Quantikine® ELISA

Human Angiopoietin-2 Immunoassay

Catalog Number DANG20
SANG20
PDANG20

For the quantitative determination of human Angiopoietin-2 (Ang-2) concentrations in cell culture supernates, serum, plasma, and saliva.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

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INTRODUCTION

Angiopoietin-2 (Ang-2) is a secreted glycoprotein that plays a complex role in vascular development. Human and mouse Ang-2 share 85% amino acid (aa) identity and Ang-2 is approximately 60% identical to its homolog, Ang-1. Ang-2 contains an N-terminal coiled-coil domain, 58-254 amino acids and a C-terminal fibrinogen-like domain, 281-467 aa, that is also found in other proteins including fibrinogen, tenascin, and ficolin (1). Located at the N-terminus are stretches of hydrophobic residues typical of secretory signal sequences. While Ang-2 is widely expressed in the mouse embryo, it is restricted postnatally to pro-angiogenic regions including the placenta, ovaries, and uterus (1-4).

Although likely playing different functional roles, both Ang-1 and Ang-2 are ligands for the endothelial cell receptor tyrosine kinase Tie-2. The regulation of Tie-2 activity by Ang-2 is complex, leading to inhibition in certain cell types or conditions, and activation in others (1, 5, 6). Initial *in vitro* studies using immortalized human umbilical vein endothelial cells (HUVECs) demonstrated that Ang-1, but not Ang-2, could stimulate Tie-2 tyrosine phosphorylation and suggested that Ang-2 could act as a competitive inhibitor of Ang-1 signaling (1). Consistent with its role as an Ang-1/Tie-2 inhibitor, transgenic overexpression of Ang-2 leads to disruption of embryonic blood vessel formation, a phenotype similar to that of Tie-2 knockouts (1, 7). In contrast, altering incubation times or elevating Ang-2 concentrations, can under some circumstances, result in Tie-2 activation in HUVECs (5, 6). It has also been proposed that Ang-2 may play a pro-angiogenic role by mediating destabilizing interactions between endothelial and perivascular cells, enhancing the effects of pro-angiogenic proteins including vascular endothelial growth factor (VEGF) (1, 8). Alone, Ang-2 promotes endothelial cell death and vessel regression, but can act in synergy with VEGF to promote new vessel formation (9-11). Mouse knockout studies have attempted to further clarify the role of Ang-2 *in vivo* (12). Ang-2 is not essential for development of the embryonic vasculature, although Ang-2$^{-/-}$ mice generally die within 2 weeks of birth. Vascular regression and sprouting that normally accompanies development of the neonatal eye does not occur in Ang-2$^{-/-}$ mice (12). Moreover, profound defects are observed in the postnatal development of lymphatic vessels. Defects in the eye capillary system, but not the lymphatic system, are rescued by replacement of the Ang-2 gene with Ang-1, further supporting the notion that Ang-2 may exhibit varying activities that are context dependent (12).

Ang-2 has been implicated in cancer development due to its role in angiogenesis. Ang-2-mediated destabilization of mature vessel structures accompanied by the presence of VEGF, may contribute to the angiogenesis associated with tumor tissues (13). Several studies have examined Ang-2 expression in an assortment of human cancers. Ang-2 is elevated in colon carcinoma (14), gastric carcinoma (15), glioblastoma (16), glioma (17), hepatocellular carcinoma (18), neuroblastoma (19) and non-small cell lung cancer (13). Furthermore, Ang-2 levels appear to correlate with advancing tumor stage progression (15, 19) and poor prognosis (13, 15).

The Quantikine Human Angiopoietin-2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Ang-2 in cell culture supernates, serum, plasma, and saliva. It contains NS0-expressed recombinant human Ang-2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Ang-2 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 human Ang-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Ang-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Ang-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Ang-2 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-2 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, 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.

PRECAUTIONS

Ang-2 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DANG20	CATALOG # SANG20	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Angiopoietin-2 Microplate	892359	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against 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.*
Angiopoietin-2 Standard	892361	3 vials	18 vials	Recombinant human Angiopoietin-2 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Angiopoietin-2 Conjugate	892360	1 vial	6 vials	21 mL/vial of a monoclonal antibody against Angiopoietin-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-76	895812	1 vial	6 vials	11 mL/vial of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-5	895485	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

* Provided this is within the expiration date of the kit.

DANG20 contains sufficient materials to run an ELISA on one 96 well plate.
SANG20 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDANG20). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

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.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as a Salivette® or equivalent.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Ang-2 Controls (optional; available from R&D Systems).

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.

Caution: *Significant levels of Ang-2 are found in fetal bovine, bovine, porcine, equine, and rabbit sera. The background level of Ang-2 in control medium should be determined and subtracted from samples of conditioned medium.*

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 heparin or 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: *Citrate plasma has not been validated for use in this assay.*

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

SAMPLE PREPARATION

Use polypropylene tubes. Serum and plasma samples require a 5-fold dilution. A suggested 5-fold dilution is 50 µL of sample + 200 µL of Calibrator Diluent RD5-5.

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

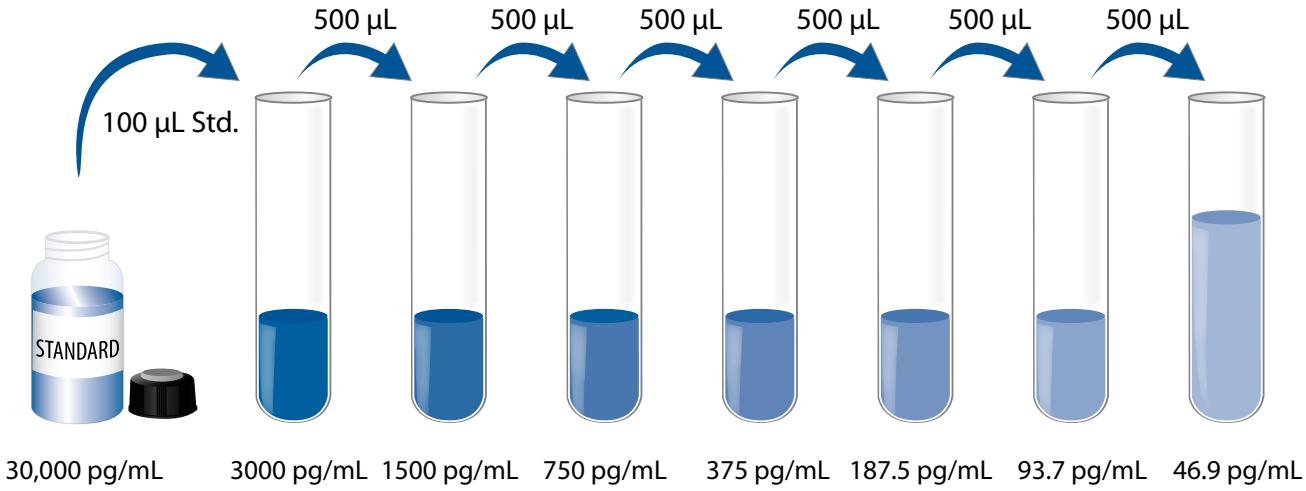
Note: *High concentrations of Ang-2 are found in saliva. We recommend using a face mask and gloves to protect kit reagents from contamination.*

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.

Angiopoietin-2 Standard - **Refer to the vial label for the reconstitution volume.** Reconstitute the Angiopoietin-2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 30,000 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 RD5-5 into the 3000 pg/mL tube. Pipette 500 µL of Calibrator Diluent RD5-5 into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 3000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-5 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, samples, and controls be assayed in duplicate.

Note: *Ang-2 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.*

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 RD1-76 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 rpm ± 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 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 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

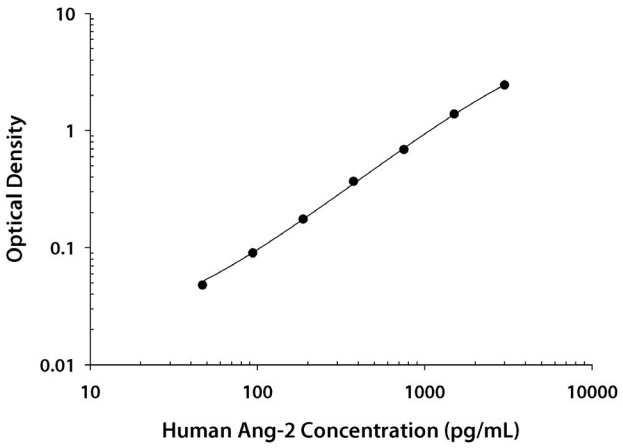
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Ang-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	0.026 0.027	0.027	—
46.9	0.074 0.076	0.075	0.048
93.7	0.114 0.120	0.117	0.090
187.5	0.197 0.206	0.202	0.175
375	0.391 0.398	0.395	0.368
750	0.701 0.728	0.715	0.688
1500	1.394 1.418	1.406	1.379
3000	2.454 2.506	2.480	2.453

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 forty separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	237	703	1301	276	805	1494
Standard deviation	16.4	45.6	54.4	28.8	73.0	111
CV (%)	6.9	6.5	4.2	10.4	9.1	7.4

RECOVERY

The recovery of Ang-2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	98-113%
Serum* (n=4)	100	90-107%
EDTA plasma* (n=4)	95	85-104%
Heparin plasma* (n=4)	94	87-104%

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Ang-2 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Saliva (n=4)
1:2	Average % of Expected	100	95	97	95	105
	Range (%)	98-102	87-103	93-103	89-101	101-110
1:4	Average % of Expected	97	95	95	94	107
	Range (%)	90-103	86-112	93-98	88-101	100-112
1:8	Average % of Expected	96	95	100	91	107
	Range (%)	87-105	85-114	89-113	87-100	104-112

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

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SENSITIVITY

Thirty-eight assays were evaluated and the minimum detectable dose (MDD) of Ang-2 ranged from 1.20-21.3 pg/mL. The mean MDD was 8.29 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 NS0-expressed recombinant human Ang-2 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of Ang-2 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=60)	2494	1065-8907	1341
EDTA plasma (n=35)	1964	1071-4389	808
Heparin plasma (n=35)	2049	1009-4973	913

Saliva - Nine samples were evaluated for the presence of Ang-2 in this assay and ranged from 247-822 pg/mL.

Cell Culture Supernates - Two cultures of HUVEC human umbilical vein endothelial cells were grown to confluency in EGM supplemented with 2% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate and bovine brain extract. Aliquots of the cell culture supernates were removed and assayed for levels of natural Ang-2.

Sample	Values (pg/mL)
HUVEC Culture 1	2326
HUVEC Culture 2	7611

Note: *Significant levels of Ang-2 are found in fetal bovine, bovine, porcine, equine, and rabbit sera. The background level of Ang-2 in control medium should be determined and subtracted from samples of conditioned medium.*

SPECIFICITY

This assay recognizes natural and recombinant human Ang-2.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-5 and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Ang-2 standard were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ANG
Ang-1
AR
BDNF
CD4
CD40
CD40 Ligand
CNTF
CT-1
CTLA-4
Epo
Fas
GDNF
GITR
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IL-10
IL-11
IL-12
IL-12 p40
IL-13
IL-15
IL-16
IL-17
Leptin
LIF
MIF
NT-3
NT-4
OPG
OSM
PTN
SCF
SLPI
SMDF
Tie-2
TNF-α
TNF-β
Tpo
TRANCE
$VEGF_{121}$
$VEGF_{165}$
VEGF/PlGF

Recombinant mouse:

CT-1
CTLA-4
Fas
Fas Ligand
GITR Ligand
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-11
IL-12
IL-12 p40
IL-13
IL-17
Leptin
LIF
OPG
OPN
OSM
SCF
TNF-α
Tpo
TRANCE

Recombinant rat:

CNTF
GDNF
IFN-γ
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-10
Leptin
MK
TNF-α

Recombinant porcine:

IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-8
IL-10
TNF-α

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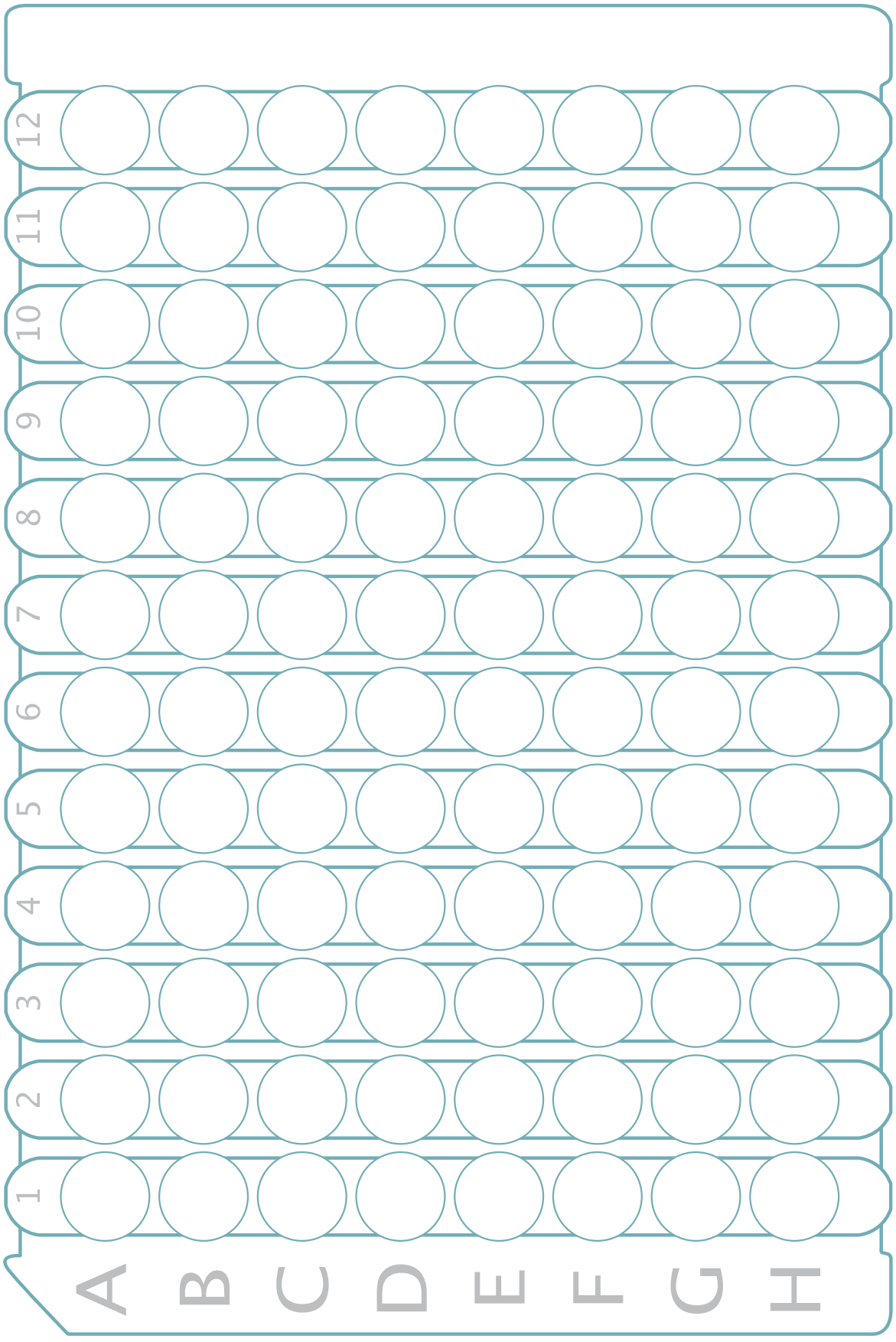
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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