Quantikine® ELISA

Mouse/Rat TNF RII/TNFRSF1B Immunoassay

Catalog Number MRT20

For the quantitative determination of mouse or rat Tumor Necrosis Factor Receptor II (TNF RII) 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 receptor I (TNF RI) and TNF RII belong to the TNF receptor superfamily which has approximately twenty members (1-4). These receptors are type I transmembrane glycoproteins characterized by multiple repeats of a highly conserved cysteine-rich motif in their extracellular ligand-binding domains. Mouse TNF RII is a 75-80 kDa, 452 amino acid (aa) residue membrane protein that contains a 188 aa residue cytoplasmic segment, a 29 aa residue transmembrane domain, and a 235 aa residue extracellular region (5). In contrast to TNF RI and some other TNF receptor family members, the cytoplasmic domain of TNF RII lacks the death domain (DD) motif critical for the cytocidal effect of TNF RI (6). Signaling molecules that interact with the cytoplasmic domain of TNF RII have been shown to be different from those that associate with TNF RI (6). Mouse TNF RII does not bind human TNF and shares only 58% aa sequence identity with human TNF RII in its extracellular region (5, 7). TNF RII is expressed broadly in a number of cells including fibroblasts (8), B cells (9), $CD4^+$ and $CD8^+$ T cells (10), adipocytes (11), microvascular endothelial cells (12), hepatocytes (13), microglia (14), $CD34^+$ stem cells (15), monocytes (16), macrophages (17), and dendritic cells (18).

TNF RII has been shown to bind both TNF-α and TNF-β with high-affinity (5, 19). Membrane-bound TNF-α, rather than soluble TNF-α, was reported to be the preferential activator of TNF RII (12, 20, 21). Although TNF RI has been shown to be the predominant mediator of TNF-α responses in numerous *in vitro* and *in vivo* studies, TNF RII has also been found to directly induce certain cellular responses independently of TNF RI (6). TNF-mediated responses that have been attributed to TNF RII include cell proliferation, IL-2 Rα expression, GM-CSF production and ICAM-1 upregulation (20-23). In other TNF-mediated cellular responses, TNF RII has been shown to enhance TNF RI effects by inducing endogenous production of TNF and by transferring TNF RII-bound ligand to TNF RI (6, 21, 24). A cooperation of both TNF RI and TNF RII is also required for TNF-mediated cytotoxicity in some cell types (25, 26). Blocking of TNF RI has been found to enhance TNF-β-mediated responses, suggesting that TNF RII may serve as a decoy receptor to attenuate TNF-β actions (19).

Soluble TNF RI and TNF RII, generated by proteolytic cleavage of the cell surface receptors, have been shown to bind TNF with high-affinity (27-30). It has been postulated that soluble TNF receptors may inhibit circulating TNF activity (31). Alternatively, soluble TNF receptors can also potentiate TNF activity by stabilizing the trimeric structure of physiologic TNF (32). Elevated levels of soluble human TNF RII have been noted in arthritis (33, 34) and colon cancer (35).

The Quantikine® Mouse/Rat TNF RII/TNFRSF1B Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse or rat TNF RII in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant mouse TNF RII and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant protein accurately. Results obtained using natural mouse or rat TNF RII 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 mouse or rat TNF RII.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse/rat TNF RII has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any TNF RII present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat TNF RII is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of TNF RII bound in the initial step. The sample values are then read off the standard curve.

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 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.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

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/Rat TNF RII Microplate	890028	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse/rat TNF RII.	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.*
Mouse/Rat TNF RII Standard	890082	Recombinant mouse TNF RII in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.*
Mouse/Rat TNF RII Control	890062	Recombinant mouse TNF RII in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse/Rat TNF RII Conjugate	890081	12 mL of a polyclonal antibody specific for mouse/rat TNF RII conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of a 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	895174	23 mL of diluted hydrochloric 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.
- Test tubes for dilution of standards and samples.

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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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 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: *Heparin and citrate plasma have not been validated for use in this assay.*

SAMPLE PREPARATION

Mouse and rat serum and plasma samples require a 10-fold dilution prior to assay. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-3.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

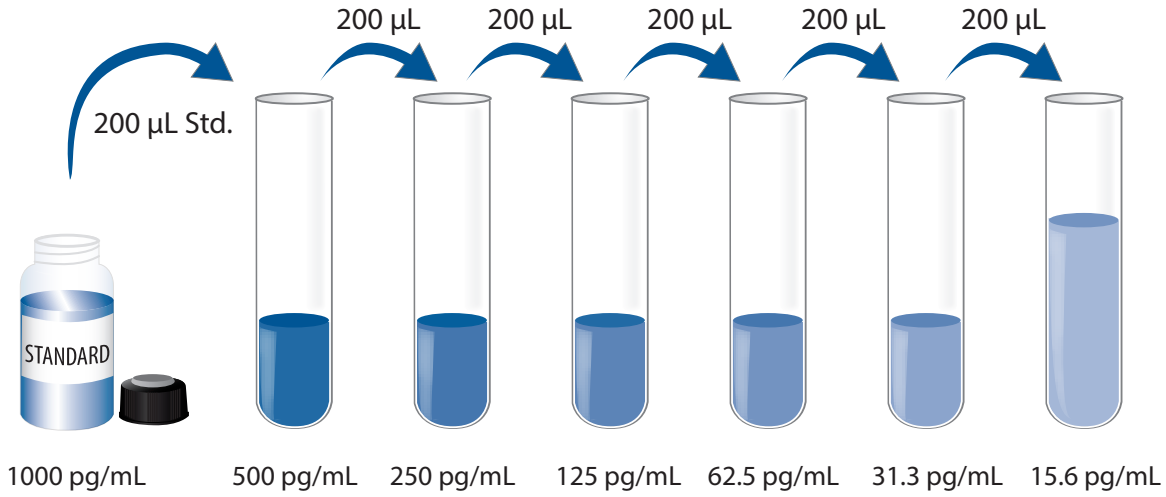
Mouse/Rat TNF RII Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

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.

Mouse/Rat TNF RII Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse/Rat TNF RII Standard with Calibrator Diluent RD5-3. Do not substitute other diluents. This reconstitution produces a stock solution of 1000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse/Rat TNF RII Standard (1000 pg/mL) serves as the high standard. Calibrator Diluent RD5-3 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, control, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, control, 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 50 µL of Assay Diluent RD1W 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 four times for a total of five 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 Mouse/Rat TNF RII Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. 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

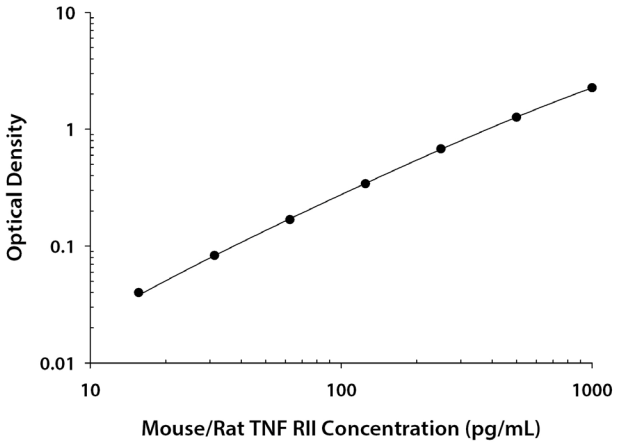
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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/rat TNF RII 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	0.022 0.023	0.022	—
15.6	0.062 0.063	0.062	0.040
31.3	0.104 0.106	0.105	0.083
62.5	0.188 0.192	0.190	0.168
125	0.364 0.365	0.364	0.342
250	0.698 0.703	0.700	0.678
500	1.260 1.317	1.288	1.266
1000	2.267 2.295	2.281	2.259

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)	37.2	112	472	38.6	119	500
Standard deviation	1.5	4.4	21.0	1.4	3.5	14.7
CV (%)	4.0	3.9	4.4	3.6	2.9	2.9

RECOVERY

The recovery of mouse/rat TNF RII spiked to levels throughout the range of the assay in various matrices was evaluated.

Mouse Samples	Average % Recovery	Range
Cell culture supernates (n=5)	102	83-119%
Serum* (n=5)	99	91-105%
EDTA plasma* (n=5)	95	80-119%

Rat Samples	Average % Recovery	Range
Cell culture supernates (n=5)	98	85-116%
Serum* (n=5)	97	84-114%
EDTA plasma* (n=5)	95	83-112%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Sixty-four assays were evaluated and the minimum detectable dose (MDD) of mouse/rat TNF RII ranged from 1.17-4.90 pg/mL. The mean MDD was 2.67 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 mouse TNF RII produced at R&D Systems®.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse/rat TNF RII were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Mouse			Rat		
		Cell culture supernates (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Cell culture supernates (n=4)	Serum* (n=4)	EDTA plasma* (n=4)
1:2	Average % of Expected	98	105	104	101	103	103
	Range (%)	89-103	103-106	101-106	94-113	100-105	102-103
1:4	Average % of Expected	96	104	102	101	104	105
	Range (%)	87-100	102-106	99-105	95-113	99-108	100-108
1:8	Average % of Expected	94	104	98	100	106	106
	Range (%)	85-98	102-106	95-102	92-111	101-112	97-115
1:16	Average % of Expected	95	104	98	96	105	104
	Range (%)	87-99	101-108	94-100	88-108	97-114	94-115

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SAMPLE VALUES

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

Mouse Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=40)	2634	1427-7031	976
EDTA plasma (n=20)	2082	1256-3728	548

Rat Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=20)	2185	1542-2933	422
EDTA plasma (n=20)	2098	1669-2825	255

Cell Culture Supernates - Mouse lung conditioned media (1 lung, 1-2 mm pieces) cultured in 10 mL RPMI supplemented with 10% fetal bovine serum was collected after culturing for 5 days. An aliquot of the cell culture supernate was removed, assayed for mouse/rat TNF RII and measured 682 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat TNF RII.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range mouse/rat TNF RII control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

C10
Eotaxin
G-CSF
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-10 R
IL-12
IL-13
IL-17
IL-18
JE/MCP-1
KC
Leptin
LIF
M-CSF
MIP-1α
MIP-1β
MIP-2
OSM
SCF
TNF-α
TNF RI
Tpo
VEGF

Recombinant human:

TNF-α
TNF-β
TNF RI

Recombinant human TNF RII does not interfere but does cross-react approximately 0.27% in this assay.

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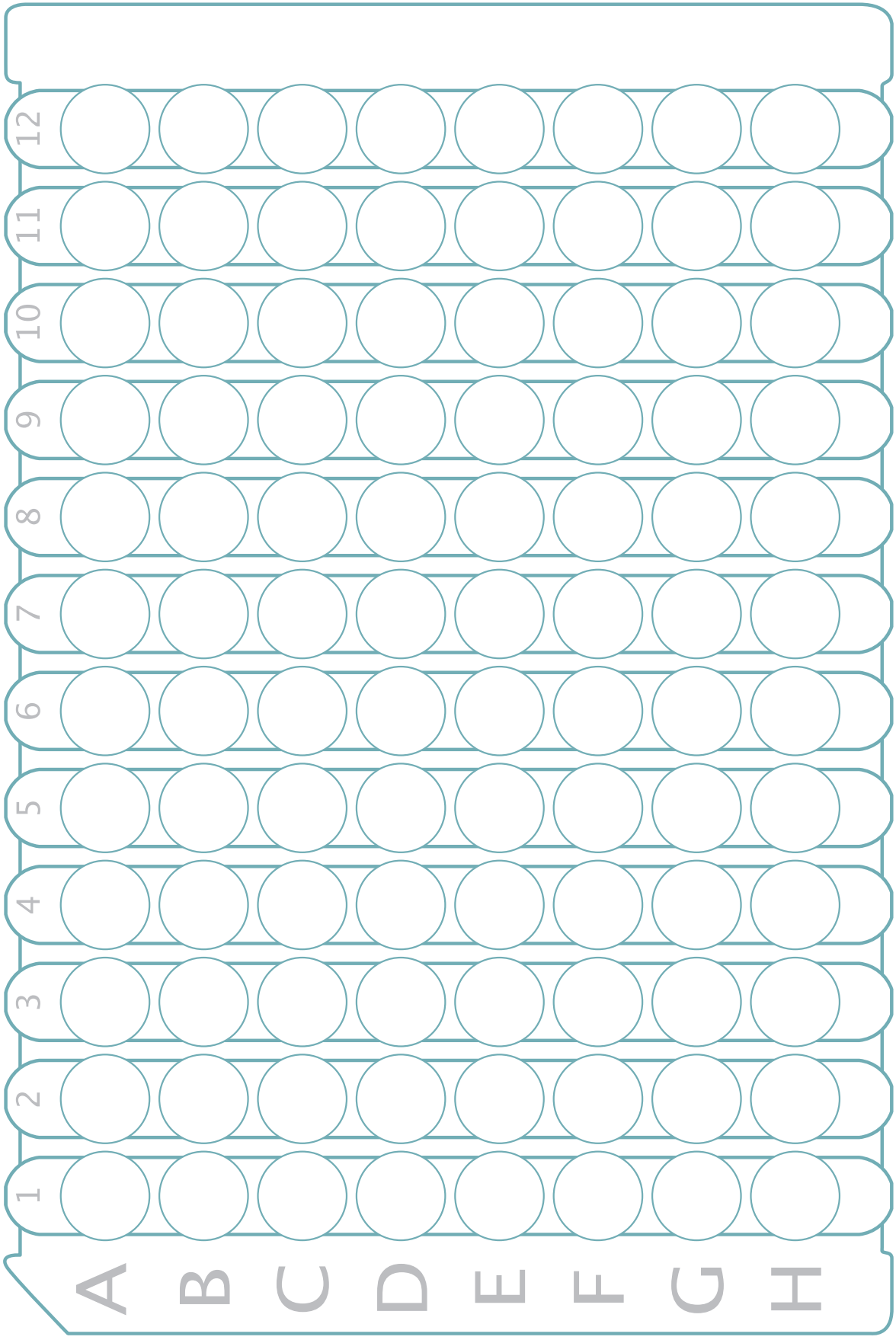
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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