QuantiGlo® ELISA

Human TNF-α Immunoassay

Catalog Number QTA00B

For the quantitative determination of human Tumor Necrosis Factor alpha (TNF-α) concentrations in cell culture supernates, serum, and plasma.

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

Tumor necrosis factor alpha (TNF-α), also known as cachectin and TNFSF1A, is the prototypic ligand of the TNF superfamily (1). It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism (2-5). TNF-α is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer (5-11).

Human TNF-α is synthesized as a 26 kDa type II transmembrane protein that consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 177 aa extracellular domain (ECD) (12, 13). Within the ECD, human TNF-α shares 97% aa sequence identity with rhesus monkey, and 71-92% aa identity with bovine, canine, cotton rat, equine, feline, mouse, porcine, and rat TNF-α. It is produced by a wide variety of immune, epithelial, endothelial, and tumor cells. TNF-α is assembled intracellularly to form a noncovalently linked homotrimer which is expressed on the cell surface (14). Cell surface TNF-α can both induce the lysis of tumor cells and virus infected cells, and generate its own downstream cell signaling following ligation by soluble TNF RI (15, 16). Shedding of membrane bound TNF-α by TACE/ADAM17 releases the bioactive cytokine, a 55 kDa soluble trimer of the TNF-α extracellular domain (17-19).

TNF-α binds the ubiquitous 55-60 kDa TNF RI (20, 21) and the hematopoietic cell-restricted 78-80 kDa TNF RII (22, 23), both of which are also expressed as homotrimers (1, 24). Both type I and type II receptors bind TNF-α with comparable affinity and can promote NFκB activation (25-28). Only TNF RI, however, contains a cytoplasmic death domain which triggers the activation of apoptosis (3, 29). Soluble forms of both types of receptors are released into human serum and urine and can neutralize the biological activity of TNF-α (30-32).

The QuantiGlo® Human TNF-α Immunoassay is a 5 hour solid phase chemiluminescent ELISA designed to measure human TNF-α in cell culture supernates, serum, and plasma. It contains *E. coli*-derived recombinant human TNF-α and antibodies raised against the recombinant factor. It has been shown to accurately quantitate recombinant human TNF-α. Results obtained using naturally occurring TNF-α samples showed linear 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 for natural human TNF-α.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TNF-α 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 TNF-α 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 the appropriate 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
Human TNF-α Microplate	892716	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human TNF-α.	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 TNF-α Standard	890387	2 vials recombinant human TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 2 weeks.*
Human TNF-α Conjugate	892921	21 mL of a polyclonal antibody specific for human TNF-α conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-27	895245	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	21 mL of a concentrated buffered protein base with preservatives. *For cell culture supernate samples. Use diluted 1:5 in this assay.*	
Calibrator Diluent RD6N	895135	21 mL of a buffered animal serum with preservatives. *For serum/plasma samples.*	
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.
- 100 mL and 1 liter graduated cylinders.
- 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 TNF-α Controls (optional; R&D Systems®, Catalog # QC196).

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: *Lipemic samples are not suitable for use in this assay.*

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 100 mL of Wash Buffer Concentrate to 900 mL of deionized or distilled water to prepare 1000 mL of Wash Buffer.

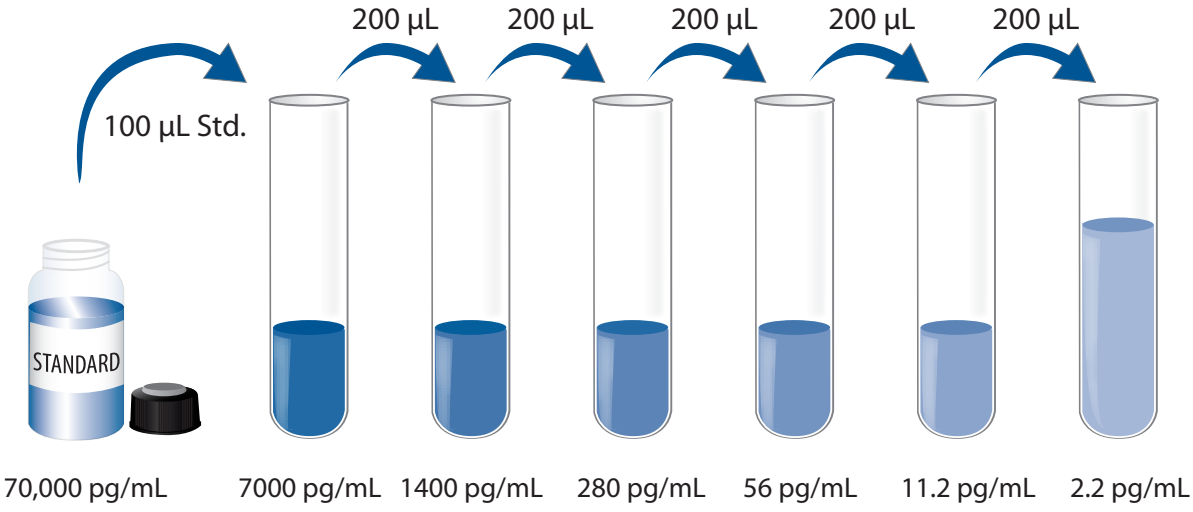
Calibrator Diluent RD5P (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5P to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Working Glo Reagent - 1 part Glo Reagent A (4.0 mL) and 2 parts Glo Reagent B (8.0 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 of Glo Reagent A and Glo Reagent B. To assay half a plate (48 wells), mix 2.0 mL of Glo Reagent A with 4.0 mL of Glo Reagent B. Working Glo Reagent should be discarded after use.*

Human TNF-α Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human TNF-α Standard with deionized or distilled water. This reconstitution produces a stock solution of 70,000 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 RD5P (diluted 1:5) (*for cell culture supernates*) or Calibrator Diluent RD6N (*for serum/plasma samples*) into the 7000 pg/mL tube. Pipette 800 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 7000 pg/mL standard serves as the high standard. The appropriate calibrator diluent 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, controls, and samples 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 RD1-27 to each well.
4. Add 100 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 3 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 TNF-α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 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 RLU.

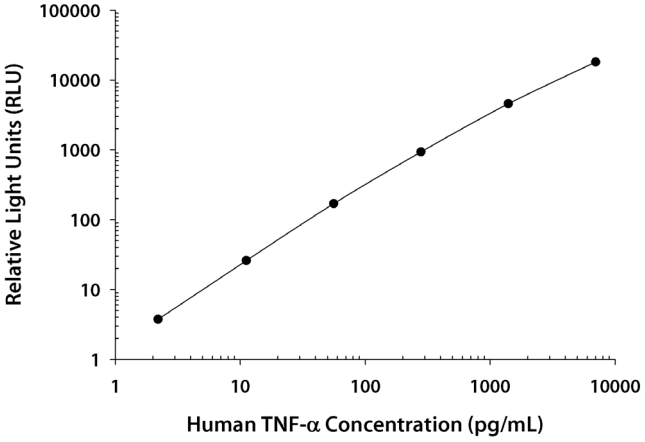
Create a standard curve by reducing the data using computer software capable of generating a cubic-spline curve fit.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

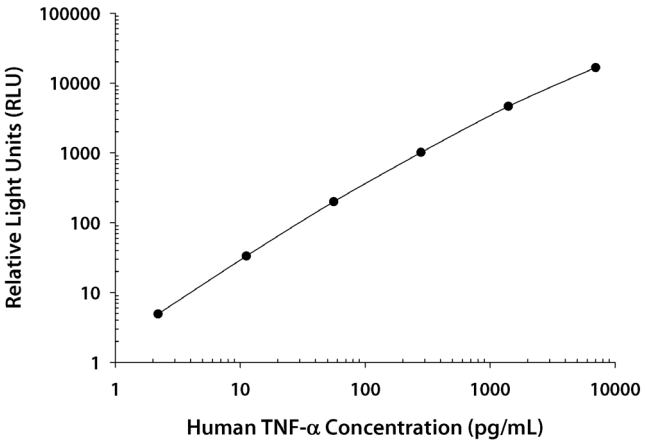
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	RLU	Average	Corrected
0	2.27 3.29	2.78	—
2.2	6.21 6.83	6.52	3.74
11.2	28.35 28.99	28.67	25.89
56	164.6 180.0	172.3	169.5
280	918.8 954.0	936.4	933.6
1400	4359 4775	4567	4564
7000	17,388 18,725	18,056	18,054

SERUM/PLASMA ASSAY



(pg/mL)	RLU	Average	Corrected
0	2.76 3.22	2.99	—
2.2	7.59 8.22	7.91	4.92
11.2	35.74 36.59	36.16	33.17
56	200.1 204.6	202.4	199.4
280	996.3 1031	1014	1011
1400	4600 4640	4620	4617
7000	16,470 16,809	16,640	16,637

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.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision				Inter-Assay Precision			
Sample	1	2	3	4	1	2	3	4
n	20	20	20	20	20	20	20	20
Mean (pg/mL)	5.94	39.7	390	3636	6.34	38.0	374	3711
Standard deviation	0.34	1.36	12.6	132	0.47	2.90	28.1	313
CV (%)	5.7	3.4	3.2	3.6	7.4	7.6	7.5	8.4

SERUM/PLASMA ASSAY

	Intra-Assay Precision				Inter-Assay Precision			
Sample	1	2	3	4	1	2	3	4
n	20	20	20	20	20	20	20	20
Mean (pg/mL)	5.17	37.7	413	3678	5.97	37.4	382	3869
Standard deviation	0.44	1.51	14.4	153	0.42	3.12	24.6	244
CV (%)	8.5	4.0	3.5	4.2	7.0	8.3	6.4	6.3

RECOVERY

The recovery of natural and recombinant human TNF-α spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	107	97-115%
Serum (n=4)	101	87-113%
EDTA plasma (n=4)	100	86-112%
Heparin plasma (n=4)	100	83-114%
Citrate plasma (n=4)	100	91-109%

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of human TNF-α ranged from 0.152-0.736 pg/mL. The mean MDD was 0.391 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.

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human TNF-α in various matrices were diluted with the appropriate 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	99	100	99	97	98
	Range (%)	96-104	98-101	95-105	94-99	90-103
1:4	Average % of Expected	102	100	97	97	100
	Range (%)	95-109	95-104	90-104	92-101	86-106
1:8	Average % of Expected	102	101	99	98	98
	Range (%)	92-107	97-108	95-106	93-102	92-104
1:16	Average % of Expected	98	100	94	95	99
	Range (%)	94-103	98-103	90-95	84-100	93-110
1:32	Average % of Expected	99	97	91	94	102
	Range (%)	92-109	90-103	87-96	84-99	92-115

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human TNF-α produced at R&D Systems®.

The NIBSC/WHO Second International Standard 88/786 (recombinant human TNF-α expressed in *E. coli*) was evaluated in this kit. The dose response curve of this First International Standard parallels the QuantiGlo® standard curve. To convert sample values obtained with the QuantiGlo® Human TNF-α kit to approximate NIBSC 88/786 International Units, use the equation below.

NIBSC (88/786) approximate value (IU/mL) = 0.096 x QuantiGlo® Human TNF-α value (pg/mL)

SAMPLES VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human TNF-α 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=41)	4.52	59	ND-9.03
EDTA Plasma (n=39)	2.88	28	ND-5.89
Heparin Plasma (n=39)	2.86	26	ND-5.76
Citrate Plasma (n=23)	2.30	4	ND-2.30

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 bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were 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 human TNF-α.

Condition	Day 1 (pg/mL)	Day 6 (pg/mL)
Unstimulated	ND	2196
Stimulated	3928	644

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant human TNF-α.

The factors listed below were prepared at 70 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 70 ng/mL in a mid-range recombinant human TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
TNF-β
TNF RI
TNF RII
TNF RII/Fc Chimera

Recombinant mouse:
TNF-α
TNF-α (truncated)
TNF RI
TNF RII

Other recombinants:
canine TNF-α
cotton rat TNF-α
porcine TNF-α
rat TNF-α

Cross-reactivity was observed with the following factors at 70 ng/mL.

Factor	% Cross-reactivity
Human pro-TNF-α	2.8
Rhesus Macaque TNF-α	0.067

Both human and mouse TNF RI/Fc Chimera showed interference at 70 ng/mL.

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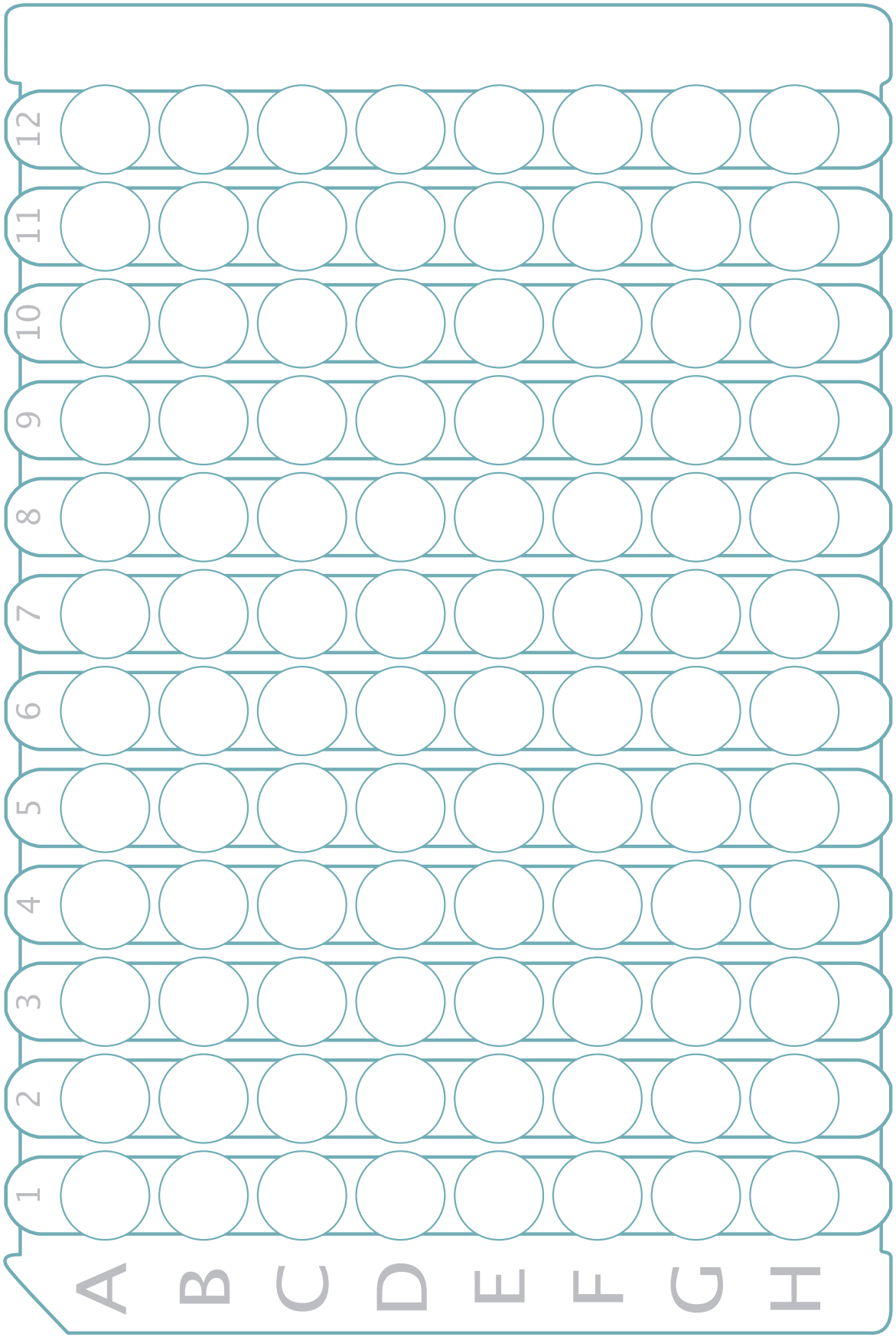
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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