Quantikine® HS ELISA

Mouse TNF-α Immunoassay

Catalog Number MHSTA50

For the quantitative determination of mouse Tumor Necrosis Factor alpha (TNF-α) concentrations in 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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24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
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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).

Mouse 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 179 aa extracellular domain (ECD) (12). Within the ECD, mouse TNF-α shares 95% aa identity with rat, and 80% aa identity with canine, equine, feline, human, rabbit, and porcine 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 (13). 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 TNFR I (14, 15). Shedding of membrane bound TNF-α by TACE/ADAM17 releases the bioactive cytokine, a 55 kDa soluble trimer containing the TNF-α extracellular domain (16-18).

TNF-α binds the ubiquitous 55 - 60 kDa TNF RI (19, 20) and the hematopoietic cell-restricted 78-80 kDa TNF RII (21, 22), both of which are also expressed as homotrimers (1, 23). Both type I and type II receptors bind TNF-α with comparable affinity and can promote NFκB activation (24-27). Only TNF RI, however, contains a cytoplasmic death domain which triggers the activation of apoptosis (3, 28). Soluble forms of both types of receptors are released into mouse serum and urine, and can neutralize the biological activity of TNF (29-31).

The Quantikine HS Mouse TNF-α Immunoassay is a 4.0 hour solid phase ELISA designed to measure mouse TNF-α levels in serum and plasma. It contains *E. coli*-expressed recombinant mouse TNF-α and antibodies raised against the recombinant factor and has been shown to accurately quantitate recombinant mouse TNF-α. Results obtained using natural TNF-α showed linear curves that were parallel to the standard curves obtained using the Quantikine HS kit standards. These results indicate that the Quantikine HS Immunoassay kit can be used to determine relative mass values for natural mouse TNF-α.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse 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, a biotinylated polyclonal antibody specific for mouse TNF-α is added to the wells. Following a wash to remove any unbound antibody-biotin reagent, an enzyme-linked streptavidin is added to the wells. After washing away any unbound streptavidin-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.
- 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

- To ensure accurate results, bring liquids to room temperature and mix to homogeneity prior to pipetting or aliquoting.
- When mixing protein solutions, always avoid foaming.
- 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse TNF-α HS Microplate	898438	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse 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.*
Mouse TNF-α HS Standard	898440	2 vials of recombinant mouse TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Mouse TNF-α HS Conjugate	898439	21 mL/vial of a polyclonal antibody specific for mouse TNF-α conjugated to biotin with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-63	895352	12 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD6-12	895214	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Stop Solution	895032	6 mL/vial of 2 N sulfuric acid.	
Color Reagent A	895000	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Streptavidin Polymer-HRP Diluent	898387	21 mL/vial of a solution with preservatives.	
Streptavidin Polymer-HRP (100X)	898350	0.3 mL/vial of Streptavidin Polymer-HRP in a buffer with preservative.	
Plate Sealers	N/A	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.
- 1000 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards.
- Mouse TNF-α Controls (optional; R&D Systems, Catalog # QC228).

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.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS 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.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 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 20 minutes at 2000 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 is not validated for use in this assay.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

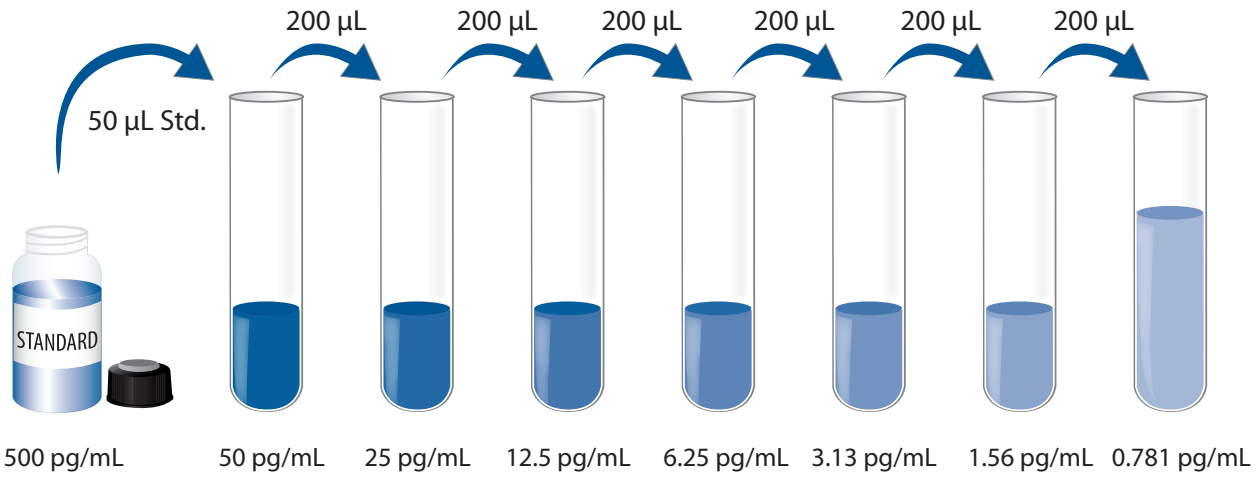
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 40 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 1000 mL of Wash Buffer.

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.

Streptavidin Polymer-HRP (1X) - Add 0.215 mL of Streptavidin Polymer-HRP (100X) directly to the Streptavidin Polymer-HRP Diluent. Mix well.

Mouse TNF-α HS Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse TNF-α HS Standard with deionized or distilled water. This reconstitution produces a stock solution of 500 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions. **Note:** *Do not use rocker.*

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD6-12 into the 50 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 50 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 samples and standards 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 RD1-63 to each well.
4. Add 50 µL of Standard, control, or sample per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for **2 hours** at room temperature. 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 Mouse TNF-α HS Conjugate to each well. Cover with a new adhesive strip. Incubate for **1 hour** at room temperature.
7. Repeat the wash as in step 5.
8. Add 200 µL of Streptavidin Polymer-HRP (1X) to each well. Cover with a new adhesive strip. Incubate for **30 minutes** at room temperature.
9. Repeat the wash as in step 5.
10. Add 200 µL of Substrate Solution to each well. Incubate for **30 minutes** at room temperature on the benchtop. **Protect from light.**
11. 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.
12. 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

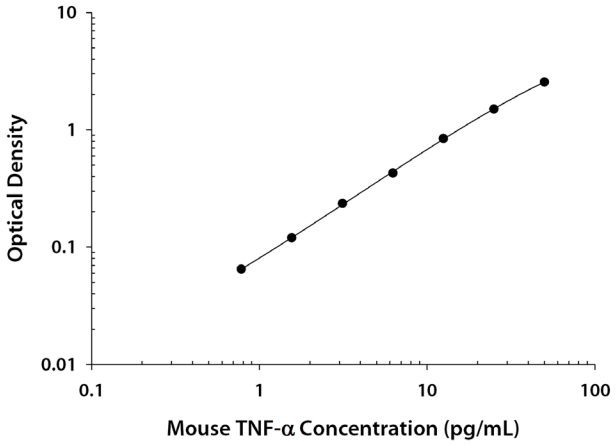
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse 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 measured concentrations 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	0.091 0.098	0.095	—
0.781	0.158 0.160	0.160	0.065
1.56	0.213 0.217	0.215	0.120
3.13	0.322 0.340	0.331	0.236
6.25	0.515 0.530	0.523	0.428
12.5	0.900 0.974	0.937	0.842
25	1.570 1.632	1.601	1.506
50	2.618 2.691	2.655	2.560

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)	6.63	15.1	29.2	6.29	15.2	28.5
Standard deviation	0.257	0.695	1.49	0.552	1.18	1.94
CV (%)	3.9	4.6	5.1	8.8	7.8	6.8

RECOVERY

The recovery of mouse TNF-α spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Serum (n=4)	86	77-99%
EDTA plasma (n=4)	91	81-104%
Heparin plasma (n=4)	90	79-106%

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of mouse TNF-α were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	100	99	103
	Range (%)	86-106	90-104	95-112
1:4	Average % of Expected	106	102	104
	Range (%)	100-111	100-105	89-113
1:8	Average % of Expected	103	108	103
	Range (%)	95-110	102-114	92-115
1:16	Average % of Expected	101	105	102
	Range (%)	83-116	96-118	92-118

SENSITIVITY

Twenty-six assays were evaluated and the minimum detectable dose (MDD) of mouse TNF-α ranged from 0.081-0.295 pg/mL. The mean MDD was 0.148 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 mouse TNF-α (aa 80-235) produced at R&D Systems.

The NIBSC TNF-α International Standard 88/532, which was intended as a potency standard, was evaluated in this kit.

The dose response curve of this International Standard parallels the Mouse TNF-α HS Standard curve. To convert sample values obtained with the Quantikine® HS Mouse TNF-α kit to approximate NIBSC 88/532 values, use the equation below.

NIBSC (88/532) approximate value (IU/mL) = 1.6882 x Quantikine® Mouse HS TNF-α value (pg/mL)

Note: *Based on data generated in June 2016.*

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse TNF-α in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	4.07	2.33-7.87	1.83
EDTA plasma (n=5)	2.09	1.59-2.66	0.520
Heparin plasma (n=7)	2.87	1.14-6.26	1.67

SPECIFICITY

This assay recognizes natural and recombinant mouse TNF-α.

The factors listed below were prepared at 500 pg/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 500 pg/mL in a mid-range recombinant mouse TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

CD40
CD40 Ligand
Fas
Fas Ligand
LIF
OPG
RANK
TNF-β
TNF RI
TNF RII
TRAIL
TROY

Other recombinants:

canine TNF-α
human TNF-α
porcine TNF-α
rhesus macaque TNF-α

Recombinant rat TNF-α cross-reacts approximately 1.1% in this assay.

Recombinant mouse TNF-α (short form) cross-reacts approximately 3.0% in this assay.

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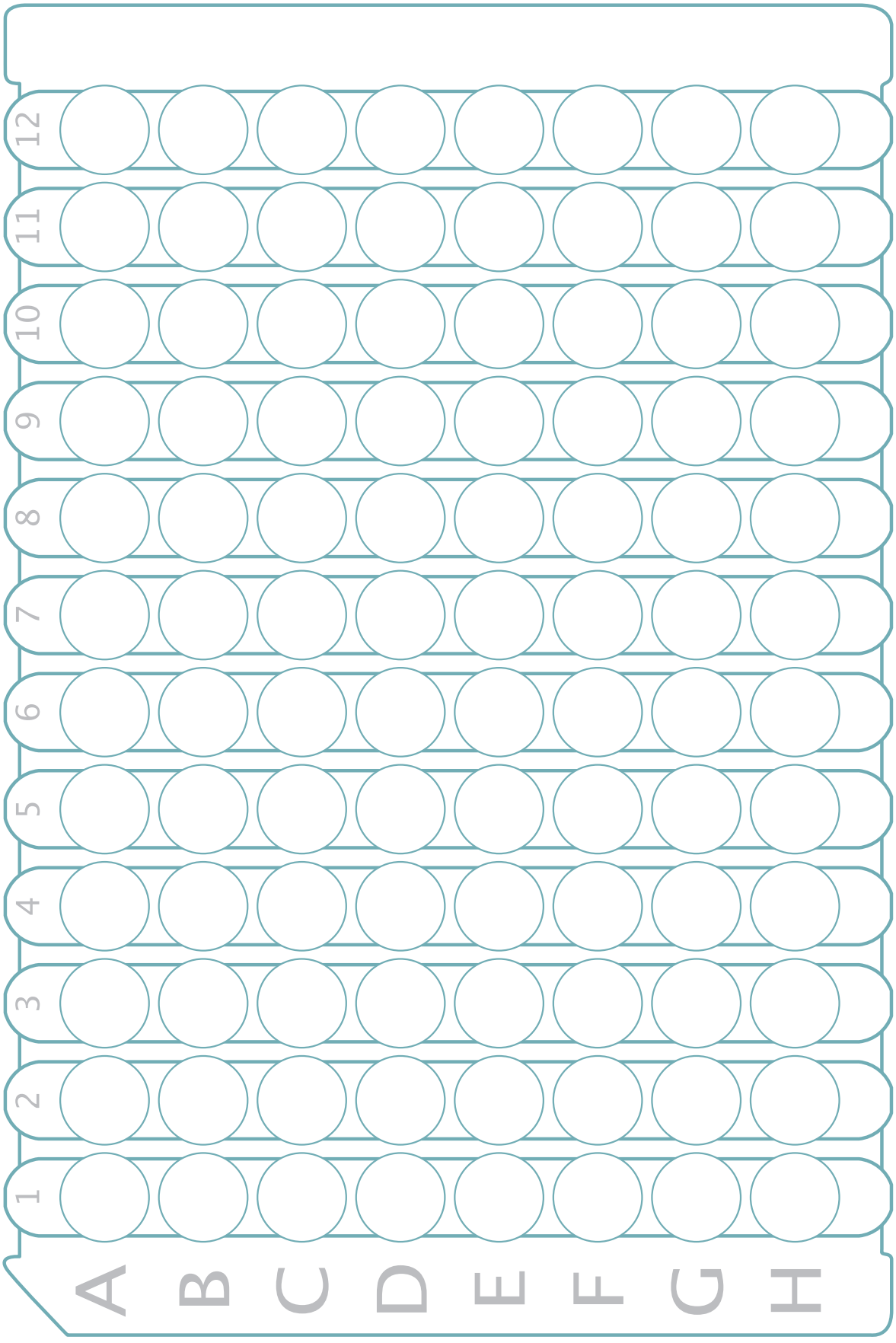
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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