Quantikine® ELISA

Mouse Vitamin D BP Immunoassay

Catalog Number MVDBP0

For the quantitative determination of mouse Vitamin D Binding Protein (Vitamin D BP) concentrations in cell culture supernates, tissue lysates, 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.

TABLE OF CONTENTS

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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 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. Vitamin D BP binds both the 25(OH) and the hormonally active $1,25(OH)_2$ forms of vitamin D (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 mouse Vitamin D BP shares 76% and 90% aa sequence identity with human and rat Vitamin D BP, respectively. 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). 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 (9). 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 (10-12). 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) (14, 15). It does not enhance movement toward the monocyte chemoattractant f-Met-Leu-Phe or function as an independent chemotactic factor (14). Vitamin D BP binding to C5a des Arg allows a greater number of C5a molecules to bind to the neutrophil (16). Neutrophil activation results in a dramatic increase of binding sites for Vitamin D BP and neutrophil chemotaxis (17). Vitamin D BP additionally interacts with the chondroitin sulfate portion of CD44 on neutrophils and monocytes. CD44 as well as Annexin A2 are required for Vitamin D BP to enhance chemotaxis (18). 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 (15). 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 (19). Vitamin D BP binds monomeric G-actin released from necrotic cells and clears it from the circulation (20, 21).

Circulating levels of Vitamin D BP are decreased in liver failure, liver disease, and cystic fibrosis due to more rapid clearance (22-24). 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 (25). This action does not alter the level of Vitamin D BP protein but prevents the formation of the anti-angiogenic MAF (25).

The Quantikine Mouse Vitamin D BP Immunoassay is a 2.5 hour solid phase ELISA designed to measure mouse Vitamin D BP levels in cell culture supernates, tissue lysates, serum, plasma, and urine. It contains NS0-expressed recombinant mouse Vitamin D BP and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant mouse Vitamin D BP accurately. Results obtained using natural mouse Vitamin D BP showed dose-response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse Vitamin D BP.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Vitamin D BP has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse Vitamin D BP present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for mouse 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. 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 mouse Vitamin D BP 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 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.
- 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 Vitamin D BP Microplate	894794	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse 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.*
Mouse Vitamin D BP Standard	894796	2 vials of recombinant mouse 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 and control for each assay.
Mouse Vitamin D BP Control	894797	2 vials of recombinant mouse Vitamin D BP in a buffered protein base with preservatives; lyophilized. The assay value of the Control should be within the range specified on the label.	
Mouse Vitamin D BP Conjugate	894795	12 mL of a monoclonal antibody specific for mouse Vitamin D BP conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Calibrator Diluent RD5P Concentrate	895151	2 vials (21 mL/vial) of a concentrated buffered protein base 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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples.

If using tissue lysate samples, the following are 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 ProClin® 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. Please 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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates - Prior to assay, tissue must be lysed according to the directions in the Sample Values section.

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 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 80,000-fold dilution. A suggested 80,000-fold dilution can be achieved by adding 10 µL of sample to 490 µL of Calibrator Diluent RD5P (diluted 1:5)*. Then add 10 µL of the diluted sample to 390 µL Calibrator Diluent RD5P (diluted 1:5). Complete the 80,000-fold dilution by adding 10 µL of the twice diluted sample to 390 µL Calibrator Diluent RD5P (diluted 1:5).

Urine samples require at least a 20-fold dilution. A suggested 20-fold dilution is 15 µL of sample + 285 µL of Calibrator Diluent RD5P (diluted 1:5).

*See Reagent Preparation section

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse Vitamin D BP Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

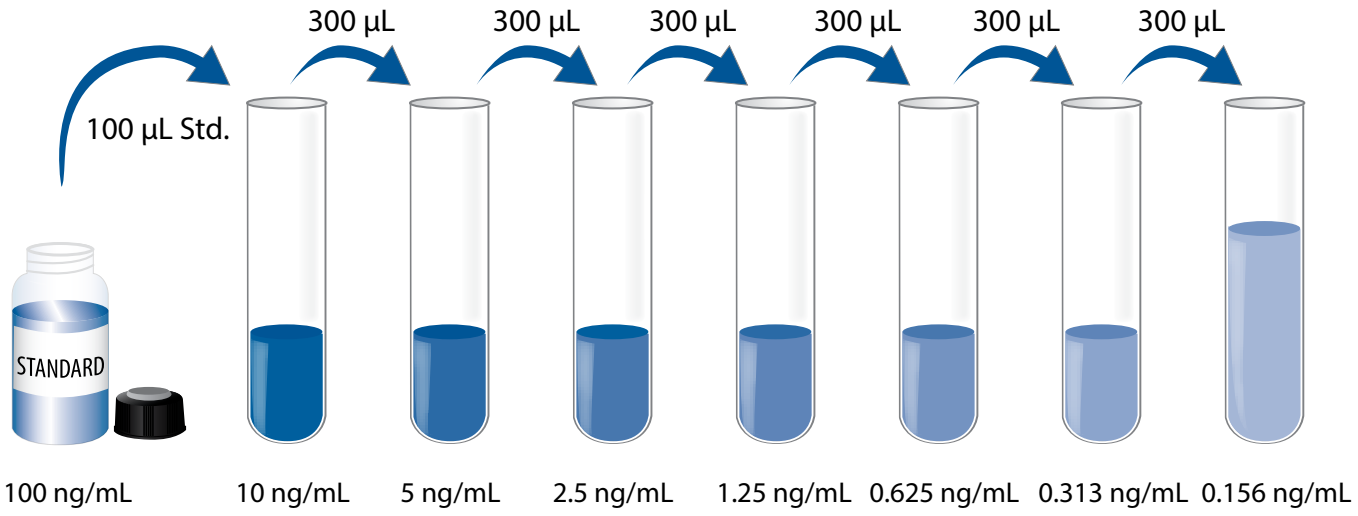
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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

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 Vitamin D BP Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse Vitamin D BP Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:5) into the 10 ng/mL tube. Pipette 300 µL into the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard 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, 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 100 µL of Standard, Control, or sample* per 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.
4. 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.
5. Add 100 µL of Mouse Vitamin D BP Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
8. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. 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

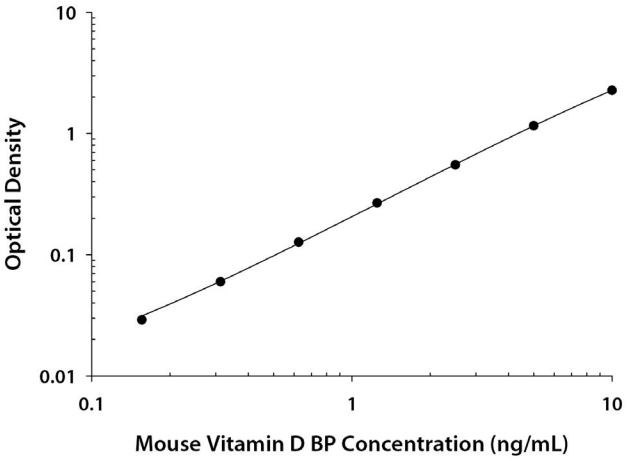
Average the duplicate readings for each standard, Control, and sample and subtract the average zero standard optical density (O.D.).

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 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	0.015 0.016	0.016	—
0.156	0.044 0.045	0.045	0.029
0.313	0.075 0.076	0.076	0.060
0.625	0.141 0.144	0.143	0.127
1.25	0.276 0.290	0.283	0.267
2.5	0.567 0.567	0.567	0.551
5	1.170 1.180	1.175	1.159
10	2.267 2.305	2.286	2.270

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)	0.404	1.13	3.30	0.444	1.22	3.47
Standard deviation	0.020	0.040	0.082	0.027	0.056	0.148
CV (%)	5.0	3.5	2.5	6.1	4.6	4.3

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	98	94-105%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse Vitamin D BP were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Tissue lysates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	100	100	98	100	100	102
	Range (%)	96-104	96-104	96-101	97-102	98-102	97-106
1:4	Average % of Expected	100	102	99	99	101	104
	Range (%)	96-104	94-105	97-102	97-101	99-103	100-110
1:8	Average % of Expected	102	102	99	99	102	107
	Range (%)	97-108	94-109	96-103	96-103	99-105	100-109
1:16	Average % of Expected	105	104	100	99	100	110
	Range (%)	98-112	90-119	98-103	97-102	99-102	102-119

SENSITIVITY

Twenty-nine assays were evaluated and the minimum detectable dose (MDD) of mouse Vitamin D BP ranged from 0.005-0.033 ng/mL. The mean MDD was 0.012 ng/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 NS0-derived recombinant mouse Vitamin D BP produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of Vitamin D BP in this assay.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum (n=10)	386	312-502	64.8
EDTA plasma (n=5)	310	276-334	25.0
Heparin plasma (n=5)	313	249-351	51.1
Urine (n=5)	73.9	46.6-102	20.5

Cell Culture Supernates - Organs from mice were removed, rinsed in PBS, and kept on ice. The organs were then homogenized using 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. Cells were cultured for 1 or 3 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse Vitamin D BP.

Tissue Type	(ng/mL)
Kidney (3 days)	257
Liver (1 day)	1058

Tissue Lysates - Organs from mice were rinsed with PBS, cut into 1-2 mm pieces, and homogenized with a tissue homogenizer in 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. Aliquots of the lysates were removed and assayed for levels of mouse Vitamin D BP.

Tissue Type	(pg/mg) of cell lysate
Kidney	397
Liver	567

SPECIFICITY

This assay recognizes natural and recombinant mouse Vitamin D BP.

The factors listed below were prepared in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors in a mid-range recombinant mouse Vitamin D BP control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinants:

mouse α-Fetoprotein
human Vitamin D BP

Other factors:

human serum albumin
bovine serum albumin
human calcifediol vitamin D3
human ercalcidiol vitamin D2

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07.14 753019.0 7/14