Quantikine® ELISA

Human t-Plasminogen Activator/tPA Immunoassay

Catalog Number DTPA00

For the quantitative determination of human t-Plasminogen Activator (tPA) concentrations in cell culture supernates, serum, plasma, saliva, urine, human milk.

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

Tissue Plasminogen Activator (tPA), also known as PLAT, is a 64-69 kDa extracellular glycoprotein that belongs to the peptidase S1 family of serine proteases. The biological effects of tPA include blood clot degradation, vascular remodeling, synaptic plasticity, and neurodegeneration in the brain following trauma. Human tPA is secreted as a 530 amino acid (aa) single chain polypeptide (36-562 aa) that contains an amino-terminal fibrin-"finger"-like domain, an epidermal growth factor-like domain, two kringle domains and a C-terminal serine protease catalytic domain (1, 2). The partially active single chain tPA is cleaved between Arg310-Ile311 by Plasmin, Kallikrein/KLKB1, and Coagulation Factor X/Xa to generate the mature two-chain disulfide-linked polypeptide. This mature form of tPA is 10-fold more catalytically active than the single chain (3, 4). From aa 36-562, human tPA shares 81% and 72% identity with mouse and rat tPA, respectively.

Human tPA is synthesized and secreted by fibroblasts, vascular endothelial cells, melanoma cells, and neural cells (5). The biological activity of tPA is tightly controlled; freely circulating tPA is sequestered by the serine protease inhibitors Serpin I1 and Serpin E1/PAI-1 (6). tPA is also rapidly cleared from the extracellular and vascular space through Low-Density Lipoprotein Receptor-related Protein-1 mediated endocytosis (7, 8).

In the vasculature, circulating tPA binds to blood clot formations. Here tPA converts its primary substrate Plasminogen into Plasmin, an enzyme that subsequently degrades the fibrin matrix of the clot (9, 10). Increased expression or activity of tPA is associated with excessive bleeding, while reduced tPA activity has been implicated in thrombosis and embolism formation. In the brain, tPA is expressed in neurons, astrocytes, microglia, and vascular parenchymal endothelial cells (5, 11). Changes in tPA expression in the brain have been shown following stroke, hypoxia, excitotoxic trauma, and stress-induced cognitive decline (5, 12, 13). The proteolytic activity of tPA is targeted against proteins in brain extracellular matrix (ECM). tPA-mediated breakdown of the ECM is involved in promoting synaptic plasticity, including neurite outgrowth and synapse remodeling (14). In addition to its proteolytic function, tPA has also been shown to have a number of critical non-proteolytic functions, including activation of microglia and modulation of neurotransmission (5, 15, 16). tPA is also involved in angiogenesis and breakdown of the blood-brain barrier (17-20).

The Quantikine Human t-Plasminogen Activator/tPA Immunoassay is a 4.5 hour solid phase ELISA designed to measure tPA levels in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains CHO cell-expressed recombinant human tPA and antibodies raised against the recombinant protein. Results obtained for naturally occurring human tPA showed linear curves that were parallel to the standard curves obtained using the Quantikine Human tPA Immunoassay standards. These results indicate that this kit can be used to determine relative mass values for natural human tPA.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human tPA has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any tPA present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human tPA 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 tPA 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 tPA Microplate	894851	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human tPA.	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 tPA Standard	894853	2 vials of recombinant human tPA in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human tPA Conjugate	894852	21 mL of a polyclonal antibody specific for human tPA 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 RD5P Concentrate	895151	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.
- 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 tPA Controls (optional; R&D Systems, Catalog # QC207).

PRECAUTIONS

tPA 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.

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, 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.

Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, 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.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum, plasma, and human milk samples require a 4-fold dilution due to matrix effects. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5P (diluted 1:5)*.

Urine samples require a 2-fold dilution due to matrix effects. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5P (diluted 1:5).

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *tPA is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

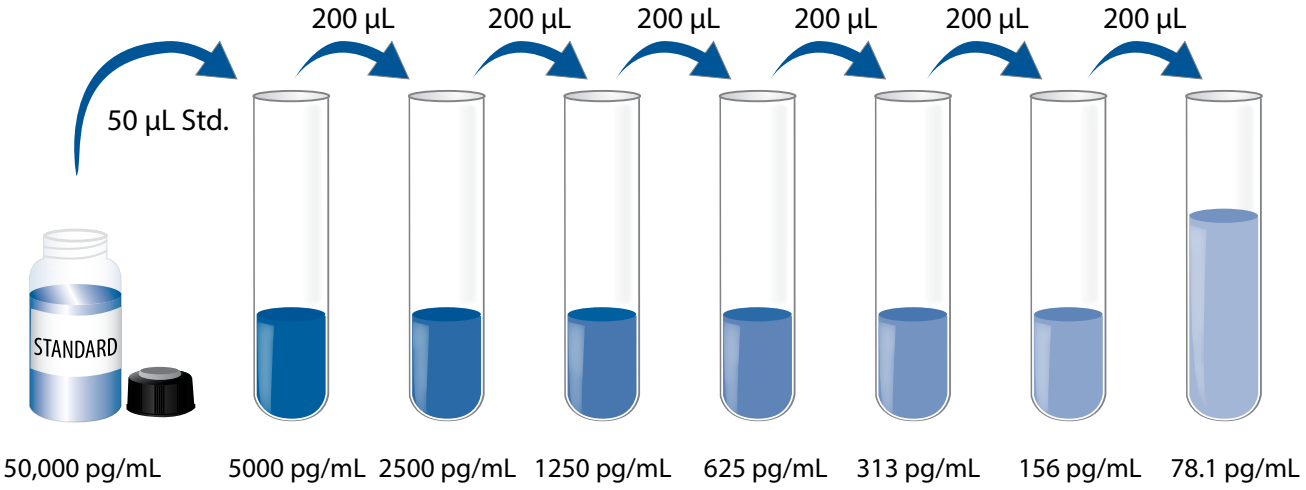
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5P (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5P Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Human tPA Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human tPA Standard with deionized or distilled water. This reconstitution produces a stock solution of 50,000 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 450 µL of Calibrator Diluent RD5P (diluted 1:5) into the 5000 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 5000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (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 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 200 µL of Human tPA 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 the Sample Preparation section.

CALCULATION OF RESULTS

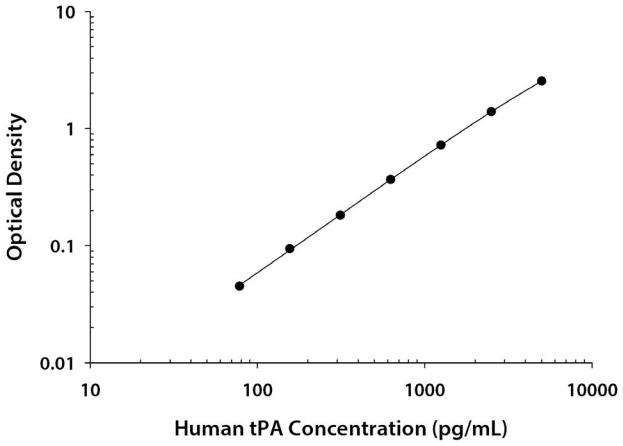
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human tPA 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 0.011	0.011	—
78.1	0.056 0.056	0.056	0.045
156	0.102 0.108	0.105	0.094
313	0.190 0.196	0.193	0.182
625	0.374 0.382	0.378	0.367
1250	0.734 0.736	0.735	0.724
2500	1.397 1.404	1.401	1.390
5000	2.546 2.554	2.550	2.539

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)	498	1475	2878	490	1460	2898
Standard deviation	36.0	48.4	88.8	32.1	62.3	115
CV (%)	7.2	3.3	3.1	6.6	4.3	4.0

RECOVERY

The recovery of human tPA spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	93	86-97%
Serum* (n=4)	95	87-109%
EDTA plasma* (n=4)	87	80-97%
Heparin plasma* (n=4)	86	79-100%
Citrate plasma* (n=4)	86	80-98%
Urine* (n=4)	97	88-110%

*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 human tPA 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)	Citrate plasma* (n-4)
1:2	Average % of Expected	102	104	101	100	103
	Range (%)	101-102	101-107	98-103	92-104	98-105
1:4	Average % of Expected	103	106	105	101	106
	Range (%)	100-104	101-111	98-109	95-107	100-115
1:8	Average % of Expected	103	100	99	97	102
	Range (%)	101-104	91-109	90-106	92-106	92-120
1:16	Average % of Expected	107	100	94	97	107
	Range (%)	102-114	94-104	93-96	90-104	94-121

		Saliva (n=4)	Urine (n=4)	Human milk* (n=4)
1:2	Average % of Expected	105	100	106
	Range (%)	101-110	98-101	103-113
1:4	Average % of Expected	105	101	103
	Range (%)	99-109	98-103	101-105
1:8	Average % of Expected	109	104	103
	Range (%)	106-112	99-110	———
1:16	Average % of Expected	———	104	———
	Range (%)	———	98-110	———

*Samples were diluted prior to assay.

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) of human tPA ranged from 1.40-16.1 pg/mL. The mean MDD was 6.17 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 highly purified CHO-cell expressed recombinant human tPA produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human tPA 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)	3295	858-8756	1637
EDTA plasma (n=35)	3662	1270-8840	1637
Heparin plasma (n=35)	3411	1190-9512	1632
Citrate plasma (n=35)	3138	1058-7576	1382
Human milk (n=10)	1657	333-6684	1853

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Saliva (n=10)	394	90	ND-1233
Urine (n=10)	531	40	ND-1094

ND=Non-detectable

Cell Culture Supernates - HUVEC human umbilical vein endothelial cells were cultured in EGM-2 and grown until confluent. An aliquot of the cell culture supernate was removed, assayed for human tPA, and measured 10,132 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human tPA. This assay also recognizes tPA complexed with PAI-1.

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

Recombinant human:

Coagulation Factor X
Fibronectin
LRP-1
LRP-1B
Serpin A1
Serpin A3
Serpin A4
Serpin A5
Serpin A6
Serpin A7
Serpin A9
Serpin A10
Serpin A11
Serpin A12
Serpin B2
Serpin B3
Serpin B8
Serpin B9
Serpin C1
Serpin D1
Serpin E1
Serpin E2
Serpin F1
Serpin F2
Serpin G1
Serpin I1
Serpin I2
uPA

Natural protein:

human Plasminogen

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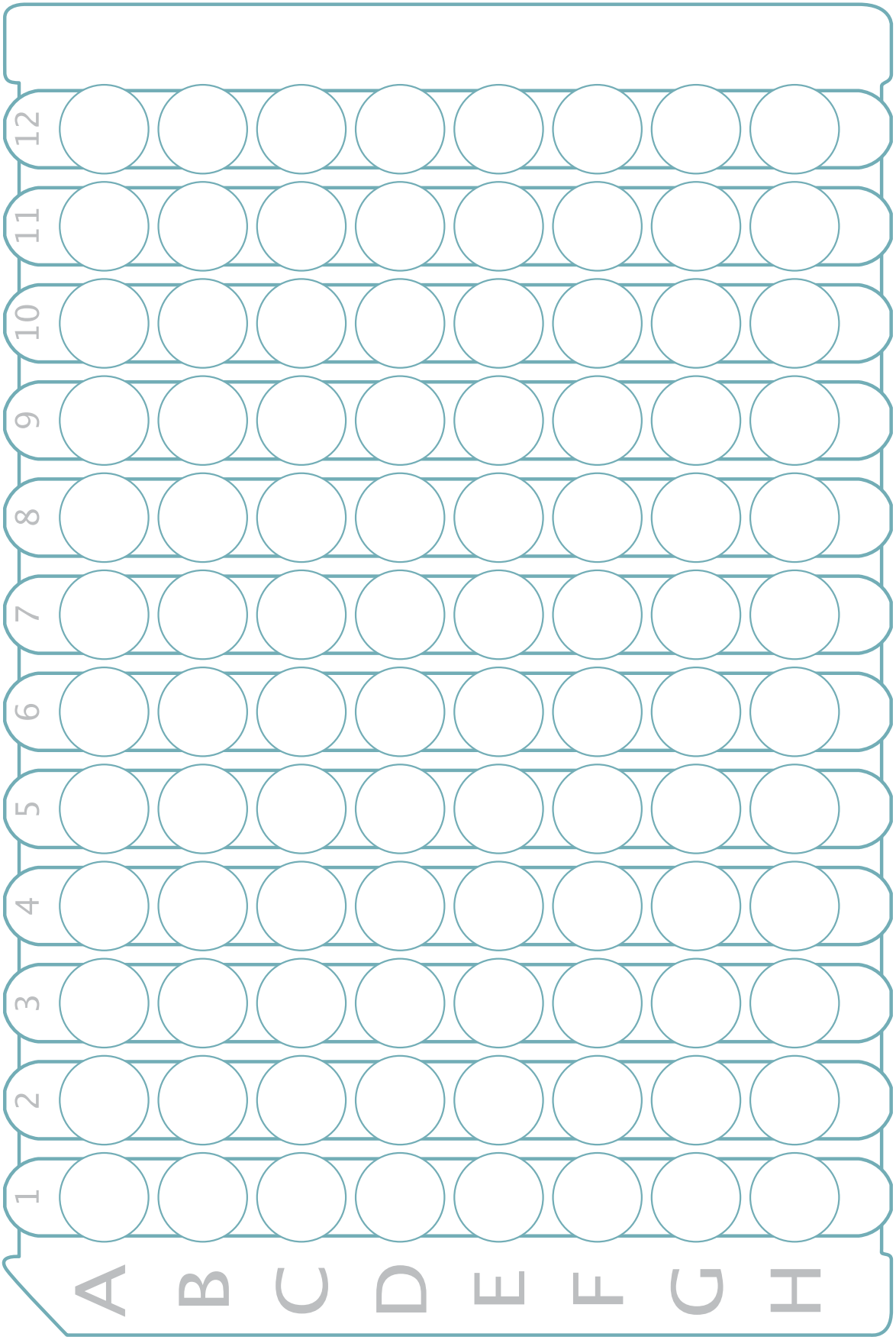
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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