QuantiGlo® ELISA

Human Endothelin-1 Immunoassay

Catalog Number QET00B

For the quantitative determination of human Endothelin-1 (ET-1) 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

Endothelin-1 (ET-1), a peptide of 21 amino acid (aa) residues, is a pleiotropic molecule best known for its action as a potent vasoconstrictor (1). Originally isolated from porcine aortic endothelial cells, ET-1 is one of a family of three proteins encoded by distinct genes that also includes Endothelin-2 (ET-2) and Endothelin-3 (ET-3) (2, 3). ET-2 and ET-3 differ from ET-1 by 2 and 6 amino acids, respectively (1, 2). All members of the Endothelin family contain two essential disulfide bridges and six conserved aa residues at the C-terminus. Human ET-1 is initially synthesized as a pre-pro-polypeptide of 212 amino acids (2, 4). It is proteolytically cleaved by a signal peptidase to produce pro-ET-1 and further processed by a Furin-like protease to yield a 38 aa peptide termed Big ET-1 (5, 6). Big ET-1 is then cleaved by the membrane-bound metalloprotease Endothelin-converting enzyme (ECE-1), producing the potent 21 aa mature form ET-1 (aa 1-21) (7, 8). Alternatively, ET-1 may exist in an active 31 aa form (ET-1 (aa 1-31)) following cleavage of Big ET-1 by chymase (9-12). The vascular endothelium is an abundant source of ET-1 (3, 13). It may also be expressed by leukocytes, smooth muscle cells, mesangial cells, cardiac myocytes, and astrocytes (14, 15). ET-1 can be induced in endothelial cells by many factors including mechanical stimulation, various hormones, and pro-inflammatory cytokines (16). Production is inhibited by nitric oxide (NO), Prostacyclin, and atrial natriuretic peptide (ANP) (17-19).

Two receptors for the Endothelin family have been cloned and designated ET_A and ET_B (20-23). ET_A and ET_B belong to the large family of heptahelical G protein-coupled receptors. The ET_A receptor shows a higher affinity for ET-1 than for ET-2 and lowest affinity for ET-3, while the ET_B receptor shows approximately equal affinity for each of the three Endothelins (21, 22, 24). ET_A is primarily responsible for the vasoconstrictor effects of ET-1 and is expressed by blood vessel smooth muscle cells (25, 26). The ET_B receptor is also present in smooth muscle and the endothelia of blood vessels, kidney, lung, and brain (27). ET-1 has the ability to activate an array of signaling cascades including classical phosphatidylinositol turnover pathways leading to downstream PKC activation and Ca^{2+} mobilization (28-32). Other potential signaling mediators activated or produced by ET-1 include PI 3-kinase/Akt, NO, FAK, and Rho GTPases (32-37). ET-1 signaling may also be mediated indirectly via transactivation of the EGF receptor leading to downstream signaling by Ras and MAP kinases (38, 39).

Injection of a single dose of ET-1 produces an initial decrease in systemic blood pressure followed by a prolonged increase in blood pressure (16, 40, 41). Blockade of Endothelin receptors with a systemic injection of an ET_A/ET_B antagonist causes progressive vasodilation, and elevated levels of ET-1 are found in some forms of human hypertension (42, 43). ET-1 also stimulates cardiac contraction and the growth of cardiac myocytes, regulates the release of vasoactive substances, and stimulates smooth muscle cell mitogenesis (32, 44-46). It also acts as a pro-survival factor for endothelial cells and regulates secretion by hypothalamic and pituitary cells (47, 48). ET-1 may control inflammatory responses by promoting the adhesion and migration of neutrophils and stimulating the production of pro-inflammatory cytokines (49-53). It has also been implicated in cancer progression at several levels including regulating the proliferation and migration of tumor cells and acting as a pro-angiogenic factor (54, 55). In addition, ET-1 has putative roles in other pathologies including septic shock, atherosclerosis, heart failure, renal insufficiency, pulmonary hypertension, and cerebrovascular conditions associated with subarachnoid hemorrhage (15, 56-64).

The QuantiGlo Human Endothelin-1 Chemiluminescent Immunoassay is a 4.5 hour solid phase ELISA designed to measure human ET-1 levels in cell culture supernates, serum, EDTA plasma, and urine without extraction. It contains synthetic human ET-1 and antibodies raised against the synthetic factor. This immunoassay has been shown to accurately quantitate human ET-1. Results obtained using natural human ET-1 showed dose-response curves that were parallel to the standard curves obtained using the QuantiGlo kit standards. These results indicate that this kit can be used to determine relative mass values of natural human ET-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ET-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ET-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for ET-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of ET-1 bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted.

TECHNICAL HINTS AND LIMITATIONS

- 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, dilute the samples with Calibrator Diluent and repeat the assay.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the QuantiGlo Immunoassay, the possibility of interference cannot be excluded.
- Variations in pipetting technique, washing technique, luminometers, incubation time or temperature can cause variations in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- 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.
- Relative light units (RLUs) may differ among luminometers. Adjust settings as recommended by the instrument manufacturer.
- Relative light units may vary within the 15 minute reading window.
- Due to limitations of many luminometers, it is recommended that the standards be assayed in duplicate from high to low beginning with the high standard in wells A_1 and A_2.

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
Endothelin-1 Microplate	892739	One 96 well black polystyrene microplate (12 strips of 8 wells) coated with a rat monoclonal antibody against ET-1.	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.*
Endothelin-1 Conjugate	892904	21 mL of a mouse monoclonal antibody against ET-1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Endothelin-1 Standard	890631	2.5 ng of synthetic human ET-1 in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1-19	895467	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-13	895309	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895222	100 mL of a 10-fold concentrated solution of buffered surfactant with preservatives.	
Glo Reagent A	895868	4 mL of stabilized enhanced luminol.	
Glo Reagent B	895869	8 mL of stabilized hydrogen peroxide.	
Plate Sealers	N/A	4 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Luminometer set with the following parameters: 1.0 minute lag time; 0.5 sec/well read time; summation mode; auto gain on, or equivalent.
- Pipettes and pipette tips.
- 1 liter graduated cylinder.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Human Endothelin-1 Controls (optional; available from R&D Systems).

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 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: *Heparin and citrate plasma are not recommended for use in this assay.*
Lipemic samples 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. Assay immediately or aliquot and store at ≤ -20° C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

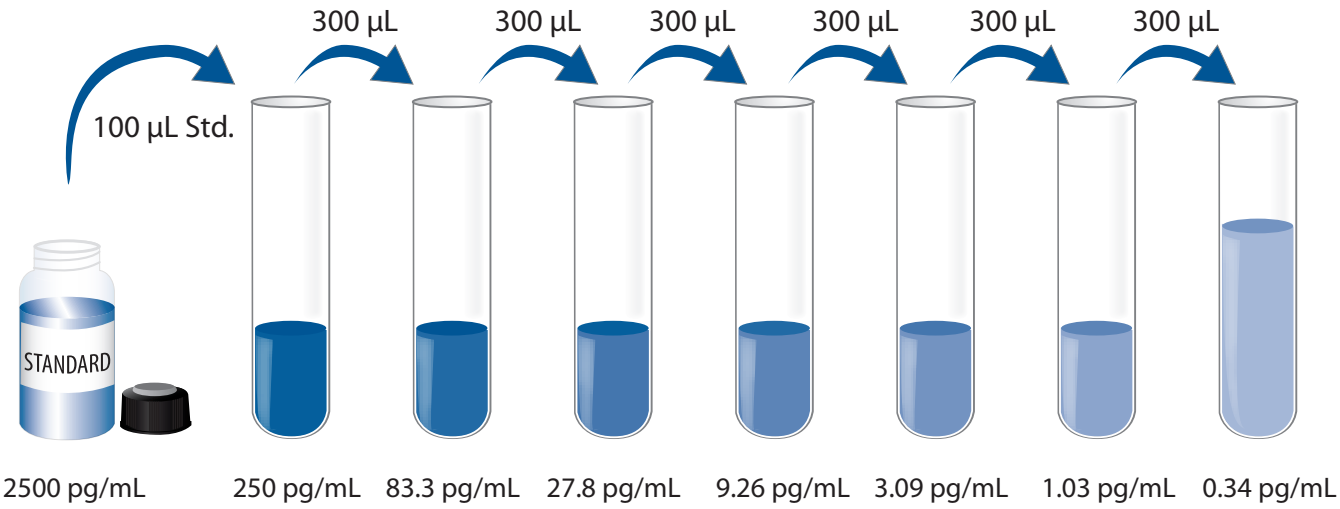
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 100 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 1000 mL of Wash Buffer.

Working Glo Reagent - 1 part Glo Reagent A (4 mL) and 2 parts Glo Reagent B (8 mL) should be mixed together 15 minutes to 4 hours before use in a capped plastic container and protected from light. 100 µL of the resultant mixture is required per well.

Note: *If running the assay in less than 96 wells, mix appropriate amounts Glo Reagent A and Glo Reagent B. To assay half a plate (48 wells), mix 2 mL of Glo Reagent A with 4 mL of Glo Reagent B. Working Glo Reagent should be discarded after use.*

Endothelin-1 Standard - Reconstitute the Endothelin-1 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 2500 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 RD5-13 into the 250 pg/mL tube. Pipette 600 µL of Calibrator Diluent RD5-13 into the remaining tubes. Use the stock solution to produce a 3-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 250 pg/mL standard serves as the high standard. Calibrator Diluent RD5-13 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 RD1-19 to each well.
4. Add 100 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 1.5 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided as a record of 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 Endothelin-1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 3 hours at room temperature on the shaker.

   Note: *Prepare Working Glo Reagent at this time.*
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Working Glo Reagent to each well. Incubate for 5-20 minutes at room temperature **on the benchtop. Protect from light.**
9. Determine the RLU of each well using a luminometer set with the following parameters: 1.0 min. lag time; 0.5 sec/well read time; summation mode; auto gain on, or equivalent.

CALCULATION OF RESULTS

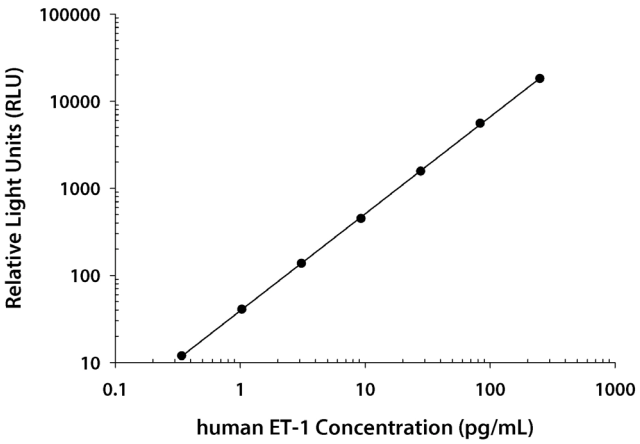
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 ET-1 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If 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)	RLU	Average	Corrected
0	12.87 14.01	13.44	—
0.34	24.32 26.47	25.39	11.95
1.03	52.42 55.84	54.13	40.69
3.09	148.6 153.0	150.8	137.4
9.26	462.7 464.6	463.6	450.2
27.8	1577 1585	1581	1568
83.3	5521 5647	5584	5571
250	18,203 18,231	18,217	18,204

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	1.78	20.5	90.9	1.76	19.8	86.8
Standard deviation	0.06	0.7	2.4	0.16	1.3	3.9
CV (%)	3.4	3.4	2.6	9.1	6.6	4.5

RECOVERY

The recovery of synthetic human ET-1 spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	93-107%
Serum (n=4)	95	87-103%
EDTA plasma (n=4)	94	88-99%
Urine (n=4)	98	92-105%

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) of ET-1 ranged from 0.023-0.102 pg/mL. The mean MDD was 0.064 pg/mL.

The MDD was determined by adding two standard deviations to the mean RLU of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This assay is standardized against synthetic human Endothelin-1.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of ET-1 in various matrices 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)	Urine (n=4)
1:2	Average % of Expected	98	103	95	97
	Range (%)	96-100	98-105	91-105	93-101
1:4	Average % of Expected	99	103	96	94
	Range (%)	93-106	96-108	90-108	88-102
1:8	Average % of Expected	90	100	91	92
	Range (%)	87-91	92-106	84-106	87-99
1:16	Average % of Expected	93	102	96	94
	Range (%)	92-94	94-110	85-114	88-100
1:32	Average % of Expected	93	97	93	92
	Range (%)	88-98	86-107	84-112	86-97

SAMPLES VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of ET-1 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=36)	1.08	100	0.401-2.83
EDTA plasma (n=36)	0.913	97.2	ND-2.48
Urine (n=35)	0.457	5.7	ND-0.495

ND=Non-detectable

Cell Culture Supernates:

Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 6 and assayed for levels of natural ET-1.

Condition	Day 1 (pg/mL)	Day 6 (pg/mL)
Unstimulated	ND	1.95
Stimulated	1.76	2.86

ND=Non-detectable

HUVEC human umbilical vein endothelial cells (3 x 10^5 cells/flask) were cultured in endothelial growth medium (EGM) until confluent. An aliquot of the cell culture supernate was removed, assayed for levels of natural ET-1, and measured 490 pg/mL.

SPECIFICITY

This assay recognizes both natural and synthetic human Endothelin-1.

ET-1 related peptides were added to the Calibrator Diluent at the concentrations listed below and assayed for cross-reactivity. The same peptides were added to a mid-level ET-1 control and evaluated for interference.

Some cross-reactivity was observed with the following related peptides:

Peptide	Concentration Tested (pg/mL)	Observed Value (pg/mL)	% Cross-reactivity
human ET-2	100	50.5	51%
human Big ET-2	10,000	0.995	0.01%
human/rat ET-3	100	8.98	9.0%
human Big ET-38	2000	0.358	0.02%
bovine Big ET-39	2000	0.354	0.02%
rat Big ET-39	2000	0.987	0.05%
Sarafotoxin S6b	50,000	146	0.29%
Sarafotoxin S6c	50,000	0.516	0.001%

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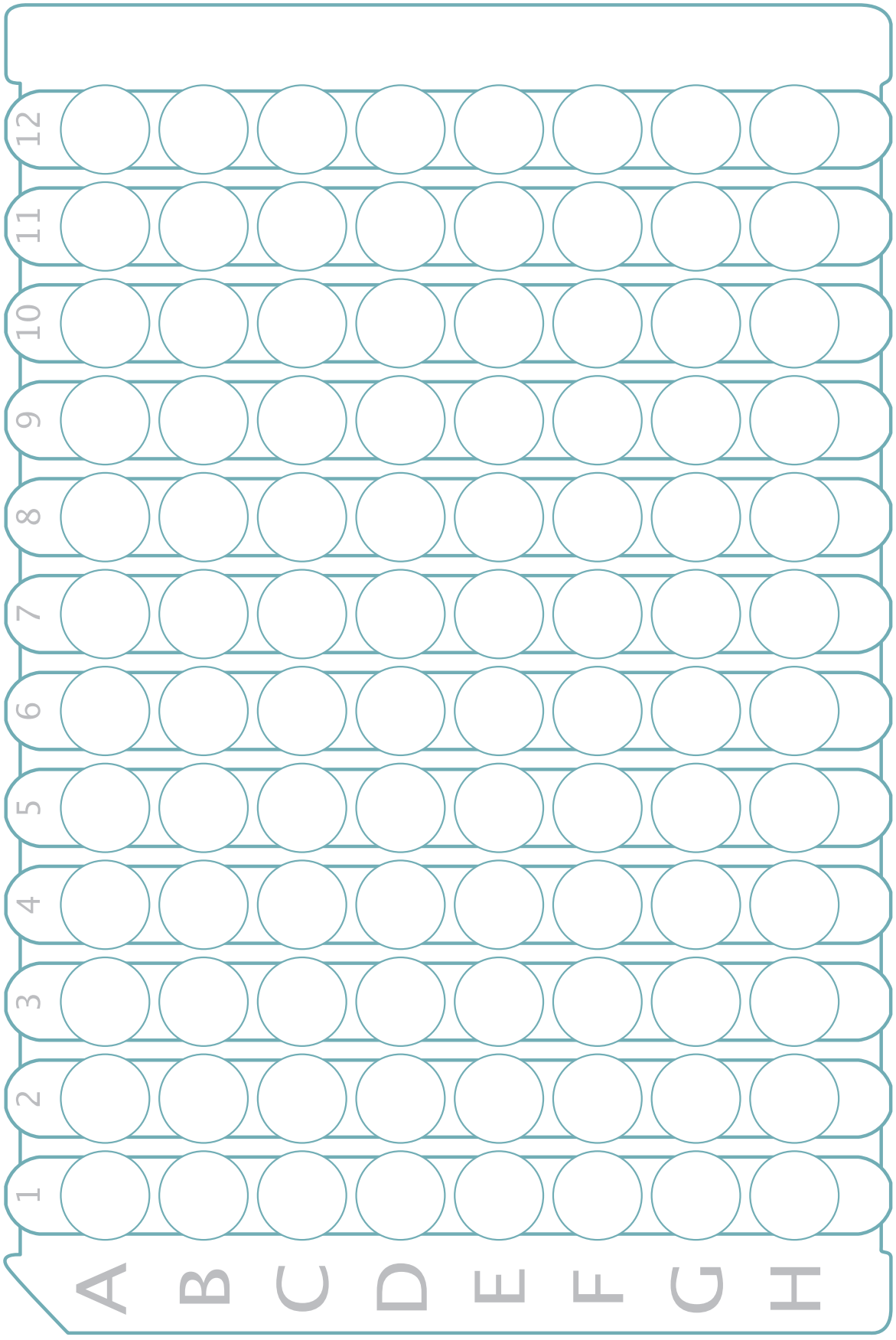
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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