Quantikine® ELISA

Human Endostatin Immunoassay

Catalog Number DNST0
SNST0
PDNST0

For the quantitative determination of human Endostatin concentrations in cell culture supernates, serum, plasma, and saliva.

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

This package insert must be read in its entirety before using this product.
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INTRODUCTION

Endostatin is well known for its anti-growth and anti-migratory effects on endothelial cells (ECs) (1). In this capacity it has received much attention for its potential use as an angiogenesis inhibitor capable of reducing the blood supply necessary for the maintenance and growth of tumors (2). Endostatin was originally isolated from murine hemangioendothelioma (EOMA) cells as a 20 kDa proteolytic fragment of type XVIII Collagen (2). Human type XVIII Collagen consists of an N-terminal region containing at least two splice variants, followed by a central triple helical domain, and a C-terminal, non-collagenous (NC1) domain from which Endostatin is derived (3, 4). The process leading to the release of human Endostatin from the NC1 domain is not fully understood (5). Cathepsin L and Elastase have been implicated as proteinases responsible for its generation in EOMA cells (6, 7). Mean concentrations in serum samples obtained from healthy human donors are somewhat variable but are estimated at approximately 20 ng/mL (8-11).

Endostatin is perhaps best known for its anti-tumor activity in animal models. Effects were first described for mouse Endostatin where an almost complete regression of several tumor types was reported including Lewis lung carcinoma, fibrosarcoma, melanoma, and EOMA (2, 12). Since then, experiments carried out using human Endostatin suggest that, like the murine form, it can also suppress tumor growth in animal models (13-18). Some studies using gene transfer techniques paradoxically report no effect on the vasculature or on implanted tumors in mice despite relatively high levels of circulating Endostatin (19, 20). The underlying reason for the conflicting results is unclear and suggests that some questions may still remain (21, 22).

In vitro studies demonstrate that Endostatin can regulate EC physiology in ways that could affect angiogenesis. For instance, soluble Endostatin inhibits EC migration and leads to rearrangements of the cytoskeleton that include the loss of Actin stress fibers and focal adhesions (13, 23, 24). This is shown to involve several binding partners including α5β1integrins, Tropomyosin, and putative heparan sulfate proteoglycans (25-27). Effects on the human EC cytoskeleton are accompanied by downregulation of Mitogen-activated Protein Kinase (MAPK), Focal Adhesion Kinase (FAK), the Urokinase Plasminogen Activator (uPA) System, and the RhoA GTPase (24, 27, 28). In addition, Endostatin inhibition of the Wnt signaling pathway has been shown to suppress VEGF and FGF-2-induced EC migration *in vitro* (29). It should be noted that differential effects on EC migration have been reported depending upon whether Endostatin was in a soluble form, or immobilized to a substrate (23). Human Endostatin has also been shown in some studies to inhibit EC proliferation. Endostatin-induced cell cycle arrest in G1 phase is accompanied by Cyclin D1 downregulation (29). Human Endostatin can initiate EC apoptosis, and in C-PAE cells, this is accompanied by a reduction in the anti-apoptotic proteins Bcl-2 and Bcl-$_{xL}$ (30, 31).

Elevated circulating levels of Endostatin are associated with many forms of cancer including breast, renal, ovarian, and endometrial cancers, soft tissue sarcoma, acute myelogenous leukemia, non-Hodgkins lymphoma, non-small cell lung cancer, hepatocellular carcinoma, and head and neck squamous cell carcinoma (9-11, 32-38). Additionally, human Endostatin treatment in murine xenograft models of rheumatoid arthritis (RA) has been effective, and increased circulating levels of Endostatin have been found in patients following the commencement of treatment for RA (39-41). Endostatin is also found upregulated in amyloid plaques associated with Alzheimer's disease (42).

INTRODUCTION *CONTINUED*

Endostatin is expressed in tissue from mouse brain, skeletal muscle, heart, kidney, testes, and liver, and is found in serum from healthy human donors (8-11, 43). However, the physiological role for Endostatin remains unclear. Collagen XVIII knockout mice display abnormal outgrowth of retinal capillaries and delays in the normal regression of hyaloid vessels that could suggest a role in development of the vasculature (44). Knockout mice do not exhibit excessive tumor growth indicating that normal circulating levels of Endostatin may not be sufficient to inhibit tumor progression (44).

In addition to ECs, Endostatin may affect other cell types as well. For instance, overexpression of mouse Endostatin can cause developmental abnormalities in early *Xenopus* embryos, potentially due to deficient Wnt signaling and subsequent promotion of β-Catenin degradation (29). In addition, the Glypicans, cell surface proteoglycans, are low affinity binding partners for mouse Endostatin (25). This interaction is important for Endostatin-mediated inhibition of renal tubular epithelial cell branching and morphogenesis, and ureteric bud branching (25, 45). Lastly, ectopic expression of the Endostatin homolog in *C. elegans* leads to neuronal migratory and axon pathfinding defects (46).

The Quantikine Human Endostatin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Endostatin in cell culture supernates, serum, plasma, and saliva. It contains *E. coli*-expressed recombinant human Endostatin and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Endostatin 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 naturally occurring Endostatin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Endostatin has been pre-coated onto a microplate. Standards, controls and samples are pipetted into the wells and any Endostatin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Endostatin 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 Endostatin bound in the initial step. The color development is stopped and the intensity of the color is measured.

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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 #	CATALOG # DNST0	CATALOG # SNST0	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Endostatin Microplate	892533	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Endostatin.	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 Endostatin Conjugate	892534	1 vial	6 vials	21 mL/vial of a monoclonal antibody specific for human Endostatin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Endostatin Standard	892535	1 vial	6 vials	Recombinant human Endostatin in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1W	895117	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD5P Concentrate	895151	1 vial	6 vials	21 mL/vial of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DNST0 contains sufficient materials to run an ELISA on one 96 well plate.
SNST0 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDNST0). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

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.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as Salivette® or equivalent.
- Test tubes for dilution of standards and samples.
- Human Endostatin Controls (optional; available from R&D Systems).

PRECAUTIONS

Endostatin 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.

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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.*

Saliva - Collect saliva into a polypropylene tube. Centrifuge for 5 minutes at 10,000 x g in microcentrifuge tubes. Collect the aqueous layer (no pellet) and assay immediately or aliquot and store samples at 2-8 °C.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

SAMPLE PREPARATION

Serum and plasma samples require a 50-fold dilution. A suggested 50-fold dilution is 20 µL of sample + 980 µL of Calibrator Diluent RD5P (diluted 1:5).*

*See Reagent Preparation section.

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of Endostatin are found in saliva. It is recommended that a face mask and gloves are used to protect kit reagents from contamination.*

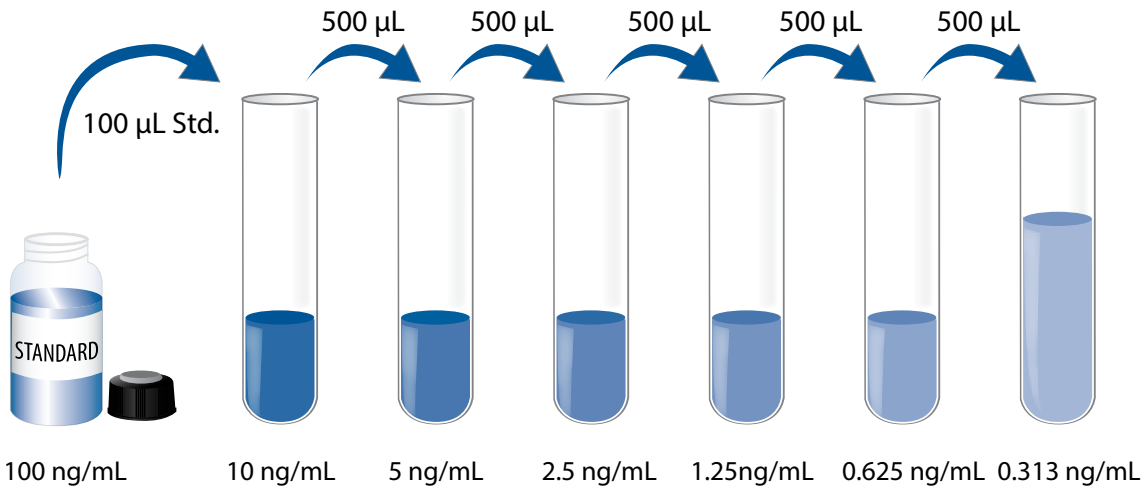
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 Endostatin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Endostatin Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/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.

Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:5) into the 10 ng/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 10 ng/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 ng/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: *High concentrations of Endostatin are found in saliva. It is recommended that a face mask and gloves are used to protect kit reagents from contamination.*

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 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 Endostatin 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 Sample Preparation section

CALCULATION OF RESULTS

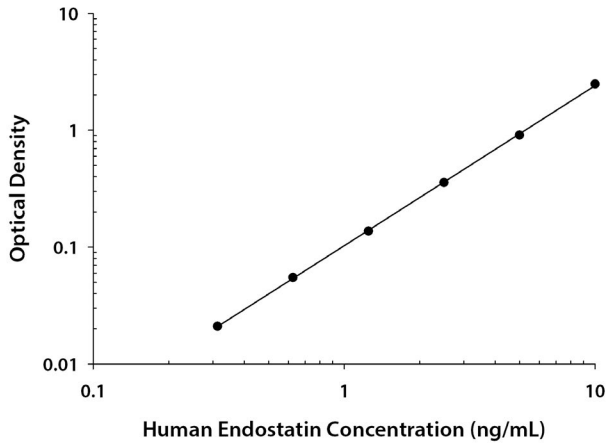
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 human Endostatin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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.



(ng/mL)	O.D.	Average	Corrected
0	0.019 0.019	0.019	—
0.313	0.039 0.041	0.040	0.021
0.625	0.074 0.074	0.074	0.055
1.25	0.152 0.159	0.156	0.137
2.5	0.366 0.383	0.375	0.356
5	0.922 0.934	0.928	0.909
10	2.433 2.573	2.503	2.484

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 (ng/mL)	0.70	2.02	4.14	0.76	2.13	4.21
Standard deviation	0.05	0.12	0.15	0.06	0.13	0.24
CV (%)	7.1	5.9	3.6	7.9	6.1	5.7

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	91 - 114%
Serum (n=4)	100	93 - 110%
EDTA plasma (n=4)	100	92 - 110%
Heparin plasma (n=4)	98	86 - 108%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Endostatin were serially 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)
1:2	Average % of Expected	95	98	97	103
	Range (%)	94-96	91-101	93-101	97-115
1:4	Average % of Expected	91	102	99	104
	Range (%)	89-92	92-109	92-105	97-115
1:8	Average % of Expected	92	101	98	103
	Range (%)	91-94	93-109	94-103	93-113

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SENSITIVITY

Fifty-one assays were evaluated and the minimum detectable dose (MDD) of human Endostatin ranged from 0.001-0.063 ng/mL. The mean MDD was 0.023 ng/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 *E. coli*-expressed recombinant human Endostatin produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Endostatin 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)
Serum (n=60)	122	58-232	30
EDTA Plasma (n=35)	120	69-172	26
Heparin Plasma (n=35)	128	65-196	28

Saliva - Seven saliva samples were evaluated for the presence of Endostatin in this assay. One of the samples measured below the lowest standard (0.31 ng/mL). The other six samples had a mean value of 0.91 ng/mL and a range of 0.39-1.87 ng/mL.

Cell Culture Supernates - HUVEC human umbilical vein endothelial cells were cultured in EGM supplemented with 2% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and bovine brain extract until confluent. Two samples were tested for the presence of human Endostatin and measured 34.0 ng/mL and 70.9 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Endostatin.

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

Recombinant human:

Angiopoietin-1
Angiopoietin-2
Angiostatin
β-ECGF
β-NGF
EGF
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
FGF-9
FGF-10
FGF-18
Flt-3/Flk-2 ligand
Flt-4
G-CSF
GM-CSF
HB-EGF
HGF
HRG-2
IGF-I
IGF-II
KGF/FGF-7
M-CSF
MSP
MSP β-chain
PDGF-AA
PDGF-AB
PDGF-BB
PD-ECGF
PlGF
$VEGF_{121}$
$VEGF_{165}$
VEGF/PlGF
VEGF-D

Recombinant mouse:

FGF-8b
FGF-8c
Flt-3/Flk-2 ligand
G-CSF
GM-CSF
M-CSF
PlGF-2
$VEGF_{120}$
$VEGF_{164}$

Recombinant rat:

GM-CSF
β-NGF
PDGF-BB

Recombinant porcine:

GM-CSF

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF

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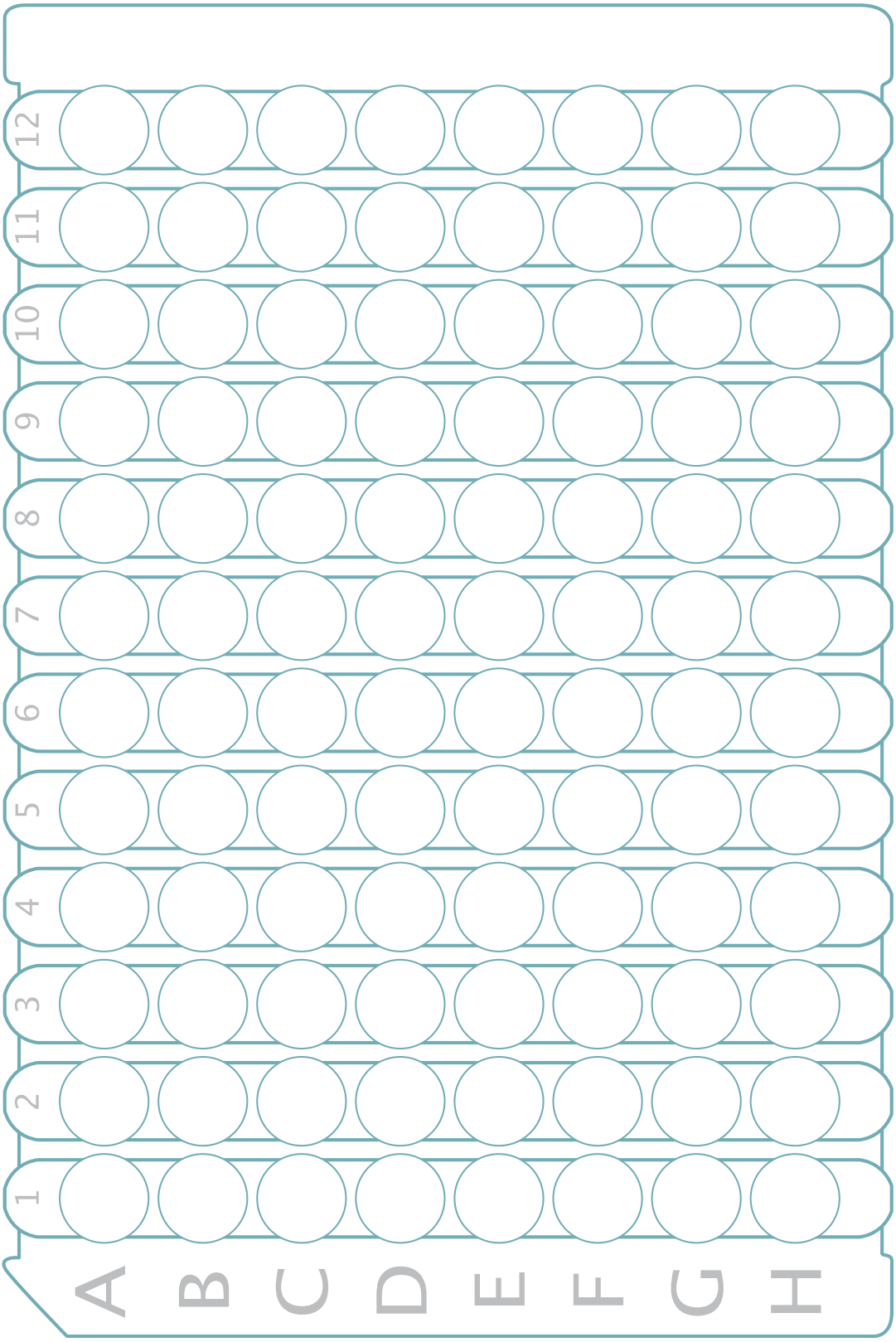
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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