Quantikine® ELISA

Human TNF-α Immunoassay

Catalog Number DTA00D
STA00D
PDTA00D

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 Quantikine® Human TNF-α Immunoassay is a 4.5 hour solid phase 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 this protein. It has been shown to accurately quantitate the recombinant factor. Results obtained with naturally occurring TNF-α samples 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 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, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-α 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, 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 # DTA00D	CATALOG # STA00D	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human TNF-α Microplate	898914	1 plate	6 plates	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 zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human TNF-α Standard	898916	2 vials	12 vials	Recombinant human TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Human TNF-α Conjugate	898915	1 vial	6 vials	21 mL/vial of 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 RD1F	895041	1 vial	6 vials	6 mL/vial of a buffered protein base with preservatives. May *contain a precipitate. Mix well before and during use.*	
Calibrator Diluent RD6-12	895214	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives.	
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.

DTA00D contains sufficient materials to run an ELISA on one 96 well plate.
STA00D (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDTA00D). 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.
- 500 mL graduated cylinder.
- 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 # QC248).

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 SDS 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.

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 was not validated for use in this assay.*

REAGENT PREPARATION

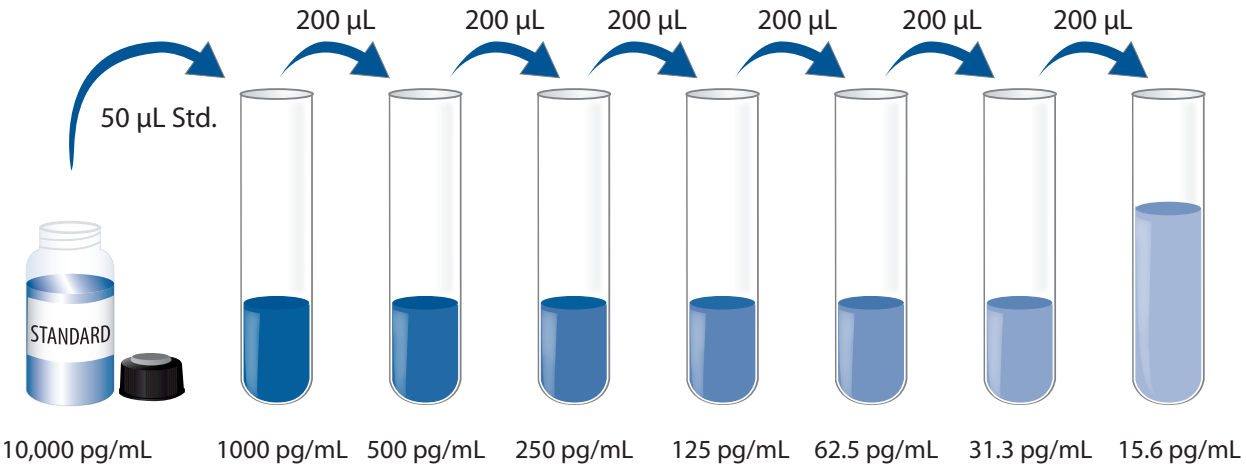
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of 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.

TNF-α Standard - **Refer to vial label for reconstitution volume.** Reconstitute the TNF-α Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD6-12 into the 1000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD6-12 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 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 50 µL of Assay Diluent RD1F to each well. *Assay Diluent RD1F may have a precipitate present. Mix well before and during use.*
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 TNF-α 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 if 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.

CALCULATION OF RESULTS

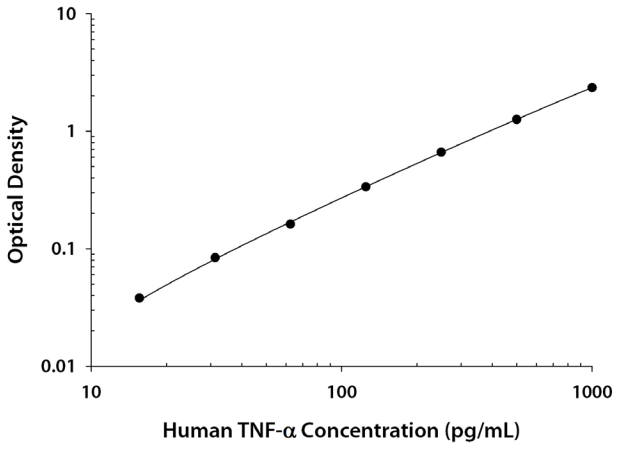
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 TNF-α 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	0.054 0.056	0.055	—
15.6	0.092 0.093	0.093	0.038
31.3	0.138 0.139	0.139	0.084
62.5	0.214 0.220	0.217	0.162
125	0.390 0.394	0.392	0.337
250	0.710 0.726	0.718	0.663
500	1.300 1.326	1.313	1.258
1000	2.382 2.432	2.407	2.352

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)	155	304	606	155	298	592
Standard deviation	4.08	6.62	18.3	11.3	21.8	49.9
CV (%)	2.6	2.2	3.0	7.3	7.3	8.4

RECOVERY

The recovery of 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)	103	98-110%
Serum (n=4)	88	79-98%
EDTA plasma (n=4)	90	75-101%
Heparin plasma (n=4)	90	82-95%

LINEARITY

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

		Cell culture supernates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	97	107	105	105
	Range (%)	93-100	103-109	103-108	103-108
1:4	Average % of Expected	98	112	108	108
	Range (%)	94-102	110-116	103-115	104-117
1:8	Average % of Expected	98	113	106	111
	Range (%)	93-107	110-118	102-113	105-119
1:16	Average % of Expected	99	119	110	116
	Range (%)	90-107	113-124	103-117	108-123

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-five assays were evaluated and the minimum detectable dose (MDD) of human TNF-α ranged from 2.09-6.23 pg/mL. The mean MDD was 4.00 pg/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 TNF-α produced at R&D Systems®.

The NIBSC/WHO 3rd International Standard 12/154 (human rDNA derived) was evaluated in this kit. The dose response curve of this International Standard parallels the Quantikine® standard curve. To convert sample values obtained with the Quantikine® Human TNF-α kit to approximate NIBSC 12/154 international units, use the equation below.

NIBSC (12/154) approximate value (IU/mL) = 0.072 x Quantikine® Human TNF-α value (pg/mL)

Note: *Based on data generated in January 2018.*

SAMPLE VALUES

Serum/Plasma - Thirty serum and plasma samples from apparently healthy volunteers were evaluated for the presence of human TNF-α in this assay. All samples measured less than the lowest standard, 15.6 pg/mL. No medical histories were available for the donors used in this study.

Cell Culture Supernates - Human peripheral blood mononuclear cells (PBMCs) (1 x 10^6 cells/mL) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were unstimulated or stimulated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin Calcium Salt (Tocris®, Catalog # 1704) for 24 hours. Aliquots of the culture supernates were removed, assayed for levels of human TNF-α, and measured 1355 pg/mL and 15,240 pg/mL, respectively.

$CD4^+$ T cells were isolated from human PBMCs using the MagCellectTM Human $CD4^+$ T cell Isolation Kit (R&D Systems®, Catalog # MAGH102) before being differentiated into Th1 cells for 5 days and then treated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin for an additional 24 hours. An aliquot of the cell culture supernate was removed, assayed for human TNF-α, and measured 81,100 pg/mL.

Human monocyte-derived macrophages (MDM) were obtained from PBMCs and attached monocytes were cultured in RPMI supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 100 ng/mL of recombinant human M-CSF for 5 days. Cells were treated with 1 µg/mL LPS and 40 ng/mL of recombinant human IFN-γ overnight. An aliquot of the cell culture supernate was removed, assayed for human TNF-α, and measured 8550 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human TNF-α.

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

Recombinant human:

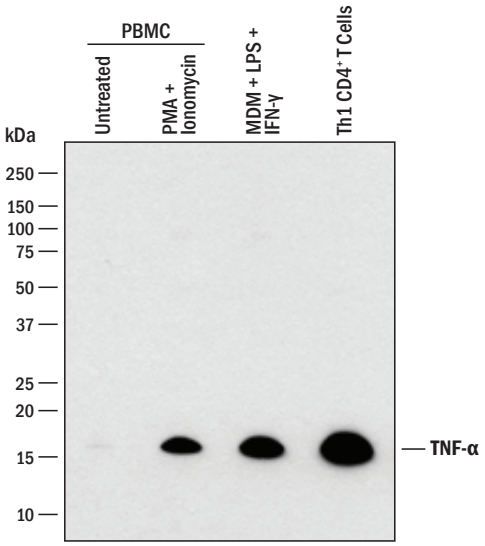
CD40
CD40 Ligand
Fas Ligand
LIGHT
TL1A
TNF-β
TNF RII
TRANCE
TRAIL

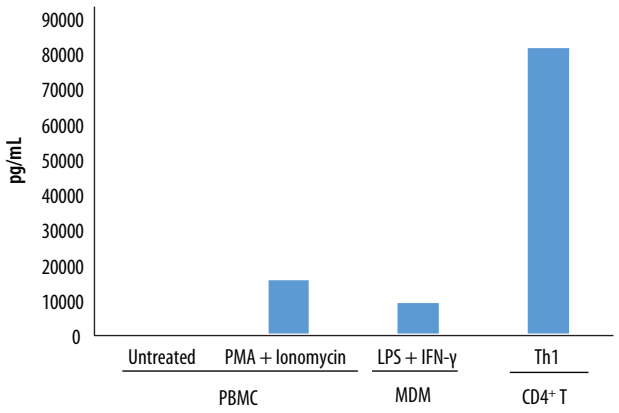
Other recombinants:

bovine TNF-α
canine TNF-α
cotton rat TNF-α
equine TNF-α
feline TNF-α
ginuea pig TNF-α
mouse TNF-α
porcine TNF-α
rat TNF-α
rhesus macaque TNF-α

Recombinant human Pro-TNF-α does not interfere but cross-reacts approximately 1.5% in this assay.

Recombinant human TNF RI does not cross-react but interferes at concentrations > 2.0 ng/mL in this assay.





Conditioned media samples were analyzed by Western blot and Quantikine® ELISA. Human PBMCs were left untreated, or treated with 10 ng/mL of PMA and 500 ng/mL Ionomycin calcium salt (Tocris®, Cat# 1704) for 24 hours. Human PBMC derived monocytes were differentiated with 100 ng/mL recombinant human M-CSF (R&D Systems®, Catalog # 216-MC/CF) for 5-6 days into MDM cells and then treated with 1 µg/mL LPS and 40 ng/mL recombinant human IFN-γ (R&D Systems®, Catalog # 285-IF/CF) for an additional 48 hours. $CD4^+$ T cells were isolated from human PBMCs using the MagCellect™ Human $CD4^+$ T cell Isolation Kit (R&D Systems®, Catalog # MAGH102) before being differentiated into Th1 cells for 5 days and then treated with 10 ng/mL of PMA and 500 ng/mL of Ionomycin for an additional 24 hours. For Western blot, all samples were initially diluted 1:2 before being resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with Goat anti-Human TNF-α (R&D Systems®, Catalog # AF-210-NA). The Western blot shows a direct correlation with ELISA value for these samples.

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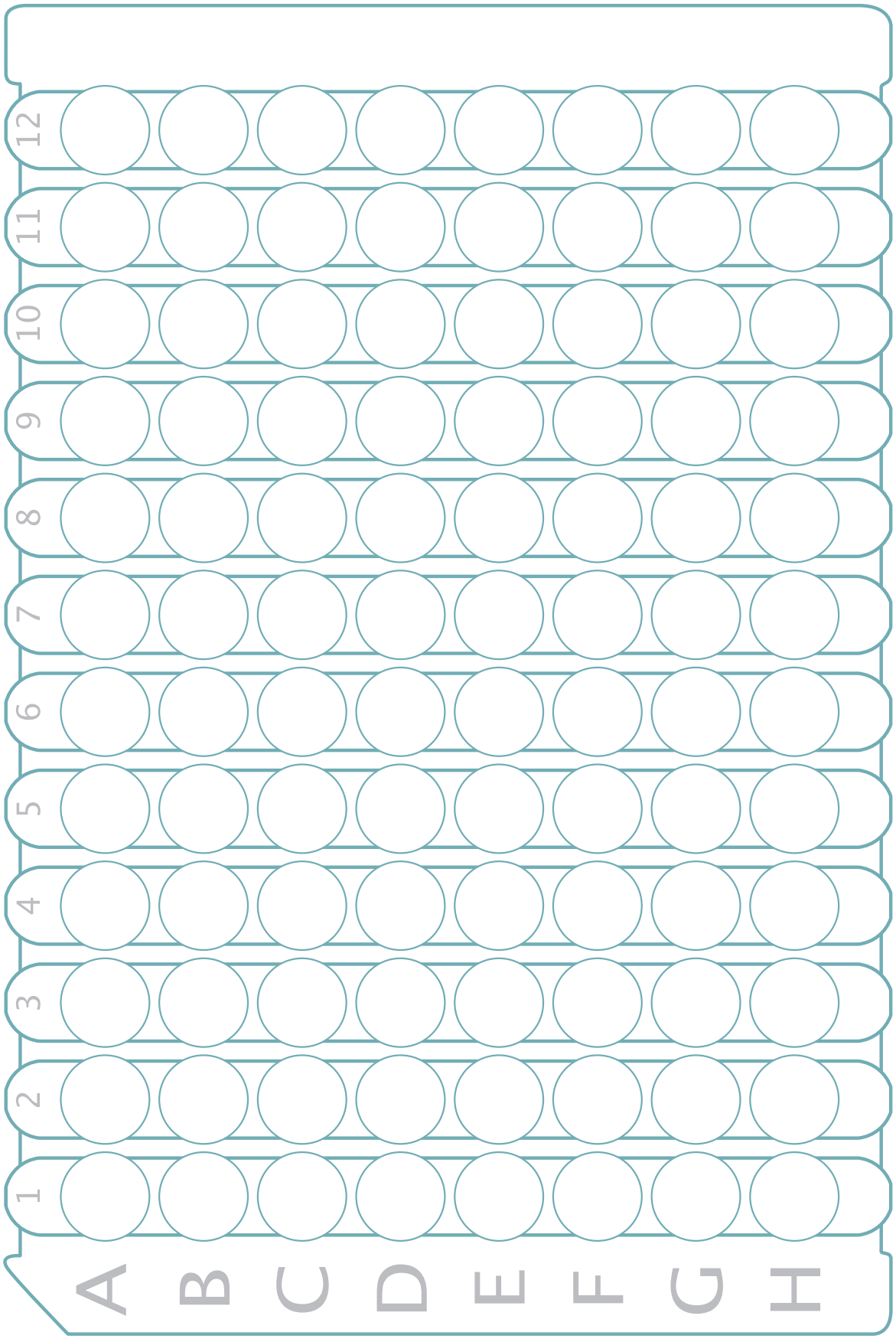
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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