Quantikine® ELISA

Human TFPI Immunoassay

Catalog Number DTFP10

For the quantitative determination of human Tissue Factor Pathway Inhibitor (TFPI) concentrations in cell culture supernates, 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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USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
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DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Blood coagulation results from a series of enzymatic reactions that involve an array of coagulation factors and their regulators. It has intrinsic and extrinsic pathways which are triggered by different coagulation factors. The extrinsic coagulation pathway plays a primary role in blood coagulation. It is initiated by the release of tissue factor from endothelial cells in response to blood vessel injury. Tissue factor then complexes with factor VII and proteolytically activates factor X and factor IX, ultimately leading to the conversion of prothrombin to thrombin and fibrin clot formation. Tissue Factor Pathway Inhibitor (TFPI or TFPI-1), also named Extrinsic Pathway Inhibitor (EPI) or Lipoprotein-associated Coagulation Inhibitor (LACI), is a key regulator of the extrinsic coagulation pathway. It inhibits coagulation through the formation of a quaternary complex with factor X, tissue factor, and factor VII, preventing factor X from activation (1, 2).

Full-length TFPI is a 40 kDa glycoprotein with 304 amino acids. It consists of an N-terminal acidic region, three Kunitz domains, and a C-terminal basic domain. The first Kunitz domain interacts with tissue factor/factor VII complex, while the second one interacts with factor X (2). TFPI is produced by endothelial cells and released by heparin stimulation (3). In the circulation, about 10% of TFPI is carried by platelets (4). Among the rest, the majority circulates as bound forms, while a small percentage exists as the free form. About 50% of the bound TFPI complexes to low density lipoprotein (LDL), 40% to high density lipoprotein (HDL), and 10% to very low density lipoprotein (VLDL) through disulfide bonds. TFPI in plasma is heterogeneous with respect to size. The LDL-bound TFPI is a truncated form that lacks the third Kunitz domain, whereas the free and HDL-bound TFPI are full length (5, 6). It is generally believed that free full-length TFPI represents the majority of its anticoagulation activity.

Abnormal TFPI concentrations in the circulation have been found to be associated with various pathological conditions characterized by hemostatic disturbance, such as thrombosis, disseminated intravascular coagulation, and sepsis (7, 8). TFPI has also been demonstrated to be beneficial when used as an anti-thrombotic remedy to treat sepsis, inflammatory diseases, and cardiovascular diseases (9-11). In addition to anticoagulation activity, TFPI also exhibits anti-angiogenic and anti-metastatic effects. It has been found that during tumor progression, increased amounts of tissue factor are produced by the tumor cells to promote angiogenesis and metastasis (12). As a potent inhibitor of tissue factor, TFPI has shown promise as a new reagent for anti-cancer therapy (13).

The Quantikine® Human TFPI Immunoassay is a 3.5 hour solid-phase ELISA designed to measure human TFPI in cell culture supernates, plasma, and urine. It contains NS0-expressed recombinant human TFPI and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human TFPI 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 TFPI.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TFPI has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TFPI present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TFPI 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 TFPI 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 TFPI Microplate	893646	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human TFPI.	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 TFPI Conjugate	893647	21 mL of a polyclonal antibody specific for human TFPI conjugated to horseradish peroxidase with preservative.	May be stored for up to 1 month at 2-8 °C.*
Human TFPI Standard	893648	Recombinant human TFPI in a buffered protein base with preservative; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-89	895881	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-20 Concentrate	895346	2 vials (21 mL/vial) of a buffered protein base with preservative. *Use undiluted 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.
- 500 mL graduated cylinder.
- 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 TFPI Controls (optional; R&D Systems®, QC167).

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.

Note: *Due to the involvement of TFPI in the coagulation pathway, serum is not recommended for use in this assay. Refer to the Sample Values section for details.*

Cell Culture Supernates - Remove particulates by centrifugation 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 room temperature at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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.

SAMPLE PREPARATION

Use polypropylene tubes.

Plasma samples require a 100-fold dilution. A suggested 100-fold dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD5-20 Concentrate. Complete the 100-fold dilution by adding 20 µL of the diluted sample to 180 µL of Calibrator Diluent RD5-20 Concentrate.

REAGENT PREPARATION

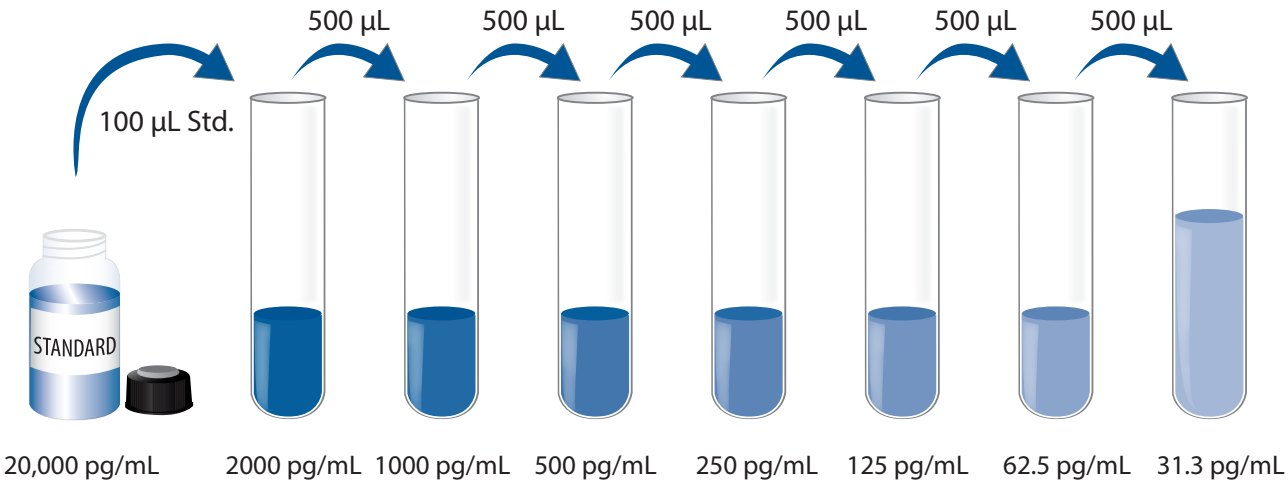
Bring all reagents to room temperature before use.

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 TFPI Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TFPI Standard with 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 RD5-20 Concentrate into the 2000 pg/mL tube. Pipette 500 µL 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 RD5-20 Concentrate 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 100 µL of Assay Diluent RD1-89 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 toweling.
6. Add 200 µL of Human TFPI Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour 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

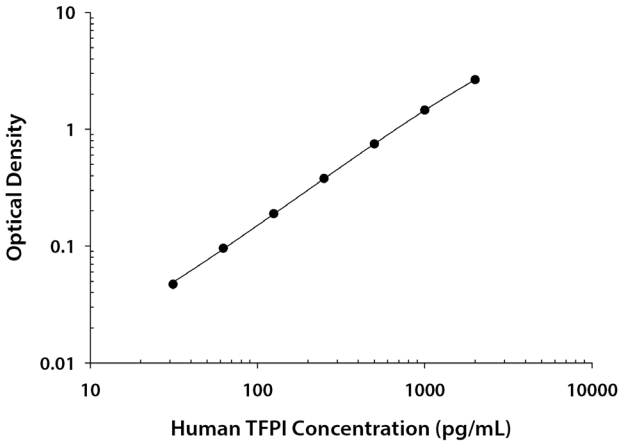
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 TFPI 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	0.007 0.008	0.008	—
31.3	0.053 0.057	0.055	0.047
62.5	0.101 0.106	0.104	0.096
125	0.191 0.203	0.197	0.189
250	0.385 0.387	0.386	0.378
500	0.750 0.761	0.756	0.748
1000	1.451 1.469	1.460	1.452
2000	2.637 2.669	2.653	2.645

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)	299	554	1108	301	569	1127
Standard deviation	13.5	22.2	40.3	18.5	30.6	66.2
CV (%)	4.5	4.0	3.6	6.1	5.4	5.9

RECOVERY

The recovery of human TFPI spiked to levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	92	85-100%
Urine (n=4)	94	85-106%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human TFPI were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=3)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Citrate plasma* (n=4)	Urine (n=4)
1:2	Average % of Expected	99	101	104	102	102
	Range (%)	94-102	98-104	99-111	101-104	99-104
1:4	Average % of Expected	98	98	108	101	99
	Range (%)	88-104	96-99	105-111	98-103	96-102
1:8	Average % of Expected	101	98	113	107	99
	Range (%)	85-113	94-100	110-115	103-109	98-100
1:16	Average % of Expected	104	93	110	105	99
	Range (%)	85-114	85-98	100-116	96-112	98-102

*Samples were diluted prior to assay.

SENSITIVITY

Thirty-eight assays were evaluated and the minimum detectable dose (MDD) of human TFPI ranged from 0.1-6.7 pg/mL. The mean MDD was 2.17 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 TFPI produced at R&D Systems®.

SAMPLE VALUES

Note: *Serum samples from 36 apparently healthy donors were evaluated for the presence of TFPI in this assay. No medical histories were available for the donors used in this study. The serum dose-response curves were parallel to standard curve, and the sample values ranged from 7.5-41.2 ng/mL with a mean of 22.6 ng/mL. Due to the involvement of TFPI in the coagulation pathway, serum is generally not recommended for use in this assay. Variations in sample processing may affect TFPI sample values.*

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

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
EDTA plasma (n=36)	29.1	13.0-44.9	9.82
Heparin plasma (n=36)	27.3	12.3-44.0	8.56
Citrate plasma (n=36)	22.0	9.7-35.4	7.77

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Urine (n=11)	174	91	ND-354

ND=Non-detectable

Cell Culture Supernates:
HUVEC human umbilical vein endothelial cells were cultured in EGM for 3 days. An aliquot of the cell culture supernate was removed, assayed for human TFPI, and measured 20.2 ng/mL.

MG-63 human osteosarcoma 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 for 4 days. An aliquot of the cell culture supernate was removed, assayed for human TFPI, and measured 720 pg/mL.

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. An aliquot of the cell culture supernate was removed, assayed for human TFPI, and measured 19.1 ng/mL.

U-87 MG human glioblastoma/astrocytoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 1 mM sodium pyruvate, and 100 µg/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for human TFPI, and measured 15.7 ng/mL.

HASMC human aortic smooth muscle cells were cultured in Medium 231 supplemented with smooth muscle growth supplement until confluent. An aliquot of the cell culture supernate was removed, assayed for human TFPI, and measured 546 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human TFPI.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range recombinant human TFPI control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
Factor VII
Factor XI
Protein C
Protein S
TFPI-2
Thrombin

Recombinant mouse:
Factor VII
Factor XI

Natural proteins:
human HDL
human LDL
human VLDL

Recombinant mouse TFPI cross-reacts approximately 0.07% in this assay.

Recombinant mouse TFPI-2 cross-reacts approximately 0.04% in this assay.

No cross-reactivity was observed with activated Factor X (Factor Xa). TFPI/Factor Xa complex, when formed *in vitro*, is poorly recognized by this kit.

Analytical gel filtration was performed to determine the specificity of this kit to detect LDL- and HDL-bound TFPI in human citrate plasma. Results indicate that the Quantikine® Human TFPI immunoassay recognizes predominantly free TFPI and a very small percentage of the LDL- and HDL-bound TFPI.

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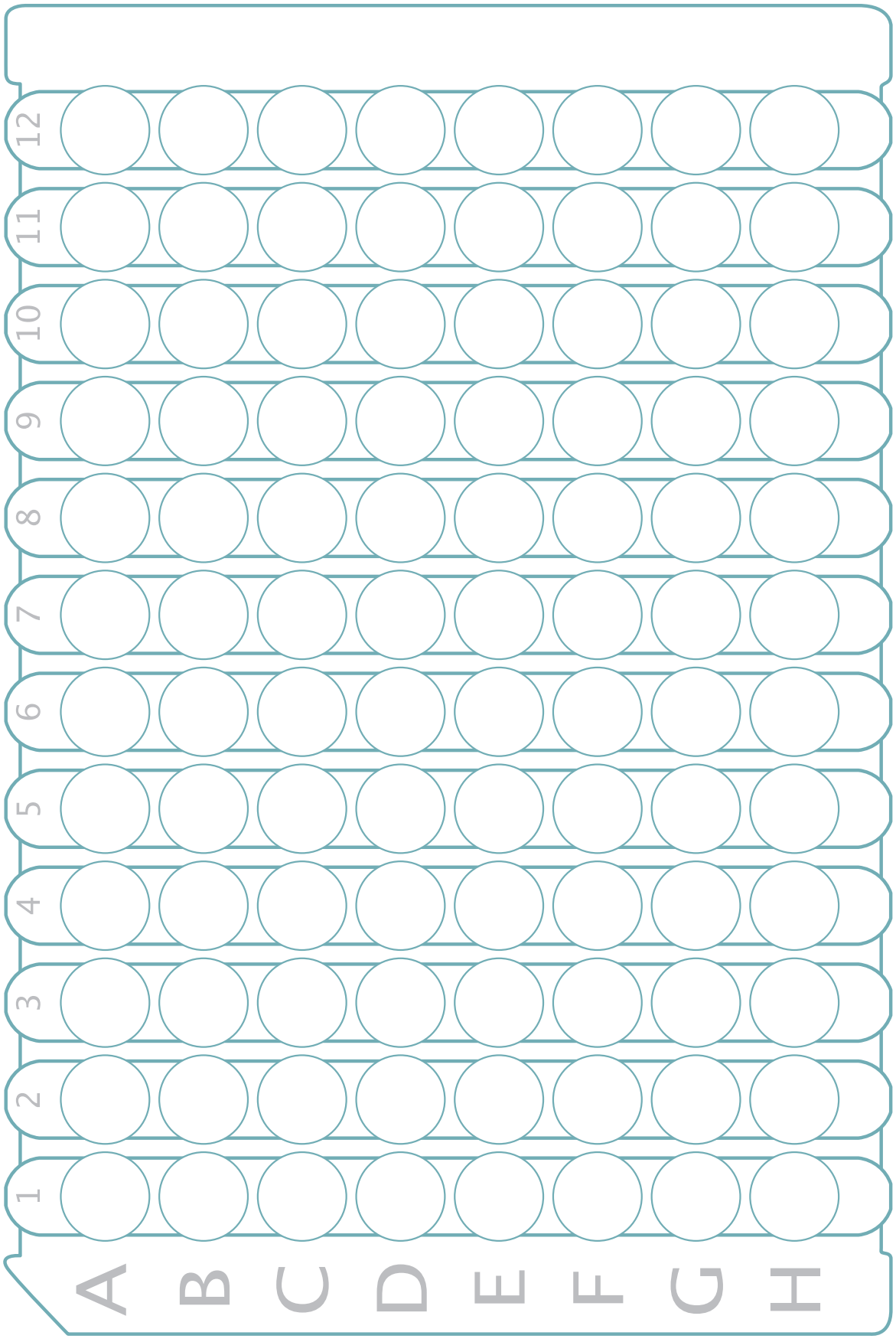
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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