Quantikine® QuickKit™ ELISA

Human TNF-α Immunoassay

Catalog Number QK210

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® QuicKit™ Human TNF-α Immunoassay is a one step, 80 minute solid phase ELISA designed to measure human TNF-α in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human TNF-α and antibodies raised against the recombinant protein. Results obtained for naturally occurring human TNF-α showed linear curves that were parallel to the standard curves obtained using the recombinant QuicKit™ 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. An anti-tag antibody has been pre-coated onto a microplate. Standards and samples are pipetted into the wells followed by an antibody cocktail. The antibody cocktail consists of an affinity tag labeled monoclonal capture antibody and an enzyme-linked polyclonal detection antibody, specific for human TNF-α. After washing away any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound. 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 from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the 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® QuicKit™ 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.
- Ensure reagent addition to plate wells is uninterrupted.
- To ensure accurate results, proper adhesion of the plate sealer during the incubation step 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
QuicKit™ Coated Microplate	899063	96 well polystyrene microplate (12 strips of 8 wells) coated with an anti-tag monoclonal antibody.	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	899069	2 vials of recombinant human TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for the reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human TNF-α Capture Ab Concentrate	899067	Lyophilized tagged monoclonal antibody specific for human TNF-α.	May be stored for up to 1 month at 2-8 °C.*
Human TNF-α Detection Ab Concentrate	899068	400 µL of a polyclonal antibody specific for human TNF-α conjugated to horseradish peroxidase with preservatives.	
Assay Diluent RD1-38	895301	12 mL of a buffered protein with preservatives.	
Calibrator Diluent RD6-12	895214	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

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 # QC259).

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.

Human TNF-α Capture Ab Concentrate - Refer to the vial label for reconstitution volume. Reconstitute the Human TNF-α Capture Ab Concentrate with Assay Diluent RD1-38. This reconstitution produces a 20X Capture Antibody stock. Allow the capture antibody to sit for a minimum of 5 minutes with gentle agitation prior to diluting. Once reconstituted, the 20X capture antibody stock can be stored for 4 weeks at 2-8 °C.

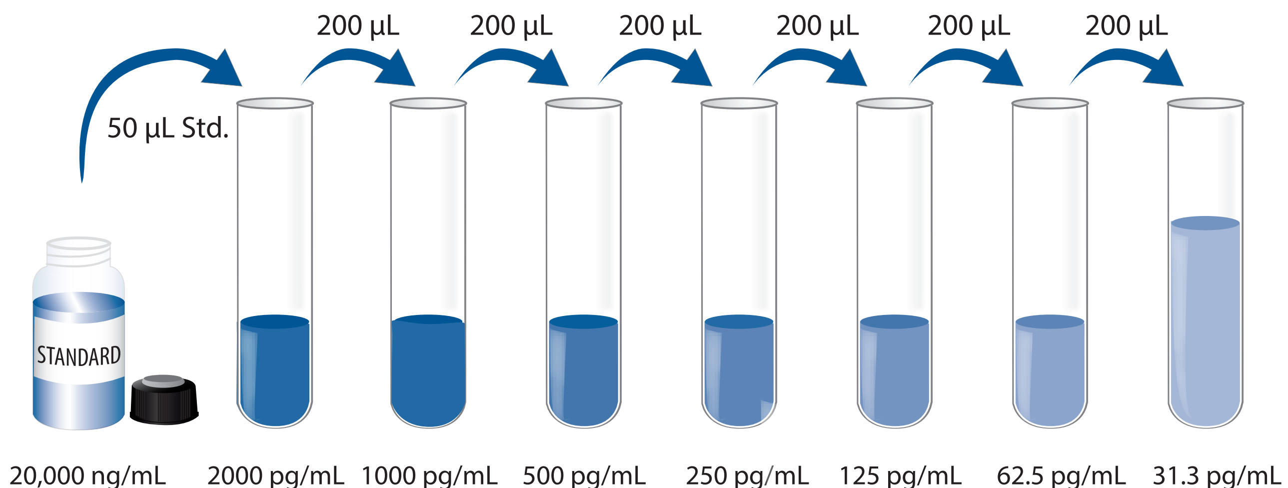
Antibody Cocktail - Dilute the reconstituted Capture Ab stock and the Detection Ab Concentrate 20-fold in Assay Diluent RD1-38. For a full plate, add 300 µL of reconstituted Human TNF-α Capture Ab stock and 300 µL of Human TNF-α Detection Ab Concentrate to 5.4 mL of Assay Diluent RD1-38.

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. 100 µL of the resultant mixture is required per well.

Human TNF-α Standard - Refer to vial label for reconstitution volume. Reconstitute the Human TNF-α Standard with Calibrator Diluent RD6-12. This reconstitution produces a stock solution of 20,000 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 2000 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 2000 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, samples, 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 standard, control, or sample per well. A plate layout is provided to record standards and samples assayed.
4. Add 50 µL Antibody Cocktail to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process twice for a total of three 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 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature **on the benchtop. Protect from light.**
7. 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.
8. 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

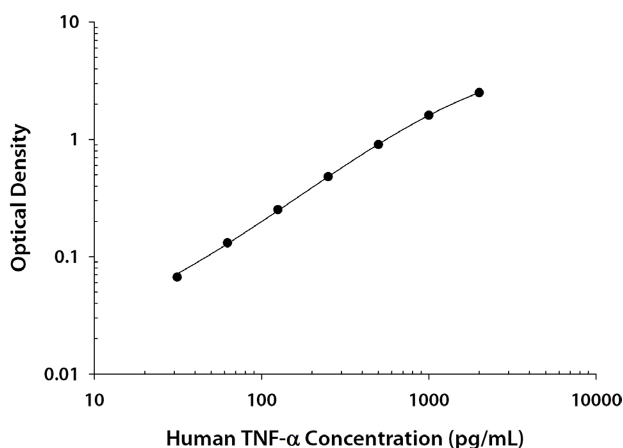
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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	0.042		—
	0.048	0.045	
31.3	0.111		
	0.112	0.112	0.067
62.5	0.174		
	0.177	0.176	0.131
125	0.291		
	0.304	0.298	0.253
250	0.525		
	0.527	0.526	0.481
500	0.930		
	0.974	0.952	0.907
1000	1.650		
	1.656	1.653	1.608
2000	2.506		
	2.596	2.551	2.506

PRECISION

Intra-Assay Precision (Precision within an assay)
Two samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Two samples of known concentration were tested in ten separate assays to assess inter-assay precision. Assays were performed by at least three technicians.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	**1**	**2**	**1**	**2**
n	20	20	10	10
Mean (pg/mL)	200	1177	214	1243
Standard deviation	3.64	28.5	13.9	67.8
CV (%)	1.8	2.4	6.5	5.5

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)	120	108-126%
Serum (n=2)	86	83-88%
EDTA plasma (n=2)	95	87-106%
Heparin plasma (n=2)	89	81-103%

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=2)	EDTA plasma (n=2)	Heparin plasma (n=2)
1:2	Average % of Expected	91	90	90	87
	Range (%)	86-93	89-90	90-90	84-90
1:4	Average % of Expected	85	94	87	86
	Range (%)	79-87	91-98	85-89	81-90
1:8	Average % of Expected	83	103	85	88
	Range (%)	77-85	102-103	85-86	84-92
1:16	Average % of Expected	81	99	77	79
	Range (%)	75-84	95-103	76-79	78-80

*Samples were diluted prior to assay.

SENSITIVITY

Eighteen assays were evaluated and the minimum detectable dose (MDD) of human TNF-α ranged from 1.58-7.02 pg/mL. The mean MDD was 3.25 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 TNF-α produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Ten 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, 31.3 pg/mL. No medical histories were available for the donors used in this study.

Cell Culture Supernates - Human peripheral blood mononuclear cells (PBMCs) (1×10^6 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 μg/mL of PHA for 5 days. Aliquots of the culture supernates were removed, assayed for levels of human TNF-α, and measured 80.9 pg/mL and 9317 pg/mL, respectively.

Human monocyte-derived macrophages (MDM) were obtained from PBMCs and attached monocytes were cultured in StemXVivo Serum-free Dendritic Cell Base Media (Catalog#, CCM003) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 100 ng/mL of recombinant human M-CSF for 5 days. Cells were unstimulated or stimulated with 1 μg/mL LPS and 40 ng/mL of recombinant human IFN-γ for 24 hours. Aliquots of the cell culture supernates were removed, assayed for levels of human TNF-α, and measured 40.5 pg/mL and 6482 pg/mL, respectively.

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 low level recombinant human TNF-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
TRANCE
TRAIL

Other recombinants:

bovine TNF-α
cotton rat TNF-α
guinea pig TNF-α
mouse TNF-α
porcine TNF-α
rabbit TNF-α
rat TNF-α

Recombinant human Pro-TNF-α does not interfere but cross-reacts approximately 6.6% in this assay.

Recombinant human TNF RI and TNF R2 do not cross-react but interfere at concentrations > 1 ng/mL in this assay.

Recombinant canine TNF-α and recombinant rhesus macaque TNF-α do not cross-react but interfere at concentrations > 25 ng/mL in this assay.

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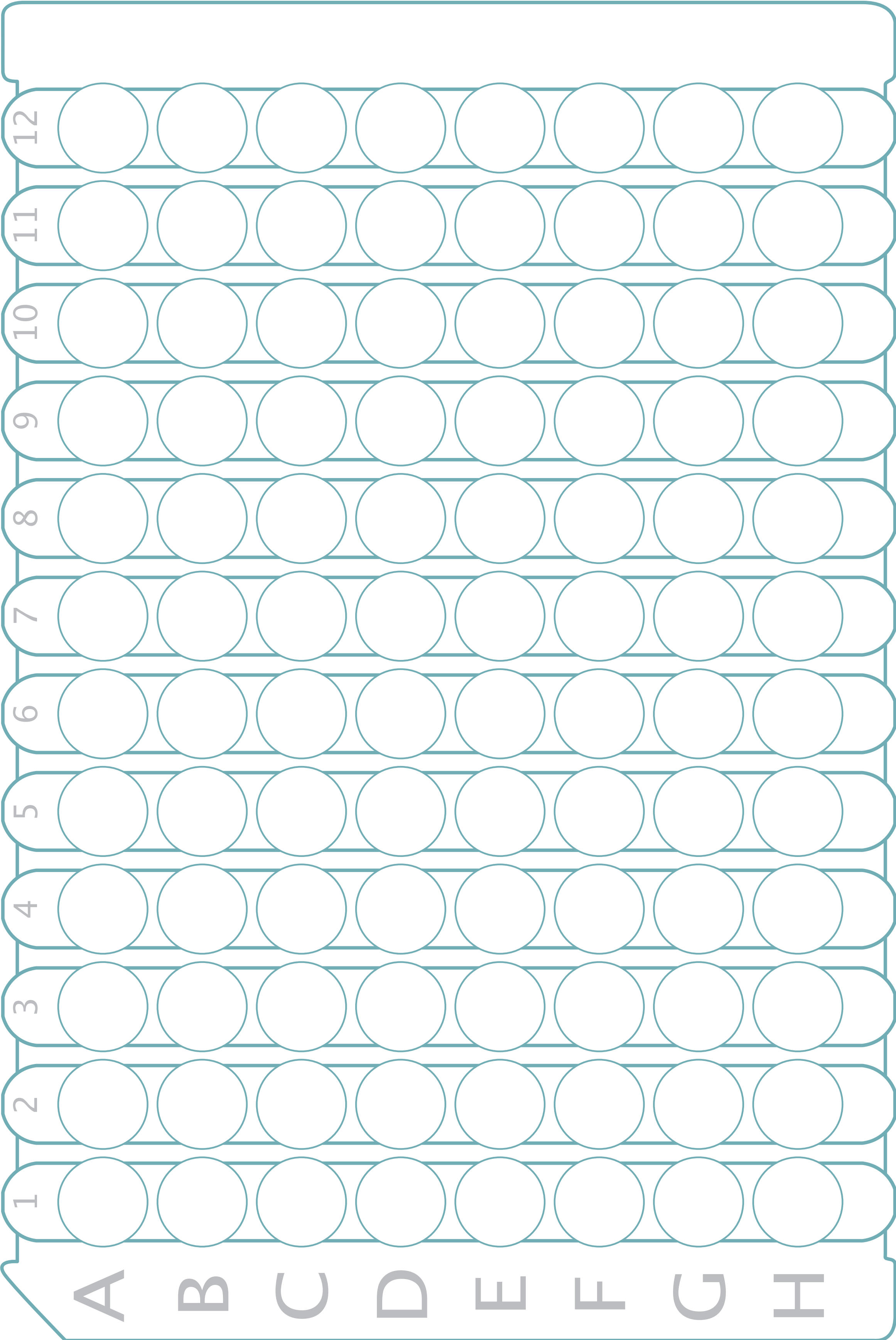
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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NOTES

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