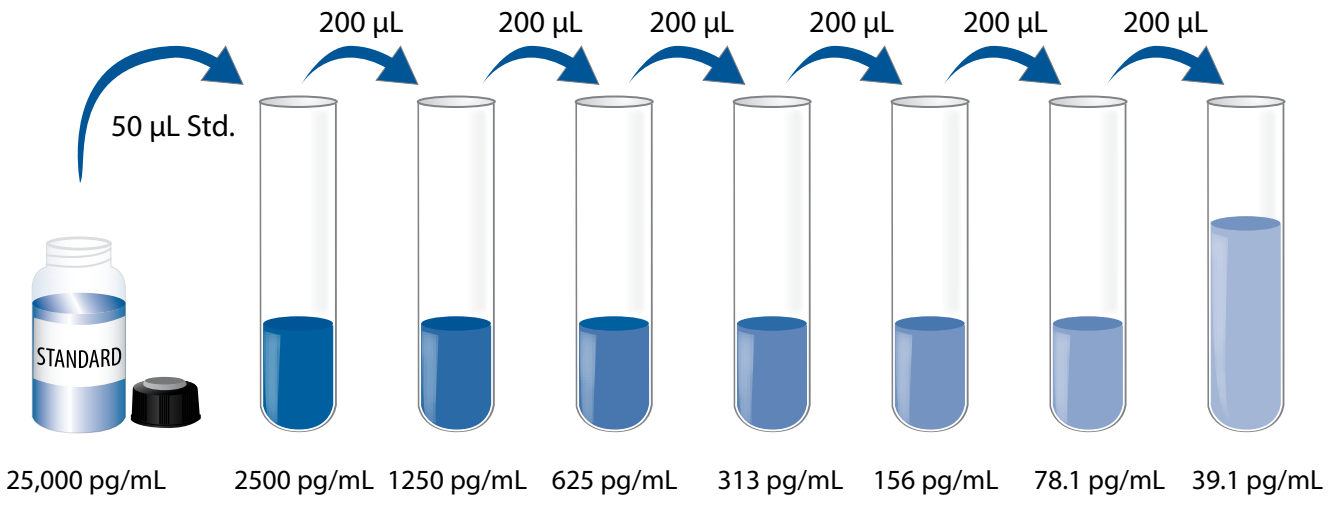
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:4) - Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Mouse/Rat Angiopoietin-2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse/Rat Angiopoietin-2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 25,000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-26 (diluted 1:4) into the 2500 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 2500 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) serves as the zero standard (0 pg/mL).

Note: *Diluted standard curve must be used within 30 minutes.*



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, control, and samples be assayed in duplicate.

1. Prepare all reagents, standards, control, and samples as directed by 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 RD1-40 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 100 µL of Mouse/Rat Angiopoietin-2 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 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 100 µL of Stop Solution to each well. 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

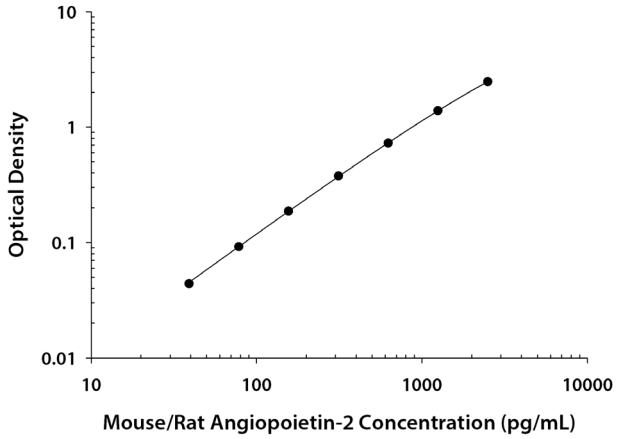
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 mouse/rat Angiopoietin-2 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	0.010 0.010	0.010	—
39.1	0.054 0.054	0.054	0.044
78.1	0.100 0.103	0.102	0.092
156	0.196 0.198	0.197	0.187
313	0.375 0.398	0.387	0.377
625	0.722 0.750	0.736	0.726
1250	1.354 1.442	1.398	1.388
2500	2.457 2.512	2.485	2.475

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 kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	119	290	831	116	283	841
Standard deviation	3.27	9.05	22.2	9.84	23.2	63.1
CV (%)	2.7	3.1	2.7	8.5	8.2	7.5

RECOVERY

The recovery of mouse/rat Angiopoietin-2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Mouse Samples	Average % Recovery	Range
Cell culture samples (n=4)	114	108-120%
Serum* (n=2)	113	109-117%
EDTA plasma* (n=2)	105	101-110%
Heparin plasma* (n=2)	107	103-111%

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse/rat Angiopoietin-2 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

Mouse Samples		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	94	97	97	100
	Range (%)	90-98	95-100	94-99	97-101
1:4	Average % of Expected	90	97	98	100
	Range (%)	84-96	95-98	95-101	94-104
1:8	Average % of Expected	90	94	98	101
	Range (%)	87-93	91-96	94-106	94-109
1:16	Average % of Expected	91	93	97	102
	Range (%)	86-92	89-95	91-110	95-119

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

Note: *Rat samples were evaluated and no significant difference in linearity or recovery was observed from the data above.*

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of mouse/rat Angiopoietin-2 ranged from 0.879-4.98 pg/mL. The mean MDD was 2.13 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 CHO cell-expressed recombinant mouse Angiopoietin-2 produced at R&D Systems™.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse/rat Angiopoietin-2 in this assay.

Mouse Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	19,340	11,440-27,254	5582
EDTA plasma (n=5)	25,258	22,072-29,617	2743
Heparin plasma (n=5)	23,890	15,858-38,240	9019

Rat Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	4904	2630-9910	2384
EDTA plasma (n=6)	3560	2711-4420	727
Heparin plasma (n=6)	5233	2840-7120	1694

Cell Culture Supernates - Lungs from individual mice and rats were removed and rinsed in PBS and kept on ice. The tissue was homogenized using a tissue homogenizer and seeded into media containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. Aliquots of the cell culture supernates were removed and assayed for mouse/rat Angiopoietin-2.

Tissue	(pg/mL)
Mouse lung	415
Rat lung	131

Tissue Lysates - Placentas from mice were chopped into 5 mm pieces. 10-15 mL of Sample Diluent Concentrate 2 (diluted 1:2) was added to 5 mL of placenta. The placenta was homogenized with a tissue homogenizer and debris was then removed by centrifugation. An aliquot of the tissue lysate was removed, assayed for mouse Angiopoietin-2, and measured 7367 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat Angiopoietin-2.

The factors listed below were prepared at 100 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 100 ng/mL in a mid-range Angiopoietin-2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Angiopoietin-1
Angiopoietin-3
Angiopoietin-like 1
Angiopoietin-like 2
Angiopoietin-like 3
Angiopoietin-like 4
Angiopoietin-like 6
Angiopoietin-like 7
Tie-2

Recombinant human:

Angiopoietin-1
Angiopoietin-2
Angiopoietin-4

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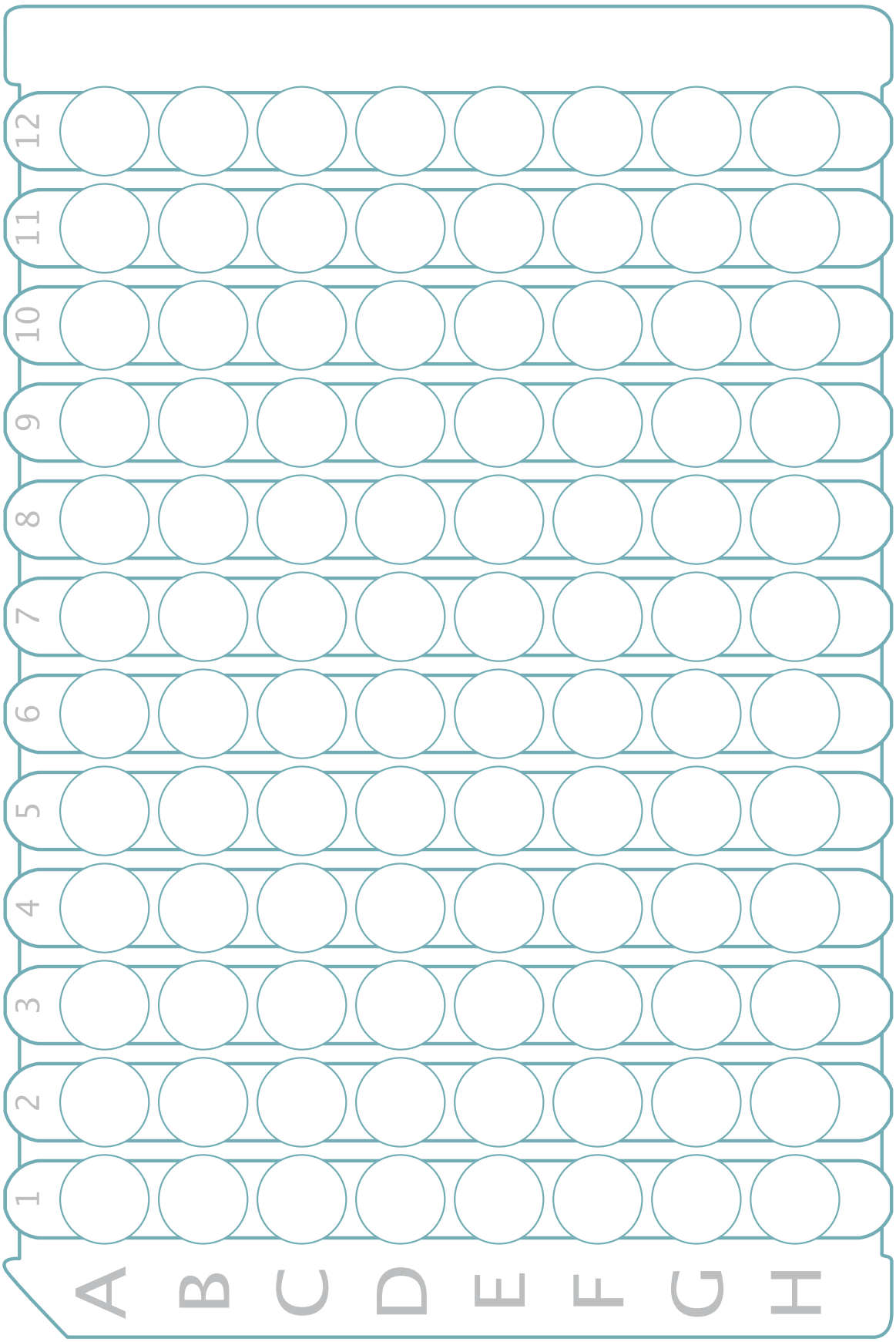
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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