Quantikine® ELISA

Human Vitamin D BP Immunoassay

Catalog Number DVDBP0B

For the quantitative determination of human Vitamin D Binding Protein (Vitamin D BP) concentrations in cell culture supernates, serum, plasma, and urine.

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

Vitamin D Binding Protein (Vitamin D BP), also known as DBP and Gc-globulin, is a 58 kDa glycoprotein that circulates at a high concentration in the serum and serves as a carrier protein for Vitamin D. The transport of Vitamin D by Vitamin D BP is important for the function of a wide variety of tissues, and alterations in Vitamin D BP activity contribute to the development of many diseases (1). A hormonally inactive form of Vitamin D, known as D2, Calcidiol, or 25(OH)D, is obtained from the diet or produced in the skin following exposure to ultraviolet radiation. Calcidiol is enzymatically hydroxylated to generate the active form of Vitamin D, known as D3 or 1,25(OH)2D. Vitamin D BP binds both the Calcidiol and Calcitriol forms. There are three dominant alleles of Vitamin D BP (Gc1f, Gc1s, and Gc2) and a large number of minor polymorphisms (1-3). Vitamin D BP is structurally related to the major serum proteins albumin and α-fetoprotein. These proteins share an internal disulfide bond pattern which divides the molecules into three domains (4, 5). Mature human Vitamin D BP shares 77% amino acid (aa) sequence identity with mouse and rat Vitamin D BP. Vitamin D BP is primarily expressed in hepatocytes and to a lesser extent in the kidney (6). It delivers Vitamin D into cells by Megalin-mediated endocytosis (7, 8). Vitamin D BP is differentially O-glycosylated depending on the isoform (9-12). A selectively deglycosylated form of Vitamin D BP known as Macrophage Activating Factor (MAF) is generated by the sequential removal of carbohydrates by B cell β-galactosidase followed by T cell sialidase (10). In addition to promoting macrophage activation and differentiation, MAF blocks the angiogenic effects of FGF basic, VEGF, and Angiopoietin-2 on vascular endothelial cells in a CD36-dependent process (14-16). MAF administration in mouse xenograft models leads to reduced neovascularization and tumor regression (13). Complete deglycosylation of Vitamin D BP destroys its anti-angiogenic effect (13).

Vitamin D BP enhances the chemotaxis of monocytes and neutrophils to the activated complement component C5a or C5a des Arg (a C-terminally processed form of C5a) (17, 18). It does not enhance movement toward the monocyte chemoattractant f-Met-Leu-Phe or function as an independent chemotactic factor (17). Vitamin D BP binding to C5a des Arg allows a greater number of C5a molecules to bind to the neutrophil (19). Neutrophil activation results in a dramatic increase of binding sites for Vitamin D BP and neutrophil chemotaxis (20). Vitamin D BP interacts with the chondroitin sulfate portion of CD44 on neutrophils and monocytes. CD44, as well as Annexin A2, is required for Vitamin D BP to enhance chemotaxis (21). Thrombospondin-1, which is released by platelets during clotting and acts through CD36, is required to develop the full chemotactic cofactor function of Vitamin D BP (18). The chemotactic cofactor property of Vitamin D BP is eliminated by binding to 1,25(OH)2 Vitamin D, but it is not altered by binding to 25(OH) Vitamin D or actin (22). Vitamin D BP binds monomeric G-actin released from necrotic cells and clears it from the circulation (23, 24).

Circulating levels of Vitamin D BP are decreased in liver failure, liver disease, and cystic fibrosis due to more rapid clearance (25-27). Patients with various cancers have an elevated serum level of alpha-N-acetylgalactosaminidase, an enzyme which removes the N-linked carbohydrates on Vitamin D BP (28). This action does not alter the level of Vitamin D BP protein but prevents the formation of the anti-angiogenic MAF (28).

The Quantikine® Human Vitamin D BP immunoassay is a 3.5 hour sandwich-type solid phase ELISA designed to measure human Vitamin D BP in cell culture supernates, serum, plasma, and urine. It contains HEK293-expressed recombinant human Vitamin D BP and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Vitamin D BP 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 Vitamin D BP.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Vitamin D BP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Vitamin D BP present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Vitamin D BP 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 Vitamin D BP 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, further 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Vitamin D BP Microplate	898513	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Vitamin D BP.	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.*
Human Vitamin D BP Standard	898514	2 vials of recombinant human Vitamin D BP in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human Vitamin D BP Conjugate	893306	21 mL of a monoclonal antibody specific for human Vitamin D BP conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-38	895301	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	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.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human Vitamin D BP Controls (optional; R&D Systems, Catalog # QC185B).

PRECAUTIONS

High concentrations of Vitamin D BP are found in saliva. Take necessary precautions to protect kit reagents.

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.

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 has not been validated for use in this assay.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require at least a 5000-fold dilution due to endogenous levels. A suggested 5000-fold dilution can be achieved by adding 10 µL of sample to 990 µL of Calibrator Diluent RD5P (diluted 1:5)*. Complete the 5000-fold dilution by adding 10 µL of the diluted sample to 490 µL of Calibrator Diluent RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of Vitamin D BP are found in saliva. Take necessary precautions to protect kit reagents.*

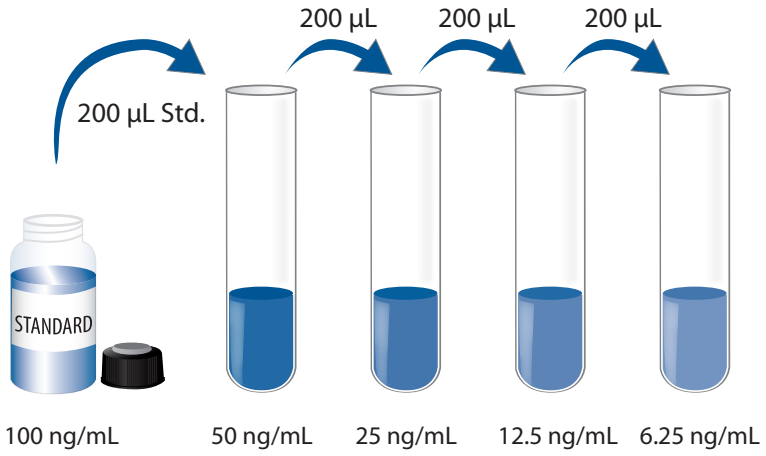
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 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 product 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Human Vitamin D BP Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Vitamin D BP Standard with Calibrator Diluent RD5P (diluted 1:5). This reconstitution produces a stock solution of 100 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation 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 thoroughly before the next transfer. The undiluted Human Vitamin D BP Standard (100 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 standards, samples, and controls be assayed in duplicate.

Note: *High concentrations of Vitamin D BP are found in saliva. Take necessary precautions to protect kit reagents.*

1. Prepare all reagents, working standards, 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 RD1-38 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 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 toweling.
6. Add 200 µL of Human Vitamin D BP Conjugate to each well. Cover with a new adhesive strip. Incubate for **1 hour** 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. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. If the color in the wells is green or 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

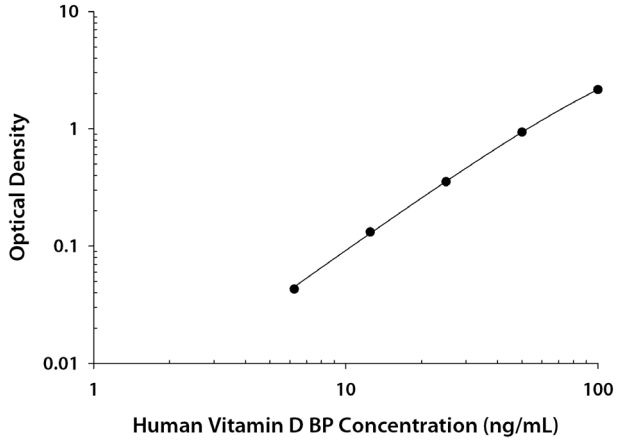
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human Vitamin D BP 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.



(ng/mL)	O.D.	Average	Corrected
0	0.009 0.011	0.010	—
6.25	0.052 0.054	0.053	0.043
12.5	0.139 0.145	0.142	0.132
25	0.354 0.374	0.364	0.354
50	0.918 0.972	0.945	0.935
100	2.110 2.231	2.171	2.161

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 (ng/mL)	10.9	31.7	63.7	10.1	30.8	61.9
Standard deviation	0.244	0.423	1.36	0.661	0.664	1.31
CV (%)	2.2	1.3	2.1	6.5	2.2	2.1

RECOVERY

The recovery of human Vitamin D BP spiked to three different levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	93-117%
Urine (n=4)	89	78-96%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Vitamin D BP were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Urine (n=4)
1:2	Average % of Expected	103	103	102	102	105
	Range (%)	96-110	96-111	95-115	98-108	99-113
1:4	Average % of Expected	106	102	103	104	108
	Range (%)	100-115	96-110	95-117	97-109	101-121
1:8	Average % of Expected	99	101	101	103	106
	Range (%)	89-112	89-111	88-119	91-110	91-122

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

SENSITIVITY

Twenty-six assays were evaluated and the minimum detectable dose (MDD) of human Vitamin D BP ranged from 0.083-0.338 ng/mL. The mean MDD was 0.180 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 a highly purified HEK293-expressed recombinant human Vitamin D BP produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Vitamin D BP in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum (n=35)	253	168-367	51.3
EDTA plasma (n=35)	224	157-348	46.4
Heparin plasma (n=35)	213	153-350	42.5

Sample Type	Mean (µg/g Creatinine)	Range (µg/g Creatinine)	Standard Deviation (µg/g Creatinine)
Urine (n=10)	22.5	10.5-38.7	8.36

Cell Culture Supernates - Hep3B human hepatocellular carcinoma cells were cultured in MEM NEAA 1X Earle's Salts supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until ~95% confluent. An aliquot of the cell culture supernate was removed, assayed for human Vitamin D BP, and measured 259 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Vitamin D BP.

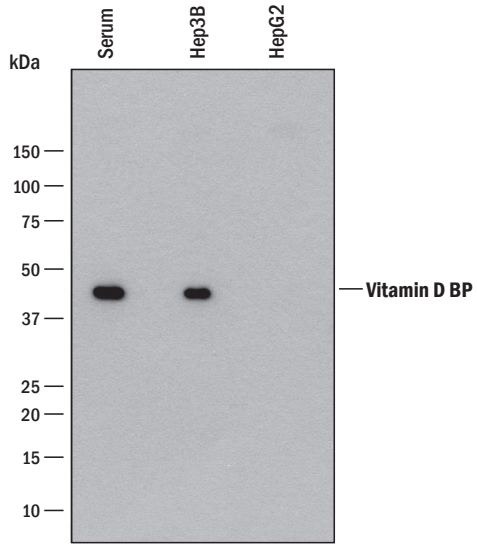
The factors listed below were prepared at 1 µg/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 1 µg/mL in a mid-range recombinant human Vitamin D BP control were assayed for interference. No significant cross-reactivity or interference was observed.

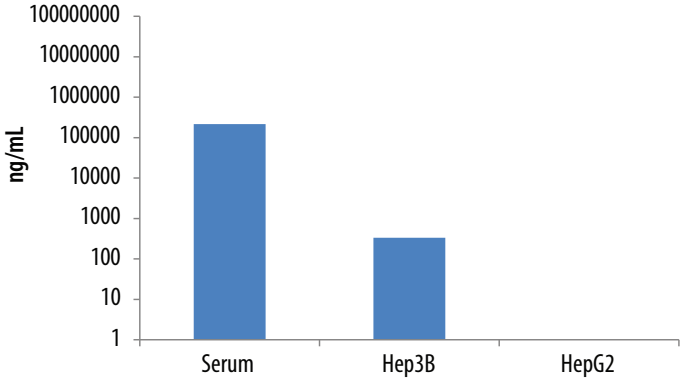
Recombinant human:

Actin α1 Skeletal Muscle
AFP
Albumin (prepared at 20 mg/mL)
CD44v2
CD44v3
CD44H

Others:

Calcifediol
Calcitriol
Vitamin D3





Human serum (diluted 1:10,000) and cell culture media (diluted 1:10) from the indicated cell lines were analyzed by Western Blot and Quantikine® ELISA. Samples were resolved under non-reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with a goat anti-human Vitamin D BP antibody. The Western Blot shows a direct correlation with the ELISA value for these samples

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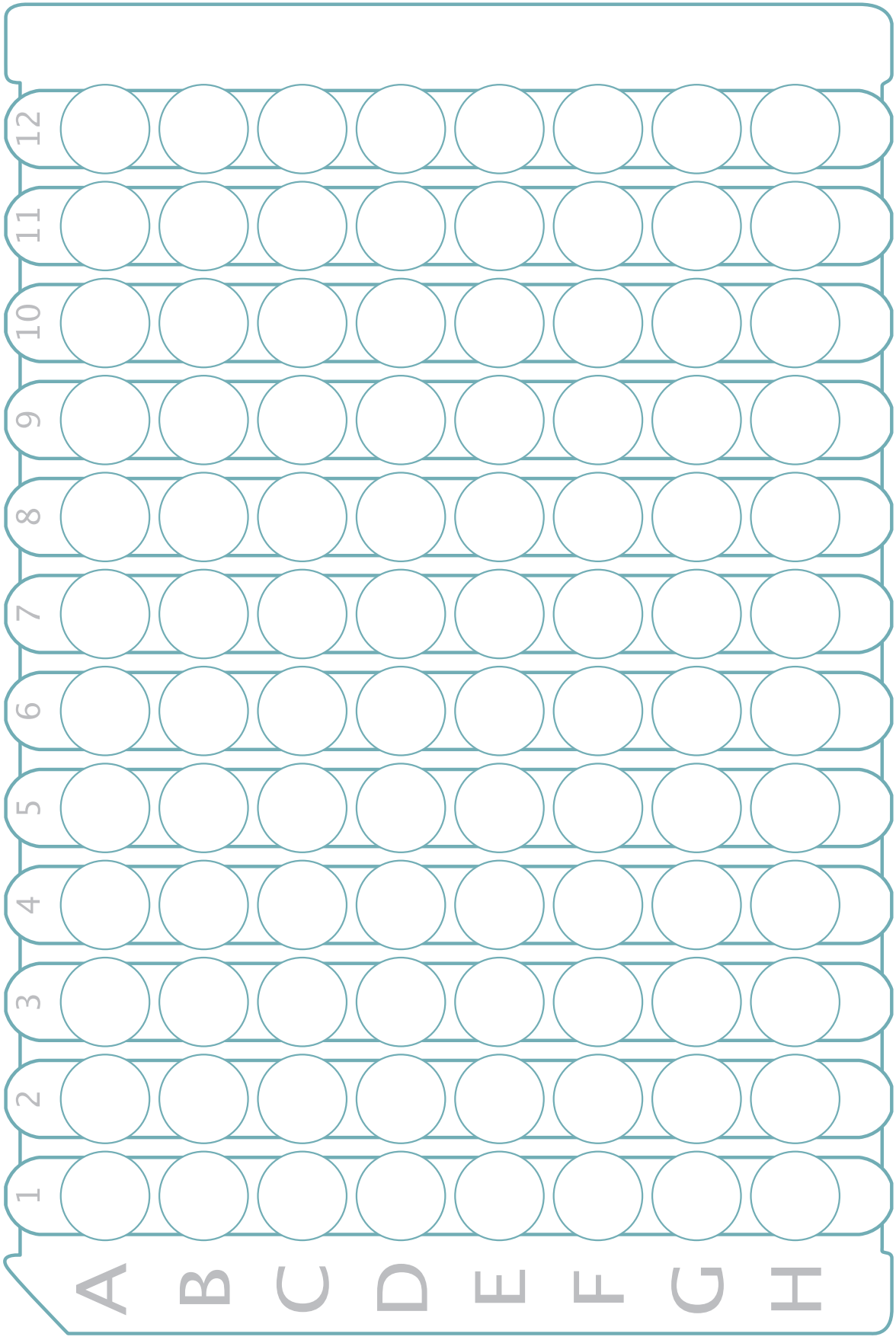
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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