

Quantikine[®] ELISA

Human u-Plasminogen Activator/Urokinase Immunoassay

Catalog Number DUPA00

For the quantitative determination of human urokinase-type Plasminogen Activator-1 (uPA) concentrations in cell culture supernates, cell lysates, 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.

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
PRINCIPLE OF THE ASSAY.....	2
LIMITATIONS OF THE PROCEDURE	2
TECHNICAL HINTS.....	2
MATERIALS PROVIDED & STORAGE CONDITIONS	3
OTHER SUPPLIES REQUIRED	3
PRECAUTION	4
SAMPLE COLLECTION & STORAGE.....	4
CELL LYSIS PROCEDURE.....	4
SAMPLE PREPARATION.....	4
REAGENT PREPARATION	5
ASSAY PROCEDURE	6
CALCULATION OF RESULTS.....	7
TYPICAL DATA.....	7
PRECISION	8
RECOVERY.....	8
SENSITIVITY	9
CALIBRATION	9
LINEARITY	9
SAMPLE VALUES.....	10
SPECIFICITY.....	10
REFERENCES	11
PLATE LAYOUT	12

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INTRODUCTION

Urokinase-type Plasminogen Activator-1 (uPA) is a secreted 54 kDa serine protease that plays an important role in extracellular matrix (ECM) remodeling, cellular migration, inflammation, and cancer. uPA and the related tissue-type Plasminogen activator (tPA) cleave and activate Plasminogen to form Plasmin. Plasmin is a serine protease that activates pro-matrix metalloproteinases and ECM proteins and dissolves the fibrin of blood clots (1, 2). In addition to cleaving Plasminogen, uPA can cleave and activate pro-HGF/SCF (3). uPA consists of an EGF-like growth factor domain (GFD), a Kringle domain (KD), a connecting peptide, and a peptidase domain (4-6). It shares 70% and 72% amino acid sequence identity with mouse and rat uPA, respectively. uPA is secreted as an inactive zymogen that is cleaved following the connecting peptide. The resulting active uPA is a disulfide-linked dimer of the N-terminal A chain (EGF-like and Kringle domains) and the C-terminal B chain (peptidase domain). A secondary cleavage releases the amino terminal fragment (ATF) which contains the EGF-like and Kringle domains. Release of the ATF produces a low molecular weight form of uPA consisting of the enzymatically active B chain disulfide-linked to a portion of the connecting peptide. uPA is irreversibly and potently inhibited by Serpin E1/PAI-1 and less effectively by Serpin-B2/PAI-2 (7). Complexes of uPA with PAI-1 are cleared from the circulation by LRP-1 and VLDL R (8, 9).

uPA binds to the widely expressed uPA Receptor (uPAR/CD87), which is a 55 kDa GPI-linked protein organized into three structural domains: D1, D2, and D3 (10). Cell surface uPA-uPAR complexes can be inhibited by PAI-1 and are even more efficient than soluble uPA at cleaving Plasminogen (11-13). The binding of uPA to uPAR induces recruitment of a second uPAR molecule to form a ternary complex (14). Soluble forms of uPAR can be generated by alternative splicing or by phospholipase-mediated shedding from the cell surface (15). When bound to uPAR, uPA can cleave its own receptor and release the ligand-binding D1 domain, leaving a 40 kDa uPAR fragment associated with the cell (16).

Full length uPAR associates *in cis* with multiple integrins and regulates their adhesive properties (17-20). Cell surface uPAR and soluble uPA-uPAR complexes can bind the ECM protein Vitronectin, and PAI-1 interferes with this interaction (14, 21). uPA also interacts with integrins in the absence of uPAR (22). Both cell surface and soluble uPAR inhibit adhesion and promote cell migration by binding and activating the formyl peptide receptors FPR and FPRL1 (23-25). In addition, the ATF of uPA functions as a chemotactic signal for uPAR expressing cells (18). uPA binding to uPAR promotes cell proliferation and migration by inducing the activation of PDGF R beta and EGF R either directly or through complexes with integrins, independently of growth factor receptor ligands (26, 27).

The effects of uPA and uPAR on cell adhesion and migration as well as functions in angiogenesis and resistance to apoptosis contribute to tumor metastasis (28). The plasma, ascites, urinary, or cerebrospinal fluid levels of uPA and/or uPAR are elevated in a variety of invasive and metastatic human cancers (15). The upregulation of uPA contributes to cardiac fibrosis (29), vascular intima thickening (30), aneurysm formation (31), and the repair of damaged skeletal muscle (32). uPA and uPAR promote immune cell activation at sites of inflammation and tissue damage (33-37). uPAR expressed on macrophages enhances their phagocytosis of apoptotic cells, whereas its expression on other non-apoptotic cells protects them from engulfment (38, 39). The isolated Kringle domain of uPA inhibits angiogenesis (40), while soluble uPAR is pro-angiogenic (24). Soluble uPAR also promotes the mobilization of hematopoietic and mesenchymal stem cells to the circulation (41, 42).

The Quantikine Human u-Plasminogen Activator/Urokinase Immunoassay is a 4.5 hour solid phase ELISA designed to measure uPA in cell culture supernates, cell lysates, serum, plasma, and urine. It contains NS0-expressed recombinant human uPA and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate recombinant human uPA. Results obtained using natural human uPA 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 uPA.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for uPA has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any uPA present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for uPA 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 uPA 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
uPA Microplate	894350	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against uPA.	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.*
uPA Conjugate	894351	21 mL of a polyclonal antibody against uPA conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
uPA Standard	894352	20 ng of recombinant human uPA in a buffer with preservatives; lyophilized.	
Assay Diluent RD1S	895137	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.	
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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human uPA controls (optional; available from R&D Systems).

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

- Cell Lysis Buffer 1 (R&D Systems, Catalog # 890713).
- PBS

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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 samples are not recommended 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.

CELL LYSIS PROCEDURE

1. Gently wash cells with cold PBS. Pour off and discard the PBS.
2. Add sufficient Cell Lysis Buffer 1 to cover the cells (for example, 10 mL if using a T75 flask) and incubate at room temperature for 1 hour with gentle agitation.
3. Centrifuge at 10,000 x g for 10 minutes to remove cell debris.
4. Collect the supernate and determine the total protein concentration of the lysate using the Bradford method (43).
5. Aliquot the lysis supernate and store at ≤ -70 °C until ready for use.

SAMPLE PREPARATION

Serum and plasma samples require a 3-fold dilution. A suggested 3-fold dilution is 50 μ L of sample + 100 μ L of Calibrator Diluent RD5-26 (1X).

Urine samples require a 20-fold dilution. A suggested 20-fold dilution is 10 μ L of sample + 190 μ L of Calibrator Diluent RD5-26 (1X).

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. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

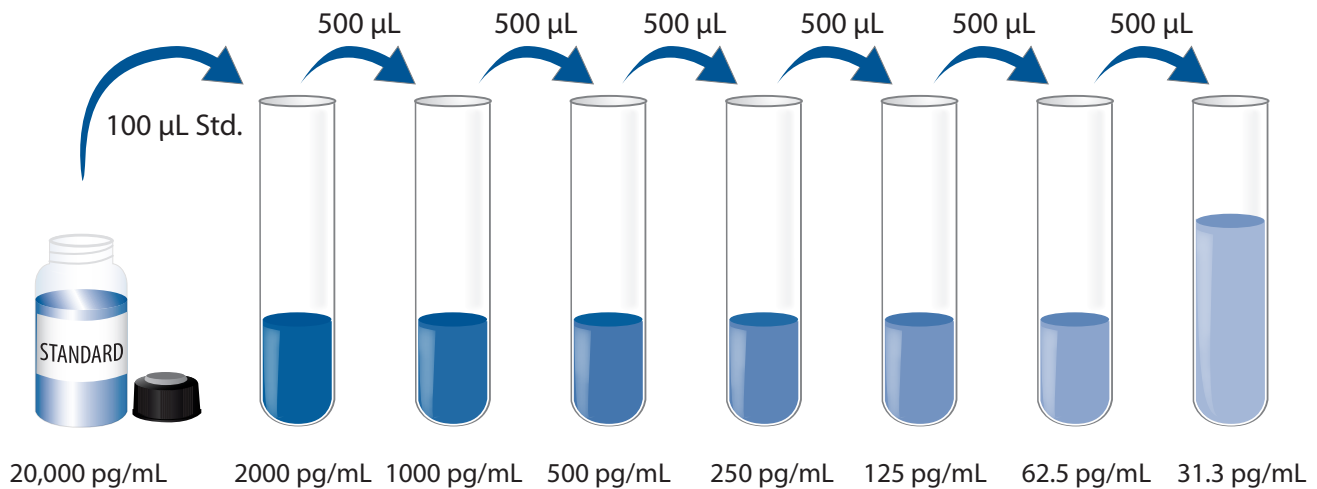
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (1X) - Add 20 mL of Calibrator Diluent RD5-26 to 60 mL of deionized or distilled water to yield 80 mL of Diluted Calibrator Diluent RD5-26 (1X).

uPA Standard - Reconstitute the uPA Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. 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 RD5-26 (1X) into the 2000 pg/mL tube. Pipette 500 μL of Calibrator Diluent RD5-26 (1X) into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (1X) serves as the zero standard (0 pg/mL).

Note: Standard curve should be added to the microplate within 10 minutes of preparation.



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 RD1S 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 \pm 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 toweling.
6. Add 200 μL of uPA 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. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. 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

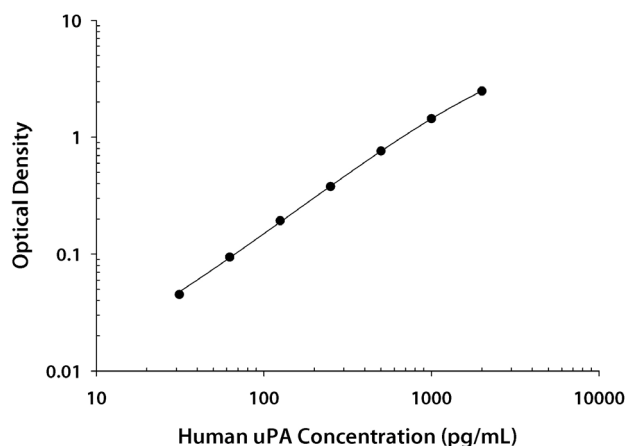
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 uPA 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	0.015 0.016	0.016	—
31.3	0.060 0.061	0.061	0.045
62.5	0.108 0.111	0.110	0.094
125	0.203 0.214	0.209	0.193
250	0.387 0.401	0.394	0.378
500	0.772 0.780	0.776	0.760
1000	1.435 1.472	1.454	1.438
2000	2.439 2.556	2.498	2.482

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.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	230	629	1219	214	614	1188
Standard deviation	4.8	8.9	29.3	15.1	39.8	82.0
CV (%)	2.1	1.4	2.4	7.1	6.5	6.9

RECOVERY

The recovery of uPA spiked to three different levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=8)	102	93-115%
Cell lysates (n=4)	97	81-116%
Serum (n=4)	87	81-93%
EDTA plasma (n=4)	87	83-94%
Heparin plasma (n=4)	89	81-110%
Urine (n=4)	98	81-119%

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) of uPA ranged from 0.931-4.17 pg/mL. The mean MDD was 1.61 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 uPA produced at R&D Systems.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of uPA were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Cell lysates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	96	98	107	108	108	96
	Range (%)	94-98	93-100	102-113	101-116	99-112	90-100
1:4	Average % of Expected	92	99	107	109	109	94
	Range (%)	89-96	94-104	99-112	103-116	103-115	86-101
1:8	Average % of Expected	93	93	108	105	109	95
	Range (%)	89-98	88-103	100-118	97-108	99-116	85-103
1:16	Average % of Expected	93	87	99	97	100	99
	Range (%)	89-100	80-96	90-105	82-104	88-113	86-108

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of uPA 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=35)	994	499-4947	1020
EDTA plasma (n=35)	958	406-4797	1023
Heparin plasma (n=35)	1054	478-5119	1069
Urine (n=12)	14,000	6020-24,300	6970

Cell Culture Supernates/Cell Lysates:

HepG2 human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. The cells were grown for 6 days.

Human peripheral blood leukocytes (PBLs) were cultured in RPMI and were unstimulated or stimulated with 100 ng/mL LPS or 10 µg/mL PHA. The cells were grown for 3 days.

Cell Line	Levels Observed	
	Cell Culture Supernates (pg/mL)	Cell Lysates (pg/mg of lysate)
HepG2	199	96.5
PBL	48.3	2533
PBL + PHA	104	630
PBL + LPS	58.4	1971

SPECIFICITY

This assay recognizes natural and recombinant human uPA. This assay also recognizes uPA complexed with PAI-1 or 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 uPA control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Plasminogen
t-Plasminogen Activator/tPA
Serpin E1/PAI-1
uPAR

Recombinant mouse:

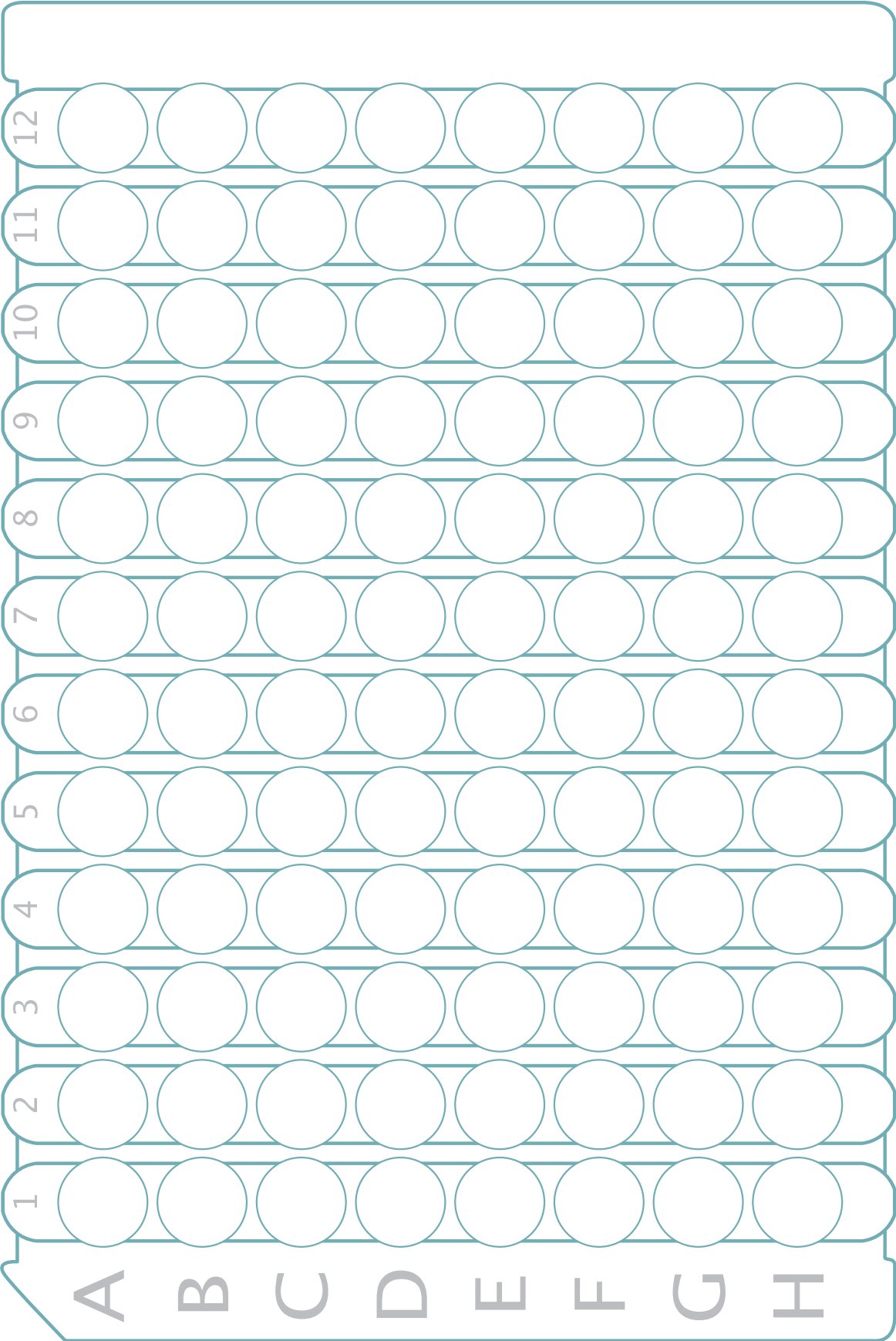
uPAR

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

NOTES