Quantikine® ELISA

Mouse RBP4 Immunoassay

Catalog Number MRBP40

For the quantitative determination of mouse Retinol-Binding Protein 4 (RBP4) concentrations in cell culture supernates, cell lysates, serum, plasma, and urine.

Note: The standard reconstitution method has changed. Read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

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INTRODUCTION

Retinol-Binding Protein 4 (RBP4), also known as plasma retinol-binding protein, is a lipocalin superfamily molecule that transports vitamin A (retinol) in the blood (1-4). Dietary retinol is metabolized to retinaldehyde, multiple isomers of retinoic acid, and retinyl esters (1, 5). Retinaldehyde is the critical chromophore in the rhodopsin photoreceptor, while both it and retinoic acid regulate a multitude of cellular differentiation and proliferation effects through the intracellular receptors RAR and RXR (6-8). RBP4 adopts a β-barrel structure with a central cavity that accommodates either retinol or retinaldehyde (9). RBP4 is synthesized primarily by hepatocytes and adipocytes as a 21 kDa non-glycosylated, non-phosphorylated, and non-sulfated molecule (10-12). Its secretion into the blood requires the presence of retinol (10). Proteolytic processing of RBP4 removes one or both C-terminal leucine residues, resulting in 181 and 180 amino acid (aa) forms (12). Mouse RBP4 shares 99.5% aa sequence identity with rat, approximately 85% with bovine, chimpanzee, human, porcine, and rabbit, and 75% with chicken RBP4.

The RBP4-retinol complex interacts with transthyretin (TTR), also known as thyroxine-binding protein and prealbumin (2, 13). Formation of this complex increases the serum half-life of RBP4 by preventing RBP4 filtration through the kidney (14). The C-terminally processed forms of RBP4, which do not bind TTR, are normally excreted into the urine but accumulate in the serum during renal failure (12, 13). Glomerular re-uptake of RBP4 is mediated by the endocytic receptor Megalin (15). RBP4 is internalized by extrahepatic tissues through a receptor mediated process (16). Vitamin A derivatives in the form of retinyl esters can also be transported in chylomicrons, consistent with the observation that RBP4 deficiency results in only minor clinical effects (5, 14, 17).

RBP4 promotes hyperglycemia through downregulation of the glucose transporter GLUT4 in adipocytes, upregulation of the hepatic gluconeogenic enzyme PEPCK, and attenuation of insulin receptor signaling in skeletal muscle (18, 19). Serum RBP4 levels are elevated in type 2 diabetes and obesity, due primarily to increased production by visceral and liver adipocytes (18, 19, 20). Increases in serum RBP4 mirror changes in several other parameters linked with those diseases (19, 22). Polymorphisms within the RBP4 gene are also associated with increased serum levels and risk of type 2 diabetes (23). The expression and secretion of adipocyte RBP4 is inhibited by TNF-α and atrial natriuretic peptide, while PPARγ agonists have been shown to have both positive and negative effects on RBP4 levels (18, 24, 25).

The Quantikine® Mouse RBP4 immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse RBP4 in cell culture supernates, cell lysates, serum, plasma, and urine. It contains NS0-expressed recombinant mouse RBP4 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate recombinant mouse RBP4. Results obtained using natural mouse RBP4 showed dose response curves that were parallel to the standard curve obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse RBP4.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse RBP4 has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any RBP4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse RBP4 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 RBP4 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.
- This assay must be performed between 20-23 °C.
- 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.
- 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 RBP4 Microplate	893980	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse RBP4.	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 RBP4 Standard	893982	2 vials of recombinant mouse RBP4 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard and control for each assay.
Mouse RBP4 Control	893983	2 vials of recombinant mouse RBP4 in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse RBP4 Conjugate	893981	12 mL of a polyclonal antibody specific for mouse RBP4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1S	895137	11 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5P	895151	21 mL of a concentrated buffered protein solution with preservatives. *Use diluted 1:5 in this assay.*	
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	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.
- 100 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.

If using cell lysate samples, the following is also required:

- Cell Lysis Buffer 2 (R&D Systems®, Catalog # 895347).
- PBS

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 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.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed before assaying. See Sample Values section for details.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging. Centrifuge 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 has not been validated for use in this assay.*

Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

SAMPLE PREPARATION

Serum and plasma samples require a 1000-fold dilution prior to assay. The suggested dilution can be achieved by adding 10 µL of sample to 990 µL of Calibrator Diluent RD5P (diluted 1:5)*. Complete the 1000-fold dilution by adding 15 µL of this solution to 135 µL of Calibrator Diluent RD5P (diluted 1:5)*.

Urine samples require a 2-fold dilution prior to assay. The suggested 2-fold dilution can be achieved by adding 75 µL of sample to 75 µL of Calibrator Diluent RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse RBP4 Control - Reconstitute the control with 1.0 mL deionized or distilled water. Mix thoroughly. Assay the control undiluted.

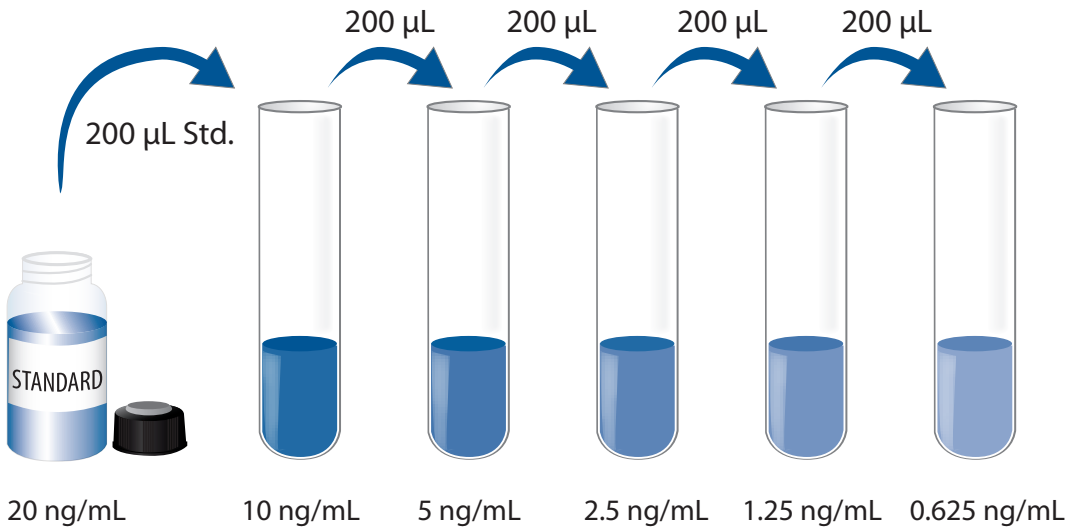
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

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

Mouse RBP4 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse RBP4 Standard with Calibrator Diluent RD5P (diluted 1:5). Do not substitute other diluents. This reconstitution produces a stock solution of 20 ng/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5P (diluted 1:5) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube gently but thoroughly before the next transfer. The undiluted Mouse RBP4 Standard (20 ng/mL) serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, control, and standards 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 RD1S to each well.
4. Add 50 µL of standard, control, or sample* per well. 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 RBP4 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 on the benchtop. **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

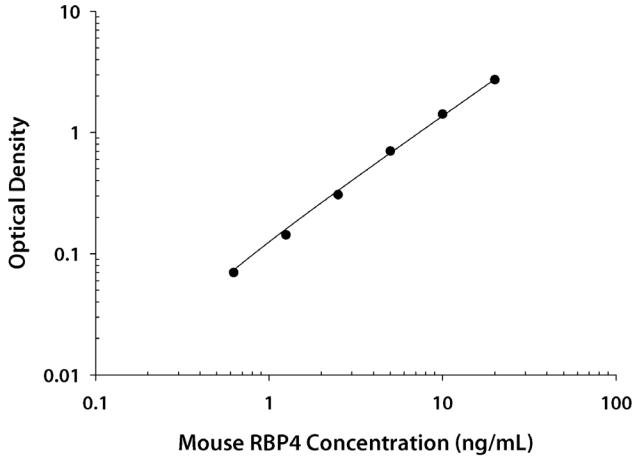
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log 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 a log/log graph. The data may be linearized by plotting the log of the mouse RBP4 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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.



(ng/mL)	O.D.	Average	Corrected
0	0.012 0.012	0.012	——
0.625	0.080 0.083	0.082	0.070
1.25	0.152 0.157	0.155	0.143
2.5	0.307 0.331	0.319	0.307
5	0.702 0.725	0.714	0.702
10	1.352 1.501	1.427	1.415
20	2.674 2.799	2.737	2.725

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 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 (ng/mL)	0.896	2.23	9.44	0.902	2.22	8.52
Standard deviation	0.036	0.12	0.51	0.089	0.15	0.64
CV (%)	4.0	5.4	5.4	9.9	6.8	7.5

RECOVERY

The recovery of mouse RBP4 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	98	90-112%
Urine (n=4)	91	80-107%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse RBP4 in each matrix were diluted with calibrator diluent and assayed. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Cell lysates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	113	107	110	106	115	113
	Range (%)	109-118	105-109	108-116	90-118	109-118	106-120
1:4	Average % of Expected	111	105	115	105	114	110
	Range (%)	110-113	92-113	108-120	88-120	106-120	100-115
1:8	Average % of Expected	109	104	102	103	110	107
	Range (%)	106-113	90-119	99-107	88-115	102-118	99-112
1:16	Average % of Expected	112	104	95	100	96	107
	Range (%)	107-119	94-120	86-100	85-118	82-110	100-112

SENSITIVITY

Thirty-five assays were evaluated and the minimum detectable dose (MDD) of mouse RBP4 ranged from 0.012-0.043 ng/mL. The mean MDD was 0.021 ng/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 highly purified NS0-expressed recombinant mouse RBP4 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine - Samples were evaluated for detectable levels of mouse RBP4 in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	12,050	8546-15,458	1754
EDTA plasma (n=20)	11,492	6204-14,832	2266
Heparin plasma (n=20)	11,355	5533-15,394	2566

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Urine (n=20)	2.39	85%	ND-11.8

ND=Non-detectable

Cell Culture Supernates:
Organs from mice were rinsed with PBS to remove excess blood, homogenized with a tissue homogenizer, and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cell culture supernates were cultured for 1 or 3 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse RBP4.

Tissue Type	(ng/mL)
Brain (1 day)	1.66
Heart (3 days)	10.3
Kidney (3 days)	58.8
Liver (3 days)	69.0
Lung (3 days)	19.2
Spleen (3 days)	4.80

LL/2 mouse Lewis lung carcinoma cells (0.5 x 10^5 cells/mL) were cultured for four days in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with either 1.0 µg/mL lipopolysaccharide (LPS), 10 µg/mL PHA and 10 ng/mL PMA, or 10 µg/mL Concanavalin A. Aliquots of the cell culture supernates were removed, assayed for mouse RBP4, and measured 40.4 ng/mL, 46.6 ng/mL, 51.3 ng/mL, and 50.0 ng/mL respectively.

Cell Lysates - Organs from mice were rinsed with 1X PBS and homogenized with a tissue homogenizer in 1X PBS. An equal volume of Cell Lysis Buffer 2 was added and tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was then removed by centrifugation. An aliquot of each cell lysate was removed and assayed for levels of mouse RBP4.

Cell Lysate	(ng/mL)
Brain	48.4
Heart	298
Kidney	1270
Liver	1509
Lung	356
Spleen	78.9

SPECIFICITY

This assay recognizes natural and recombinant mouse RBP4.

The factors listed below were prepared at 200 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range mouse RBP4 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:	Recombinant human:	Natural protein:	Others:
Lipocalin-2	RBP4	human Prealbumin	Retinol Thyroxine

Rat serum samples were tested for RBP4 and no detectable levels were observed.

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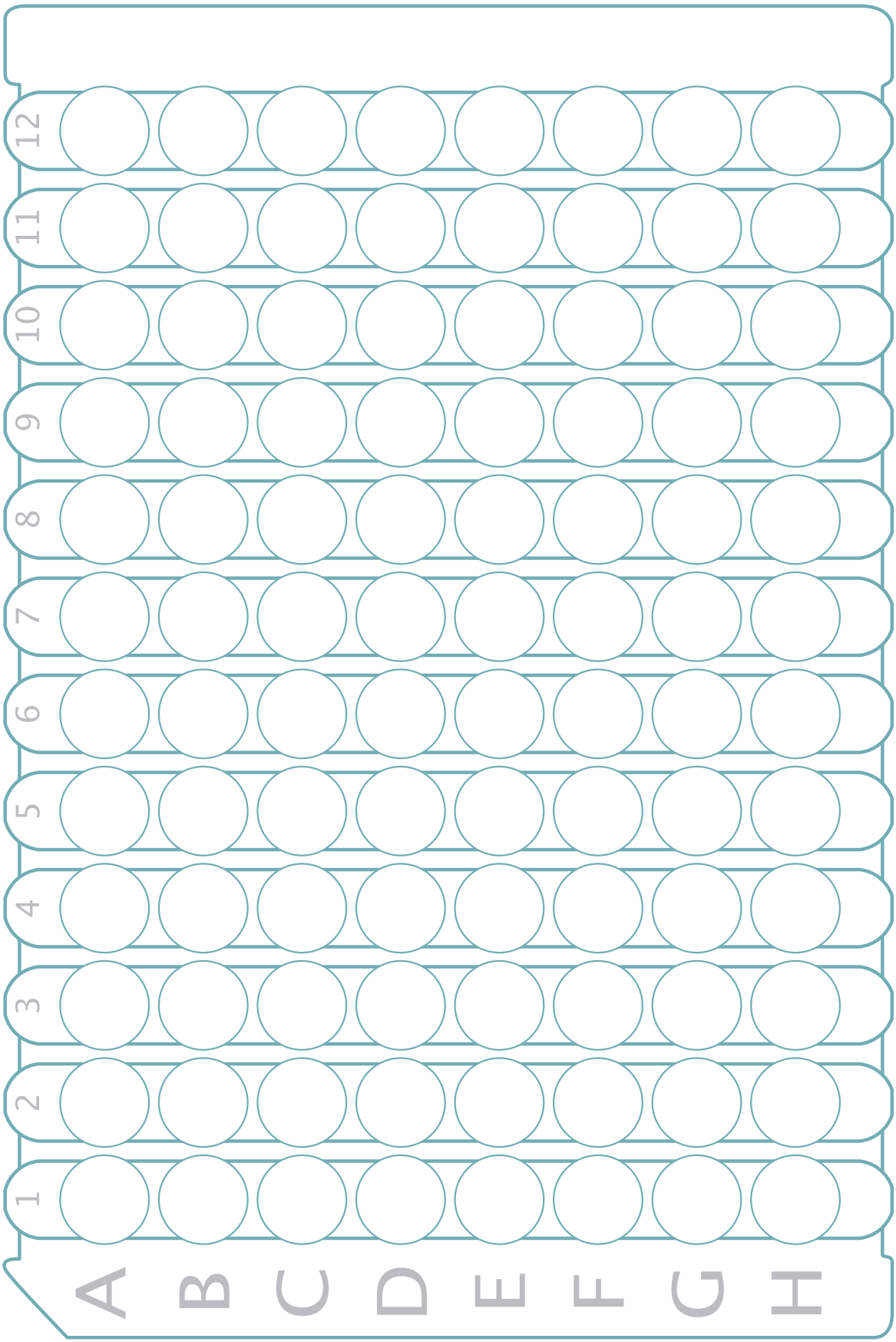
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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