Quantikine® HS ELISA

Human PlGF Immunoassay

Catalog Number HSPG00

For the quantitative determination of human Placenta Growth Factor (PlGF) concentrations in serum and plasma.

Note: The standard reconstitution method has changed. Read this package insert in its entirety before using this product.

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

Placenta growth factor (PlGF, also called PGF) is a member of the PDGF/VEGF family of growth factors that share a conserved pattern of eight cysteines (1-4). Alternate splicing may result in human mature PlGF forms containing 131 (PlGF-1), 152 (PlGF-2), 203 (PlGF-3), and 224 (PlGF-4) amino acids (aa) (1-6). PlGF-2 and PlGF-4 contains a highly basic heparin-binding 21 amino acid insert at the C-terminus that is not present in PLGF-1 or -3 (2-5). In the mouse, only one PlGF that is the equivalent of human PlGF-2 has been identified (1, 5, 6). Human PlGF-1 shares 56%, 55%, 74%, and 95% aa identity with the appropriate isoform of mouse, rat, canine and equine PlGF. PlGF is mainly found as variably glycosylated, secreted, 55-60 kDa disulfide-linked homodimers (2, 4). Mammalian cells expressing PlGF include villous trophoblasts, decidual cells, erythroblasts, keratinocytes, and some endothelial, smooth muscle, alveolar epithelial and cancer cells (3-5, 7-9). PlGF expression is generally low, but is upregulated in situations that require angiogenesis and inflammatory cell recruitment such as pregnancy, ischemia, wound healing, bone fracture repair, and cancer metastasis (5, 10-12).

PlGF binds to and signals through VEGF R1/Flt-1, but not VEGF R2/Flk-1/KDR (5, 6, 13, 14). In contrast, VEGF binds both but sends angiogenic signals only through VEGF R2 (13-15). PlGF and VEGF therefore compete for binding to VEGF R1, and increased PlGF expression discourages VEGF/VEGF R1 binding and promotes VEGF/VEGF R2-mediated angiogenesis (3, 4, 13, 14). PlGF (especially PlGF-1) and some forms of VEGF can form dimers, which are reported to have angiogenic activity that is anywhere from very little to being almost as potent as VEGF homodimers (4, 12-17). PlGF-2, but not PlGF-1, shows heparin-dependent binding of neuropilin (Npn)-1 and Npn-2 (4, 18, 19).

PlGF mediates compensatory responses to injury, but can also contribute to pathologies (12). PlGF induces migration of cells such as monocytes, macrophages, endothelial cells, osteogenic precursors, and dermal fibroblasts, and induces monocyte production of inflammatory cytokines and VEGF (2-5, 7, 20-23). These activities facilitate wound and bone fracture healing (4, 10, 11, 22). PlGF induction is reduced in diabetic wounds, causing impaired healing (23). PlGF may also promote chronic inflammation, which contributes to plaque formation in atherosclerosis (5, 7, 8). In active sickle cell disease, PlGF produced by erythroblasts in response to hypoxia creates a chronic inflammatory state. Increased circulating PlGF also correlates with pulmonary hypertension and vascular occlusion in sickle cell disease (7, 24-26). Increased PlGF expression by some tumors, such as breast and gastric carcinomas, generally correlates with tumor stage and metastasis, and supports tumor angiogenesis and differentiation of recruited macrophages to the tumor-associated M2 phenotype (4, 27-30). PlGF can also be immunosuppressive by inhibiting dendritic cell function, which subsequently downregulates Th1 immune response (31).

PlGF cooperates with VEGF to induce endothelial cell growth and inhibit apoptosis, thus supporting angiogenesis (11, 17, 32, 33). Circulating PlGF increases during pregnancy, reaching a peak in mid-gestation (35). Attenuation of this increase is found in preeclampsia and is thought to contribute to its pathology (9, 12, 34-37). However, deletion of PlGF in the mouse does not affect development or reproduction (4, 13). Mice lacking PlGF show normal embryonic development, but postnatal angiogenic response to ischemia is impaired (4, 13). PlGF mediates beneficial angiogenesis and cardiac hypertrophy in response to myocardial infarction; however, highest levels of plasma PlGF may correlate with the severity of heart failure (33, 38-40).

The Quantikine® HS Human PlGF Immunoassay is a 3.5 hour solid phase ELISA designed to measure human PlGF levels in serum and plasma. It contains *E. coli*-expressed recombinant human PlGF and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate recombinant human PlGF accurately. Results obtained using natural human PlGF showed linear curves that were parallel to the standard curves obtained using the Quantikine® HS kit standards. These results indicate that this kit can be used to determine relative mass values for natural human PlGF.

PRINCIPLE OF THE ASSAY

DUE TO THE HIGH SENSITIVITY OF THIS KIT, PLEASE NOTE THE FOLLOWING PRECAUTIONS.

- **Alkaline phosphatase is detectable in saliva.** Take precautionary measures (*e.g. wear a mask and gloves*) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Stop Solution).
- Inorganic phosphate is a strong competitive inhibitor of alkaline phosphatase; avoid the use of PBS based wash buffers and other sources of inorganic phosphate contamination.
- Use separate pipettes and glassware for dispensing all reagents to avoid cross-contamination.
- Careful washing of the microplate is essential to minimize nonspecific binding of the conjugate.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human PlGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any PlGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human PlGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an incubation period, an amplifier solution is added to the wells and color develops in proportion to the amount of PlGF 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.
- Samples, controls, and standards must be pipetted within 15 minutes.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in 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

- To ensure accurate results, bring liquids to room temperature and mix to homogeneity prior to pipetting or aliquoting.
- When mixing 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.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Neither the addition of the Substrate Solution nor the Stop Solution will result in a color change.
- Addition of the Amplifier Solution will result in a color change (colorless to shades of maroon).
- Amplifier Solution and Stop Solution should be added to the plate in the same order as 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 PlGF HS Microplate	894541	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human PlGF.	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 PlGF HS Conjugate	894542	21 mL of a polyclonal antibody specific for human PlGF, conjugated to alkaline phosphatase with preservatives.	Store for up to 1 month at 2-8 °C.*
Human PlGF HS Standard	894543	Recombinant human PlGF in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-22	895490	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-11	895489	21 mL of a buffered protein base with preservatives.	
Wash Buffer 10X Concentrate	895188	100 mL of a 10-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Substrate	895884	Lyophilized NADPH with stabilizers.	**Store in an upright position** for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Substrate Diluent	895885	7 mL of a buffered solution with stabilizers and preservative.	
Amplifier	895886	Lyophilized amplifier enzymes with stabilizers.	
Amplifier Diluent	895887	7 mL of a buffered solution containing INT-violet with stabilizer and preservative.	
Plate Sealers	N/A	8 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 490 nm, with the correction wavelength set at 650 nm or 690 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser or autowasher.
- 1000 mL graduated cylinder for preparation of Wash Buffer.
- Test tubes for dilution of standards.
- Human PlGF Controls (Optional; R&D Systems®, Catalog # QC144).

PRECAUTIONS

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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.

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.

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.*
Grossly hemolyzed or icteric samples are not suitable for use in this assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: ***Alkaline phosphatase is detectable in saliva.*** *Take precautionary measures (e.g. wear a mask and gloves) to protect reagents during preparation and use and while running the assay (reagent preparation through the addition of Stop Solution).*

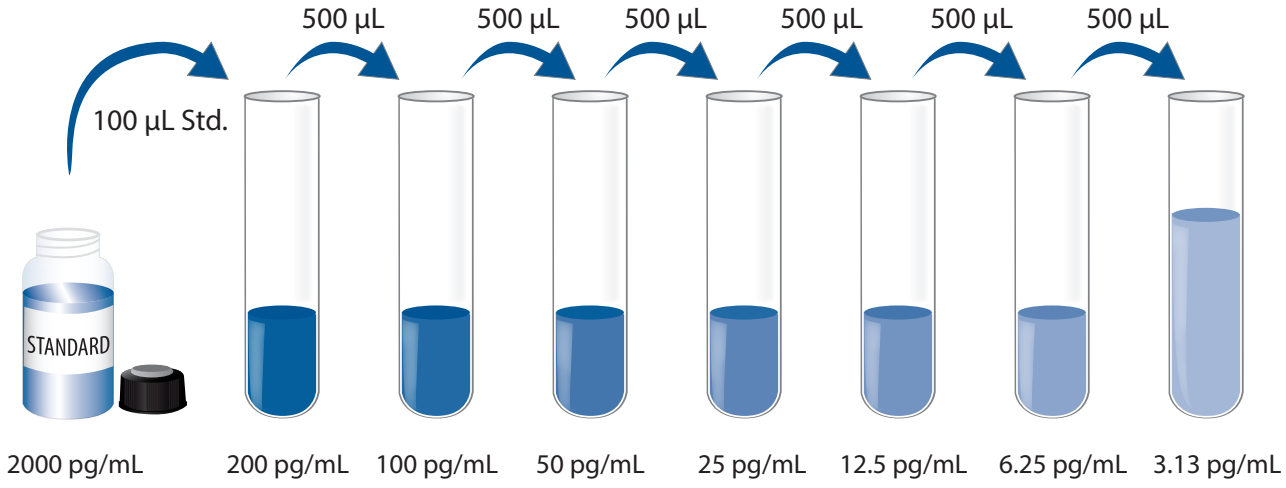
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 100 mL of Wash Buffer 10X Concentrate to deionized or distilled water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Reconstitute the lyophilized Substrate in 6.0 mL of Substrate Diluent at least 10 minutes before use. **Re-stopper and re-cap the vial**, and mix thoroughly. Avoid contamination.

Amplifier Solution - Reconstitute the lyophilized Amplifier in 6.0 mL of Amplifier Diluent at least 10 minutes before use. **Re-stopper and re-cap the vial**, and mix thoroughly. Avoid contamination.

Human PlGF HS Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the PlGF HS Standard with deionized or distilled water. This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6-11 into the 200 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 200 pg/mL standard serves as the high standard. Calibrator Diluent RD6-11 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: *Alkaline phosphatase is detectable in saliva. Take precautionary measures to protect reagents (e.g. wear a mask and gloves).*

1. Prepare all reagents and working standards 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 RD1-22 to each well.
4. Add 100 µL of standard, control, or sample per well. Securely cover with a plate sealer and incubate for 1 hour at room temperature. Gently tap the plate to ensure thorough mixing. A plate layout is provided to record standards and samples assayed.

   Note: Standard, control, and samples must be pipetted within 15 minutes.
5. Aspirate each well and wash, repeating the process five times for a total of six 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 PlGF HS Conjugate to all wells. Securely cover with a plate sealer and incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 50 µL of Substrate Solution to each well. Securely cover with a plate sealer and incubate for 1 hour at room temperature. **Protect from light. Do not wash the plate.**
9. Add 50 µL of Amplifier Solution to each well. Securely cover with a plate sealer and incubate for 30 minutes at room temperature.

   Note: *Addition of Amplifier Solution initiates color development.*
10. Add 50 µL of Stop Solution to each well. Addition of Stop Solution does not affect color in wells.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 490 nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

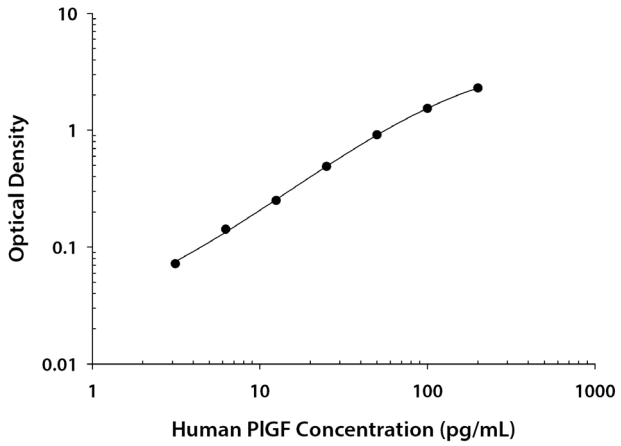
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 PlGF 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	0.047 0.047	0.047	—
3.13	0.116 0.122	0.119	0.072
6.25	0.183 0.195	0.189	0.142
12.5	0.296 0.299	0.298	0.251
25	0.520 0.551	0.536	0.489
50	0.952 0.965	0.959	0.912
100	1.554 1.617	1.586	1.539
200	2.303 2.386	2.345	2.298

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)	27.2	57.4	108	27.3	57.7	108
Standard deviation	0.72	1.87	2.84	1.65	3.88	7.37
CV (%)	2.6	3.3	2.6	6.0	6.7	6.8

RECOVERY

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

Sample Type	Average % Recovery	Range
Serum (n=4)	92	86-97%
EDTA plasma (n=4)	98	85-115%
Heparin plasma (n=4)	90	86-97%

LINEARITY

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

		Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	101	99	104
	Range (%)	98-106	96-104	100-109
1:4	Average % of Expected	103	102	104
	Range (%)	98-109	97-107	96-113
1:8	Average % of Expected	105	105	105
	Range (%)	102-107	99-107	99-113
1:16	Average % of Expected	105	101	103
	Range (%)	96-111	98-105	100-110

SENSITIVITY

Twenty-eight assays were evaluated and the minimum detectable dose (MDD) of human PlGF ranged from 0.065-0.326 pg/mL. The mean MDD was 0.137 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 *E. coli*-expressed recombinant human PlGF produced at R&D Systems®.

The NIBSC PlGF reference standard 09/272 was evaluated in this kit. The dose response curve of this standard parallels the Quantikine® HS standard curve. To convert sample values obtained with the Quantikine® HS Human PlGF kit to approximate NIBSC units, use the equation below.

NIBSC (09/272) approximate value (U/mL) = 0.0008 x Quantikine® HS PlGF value (pg/mL)

Note: *Data was generated in August 2013.*

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human PlGF 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=20)	18.8	13.9-26.5	3.2
EDTA plasma (n=20)	17.6	12.0-24.0	3.0
Heparin plasma (n=20)	17.1	13.0-24.2	3.0

SPECIFICITY

This assay recognizes natural and recombinant human PlGF.

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

Recombinant human:

$VEGF_{111}$
$VEGF_{121}$
$VEGF_{162}$
$VEGF_{165}$
$VEGF_{165b}$
$VEGF\text{-}B_{167}$
VEGF-C
VEGF-C (Cys156Ser)
VEGF-D
VEGF R2/KDR
VEGF R3/Flt-4

Recombinant mouse:

$VEGF_{120}$
$VEGF_{164}$
$VEGF\text{-}B_{167}$
$VEGF\text{-}B_{186}$
VEGF-D
VEGF R2/Flk-1
VEGF R3/Flt-4

Other recombinants:

canine VEGF
feline VEGF
rat $VEGF_{164}$
zebrafish $VEGF_{165}$

Cross-reactivity was observed with the following:

Recombinant Factor	% Cross-reactivity
human PlGF-2	34
human PlGF-3	8.9
human VEGF/PlGF Heterodimer	0.30
mouse PlGF-2	0.01

Interference was observed with the following:

Recombinant Factor	Concentration Tested (ng/mL)
human VEGF R1/Flt-1	> 1.00
human VEGF R1/Flt-1 (aa 27-328)	> 10.0
mouse VEGF R1/Flt-1	> 1.00

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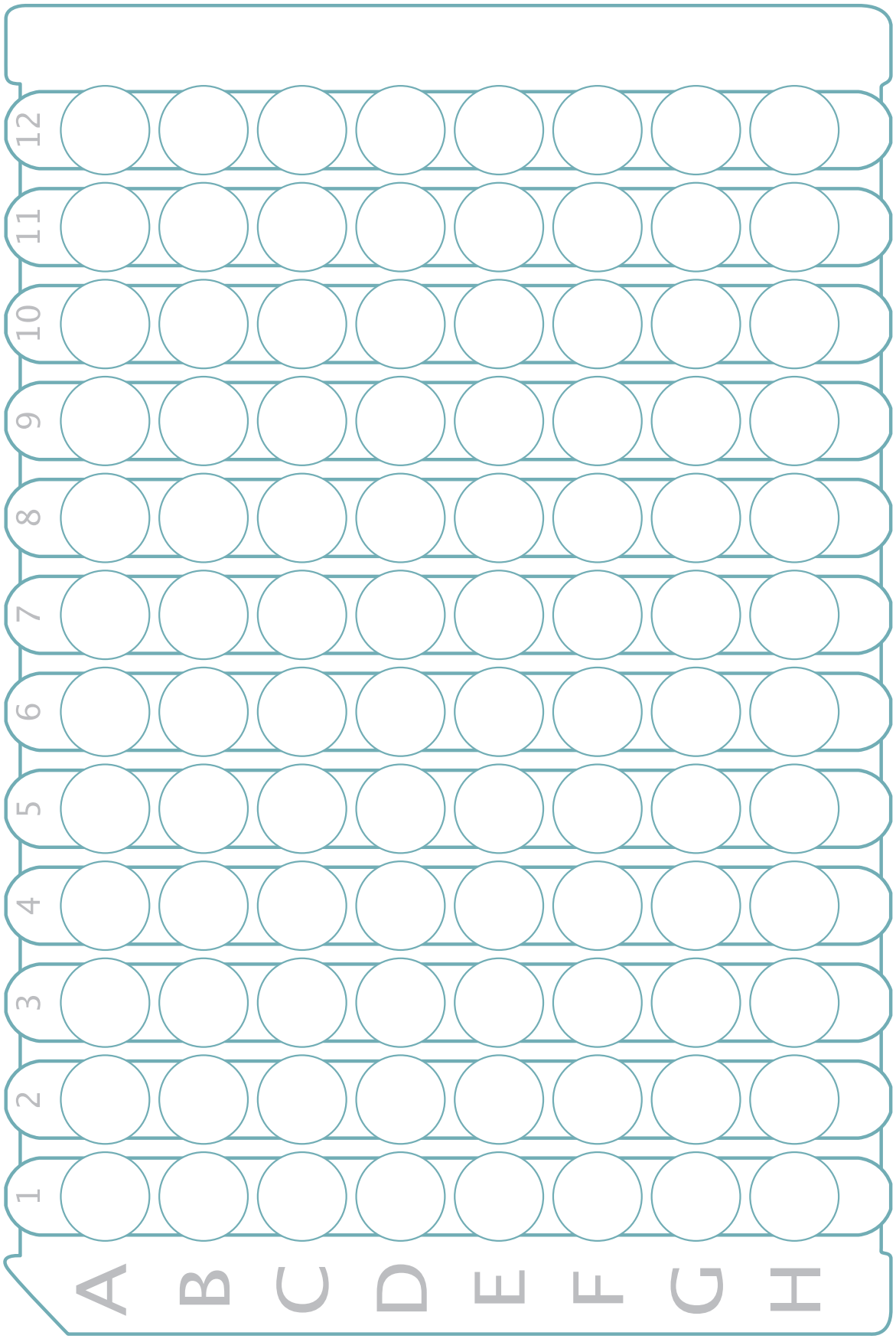
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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