Quantikine® ELISA

Human uPAR Immunoassay

Catalog Number DUP00

For the quantitative determination of human Urokinase-type Plasminogen Activator Receptor (uPAR) concentrations in cell culture supernates, serum, plasma, 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

Urokinase-type Plasminogen Activator Receptor (uPAR), also known as CD87, is a cell surface receptor that binds urokinase-type plasminogen activator (uPA) with high affinity, thereby facilitating the pericellular activation of plasminogen (see references 1 and 2 for reviews). uPAR, uPA and plasminogen activator inhibitor-1 (PAI-1), form a triad with multiple functions, including regulation of cell attachment, migration, proliferation and differentiation, by both proteolytic and nonproteolytic mechanisms (2).

uPAR is anchored to extracellular surfaces through a glycosyl phosphatidylinositol (GPI) linkage, with no transmembrane domain (3). uPAR is synthesized as a 335 amino acid (aa) polypeptide that includes a 22 aa residue signal peptide (4). A 30 aa residue peptide is cleaved from the C-terminus during addition of the GPI anchor (3). With loss of the signal peptide and the C-terminal peptide, the mature protein has 283 amino acids. It is variably glycosylated, increasing its mass from about 31 kDa for the protein backbone to as much as 55 kDa for the mature glycoprotein (5).

Pro-uPA, a single-chain protein, is activated to a disulfide-linked, two-chain protein by proteolytic cleavage by plasmin (1, 2, 6). Either pro-uPA or the active two-chain uPA bind with high affinity to uPAR. Traces of plasmin activate pro-uPA to uPA, leading to increasing generation of plasmin in a positive feedback loop that is amplified by uPAR. While the initiating event is not clear, the effect is the generation of plasmin at the cell surface, where it degrades the extracellular matrix by activating matrix metalloproteinases. This appears to be a key event in tumor invasiveness and metastasis, and in migration of cells in general (1, 2, 7).

The functions of the uPA/uPAR system are, however, more extensive than mediation of plasmin formation, and they include non-proteolytic functions (1, 2). uPA/uPAR is localized to focal contact points of cells within the substratum. These sites include intracellular vinculin and the extracellular adhesion molecule vitronectin, suggesting direct adhesive functions and intracellular signalling functions for uPAR. uPAR binds to integrins, and it contains chemotactic activity in its single protease-sensitive region.

uPAR has been measured in human plasma (7-9). Soluble uPAR is generated by removal of the GPI anchor by an endogenous phospholipase D, freeing uPAR of its surface attachment (10). It is elevated in the plasma of patients with paroxysmal nocturnal hemoglobinuria (7, 8), a manifestation of an inability to add GPI anchors to proteins. It has been postulated that there also may be soluble uPAR due to alternative splicing of the primary transcript (1), as has been demonstrated for mouse uPAR (11). uPAR has been identified in urine, where the level is a consistent fraction of creatinine concentration (12).

The Quantikine® Human uPAR Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human uPAR in cell culture supernates, serum, plasma, and urine. It contains NS0-expressed recombinant human uPAR and antibodies raised against the recombinant factor. It has been shown to accurately quantitate the recombinant factor. Results obtained using natural human uPAR 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 of natural human uPAR.

PRINCIPLE OF THE ASSAY

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

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 uPAR Microplate	890714	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human uPAR.	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 uPAR Conjugate	890715	21 mL of a polyclonal antibody specific for human uPAR conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human uPAR Standard	890716	Recombinant human uPAR in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6P	895118	21 mL of animal serum 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.
- Test tubes for dilution of standards and samples.
- Human uPAR controls (optional; R&D Systems®, Catalog # QC21).

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 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 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.*
Hemolyzed, icteric, and lipemic samples are not suitable for use in this assay.
Samples containing high levels of human serum albumin are not suitable for use in this assay.

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.

SAMPLE PREPARATION

Cell culture supernates, serum, plasma, and urine samples require at least a 5-fold dilution. A suggested 5-fold dilution is 25 µL of sample + 100 µL of Calibrator Diluent RD6P.

REAGENT PREPARATION

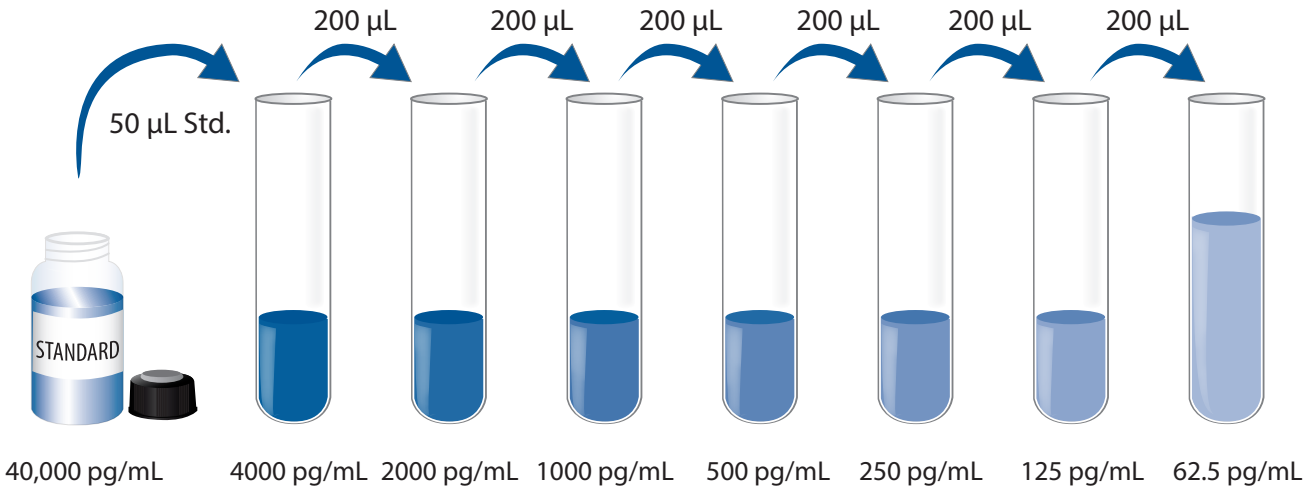
Bring all reagents to room temperature before use.

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.

Human uPAR Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human uPAR Standard with deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD6P into the 4000 pg/mL tube. Pipette 200 µL of Calibrator Diluent RD6P into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent RD6P 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.

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 RD1W 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. 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 Human uPAR Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
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. **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 if 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 require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

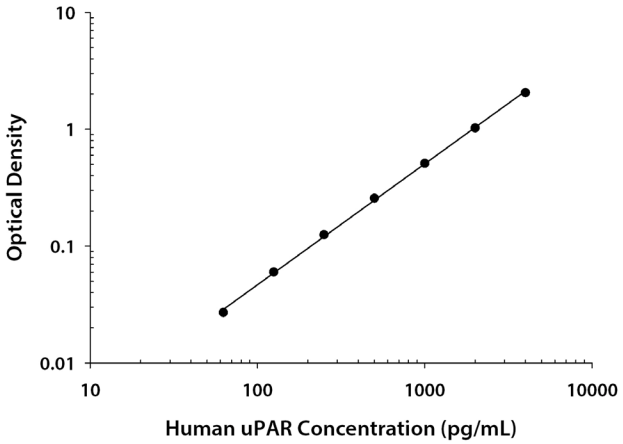
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 human uPAR concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

Since 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	0.044 0.046	0.045	—
62.5	0.072 0.073	0.072	0.027
125	0.105 0.105	0.105	0.060
250	0.170 0.171	0.170	0.125
500	0.300 0.304	0.302	0.257
1000	0.545 0.565	0.555	0.510
2000	1.066 1.075	1.070	1.025
4000	2.076 2.122	2.099	2.054

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. 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	40	40	40
Mean (pg/mL)	836	1593	2412	796	1546	2300
Standard deviation	17.3	65.4	181	44.3	78.9	136
CV (%)	2.1	4.1	7.5	5.6	5.1	5.9

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human uPAR were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture media (n=4)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)	Urine (n=5)
1:2	Average % of Expected	103	104	101	104	102
	Range (%)	96-112	101-105	94-105	101-106	99-105
1:4	Average % of Expected	109	106	105	107	104
	Range (%)	101-115	103-111	101-108	104-110	95-112
1:8	Average % of Expected	109	106	106	106	108
	Range (%)	101-111	103-112	103-113	102-110	104-113
1:16	Average % of Expected	109	105	104	105	108
	Range (%)	99-114	99-111	98-111	98-110	103-112

SENSITIVITY

The minimum detectable dose (MDD) of human uPAR is typically < 33 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 NS0-expressed recombinant human uPAR produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human uPAR in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)
Serum (n=60)	2370	1195-4415
EDTA plasma (n=35)	1871	864-3829
Heparin plasma (n=35)	2165	977-5347
Urine (n=35)	2975	691-6098

Cell Culture Supernates - Human peripheral blood mononuclear cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for levels of human uPAR.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	1228	2123
Stimulated	10,005	6895

SPECIFICITY

This assay recognizes natural and recombinant human uPAR.

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

Recombinant human:

ANG
AR
CNTF
β-ECGF
EGF
Epo
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
Flt-3 Ligand
G-CSF
GM-CSF
gp130
GROα
GROβ
GROγ
HB-EGF
HGF
IFN-γ
IGF-I
IGF-II
IL-1α
IL-1β
IL-1ra
IL-1 RI
IL-1 RII
IL-2
IL-2 Rα
IL-3
IL-3 Rα
IL-4
IL-4 R
IL-5
IL-5 Rα
IL-5 Rβ
IL-6
IL-6 R
IL-7
IL-8
IL-9
IL-10
IL-11
IL-12
IL-13
KGF/FGF-7
LAP (TGF-β1)
LIF
M-CSF
MCP-1
MIP-1α
MIP-1β
β-NGF
OSM
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PTN
RANTES
SCF
SLPI
TGF-α
TGF-β1
TGF-β2
TGF-β3
TGF-β RII
TNF-α
TNF-β
TNF RI
TNF RII
VEGF

Recombinant mouse:

GM-CSF
IL-1α
IL-1β
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-13
LIF
MIP-1α
MIP-1β
SCF
TNF-α
uPAR

Recombinant amphibian:

TGF-β5

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
porcine TGF-β1

Recombinant human uPA interferes at concentrations > 3000 pg/mL in this assay.

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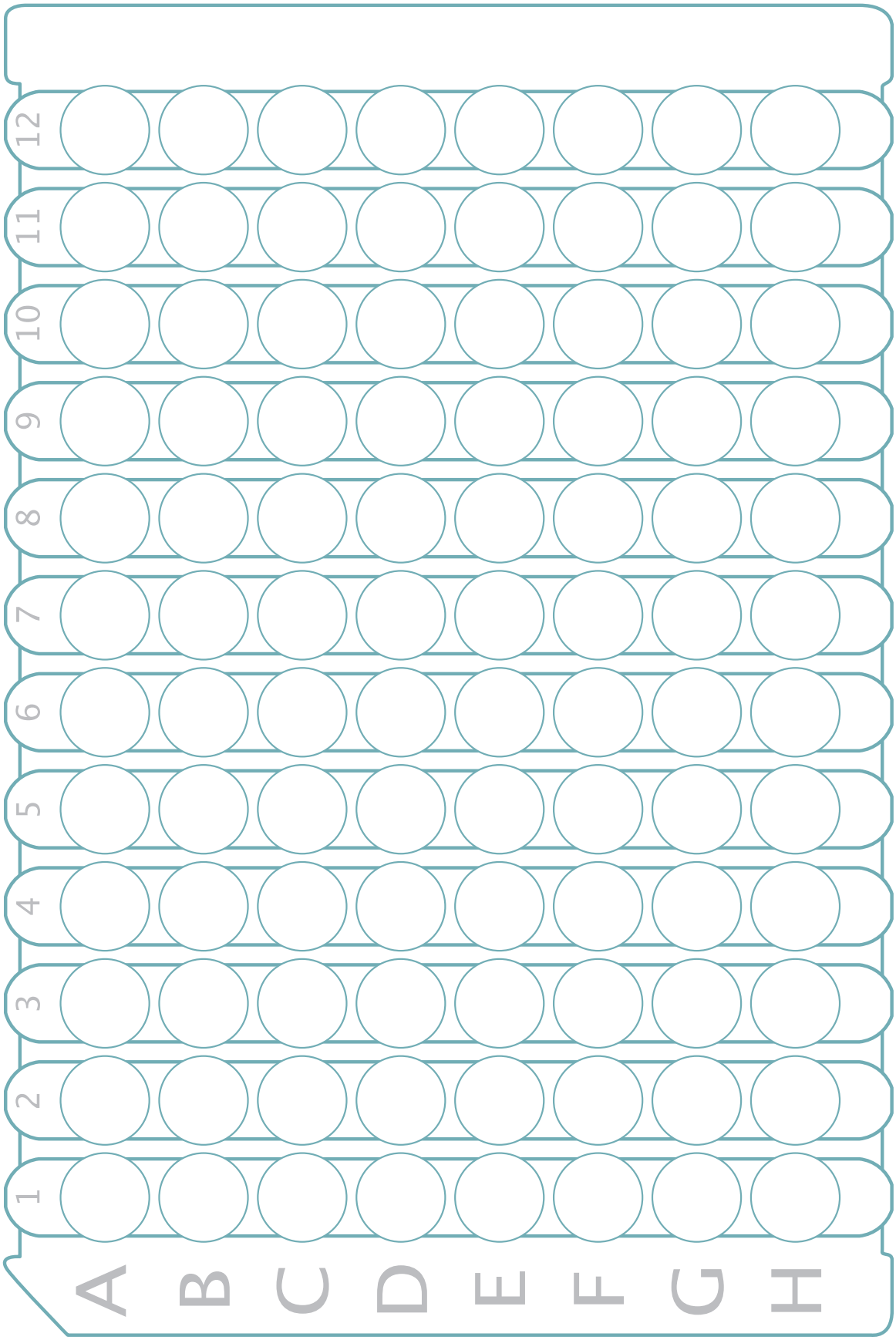
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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