

## **Human Vitamin D-binding protein**

ELISA Kit **Cat #: orb1289433 (manual)**

Sandwich Enzyme-Linked Immunosorbent Assay for Quantitative Detection of Human Vitamin D-binding protein Concentrations in Cell Culture Supernates, Serum, Plasma, Cell Lysates, Tissue Homogenates.

*For research use only. Not for diagnostic or therapeutic procedures.*

## INTRODUCTION

Vitamin D binding protein (VitD 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 VitD BP is important for the function of a wide variety of tissues. VitD BP binds both the 25 (OH) and the hormonally active 1, 25 (OH) 2 forms of vitamin D. VitD BP is primarily expressed in hepatocytes and to a lesser extent in the kidney. It delivers vitamin D into cells by Megalin-mediated endocytosis. A selectively deglycosylated form of VitD BP known as macrophage activating factor (MAF) blocks the angiogenic effects of FGF basic, VEGF, and Angiopoietin 2. VitD 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). The chemotactic cofactor property of VitD 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.

## ASSAY PRINCIPLES

The Biorbyt Human Vitamin D-binding protein ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of Human Vitamin D-binding protein in Cell Culture Supernates, Serum, Plasma, Cell Lysates, Tissue Homogenates. This assay employs an antibody specific for Human Vitamin D-binding protein coated on a 96-well plate. Standards and samples are pipetted into the wells and Vitamin D-binding protein present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human Vitamin D-binding protein antibody is added. After washing away unbound biotinylated antibodies, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of Vitamin D-binding protein bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

## KIT COMPONENTS

Component	Volume
96-well Plate Coated with Anti-Human Vitamin D-binding protein Antibody	8 wells x 12 Strips
Human Vitamin D-binding protein Standard	3 ng x 2
Biotin-Labeled Detection Antibody (100X)	120 $\mu$ l
Streptavidin-HRP (100X)	120 $\mu$ l
Standard/Sample Diluent	30 ml
Detection Antibody Diluent	12 ml
Streptavidin-HRP Diluent	12 ml
Wash Buffer (20X)	30 ml
TMB Substrate Solution	12 ml
Stop Solution	12 ml
Plate Adhesive Strips	3 Strips
Technical Manual	1 Manual

## STORAGE AND STABILITY

All kit components are stable at 2 to 8 °C. Standard (recombinant protein) should be stored at -20 °C or -80 °C (recommended at -80 °C) after reconstitution. Opened Microplate Wells or reagents may be stored for up to 1 month at 2 to 8 °C. Return unused wells to the pouch containing the desiccant pack, and reseal along the entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20 °C. Avoid repeated freeze-thaw cycles.

### **MATERIALS REQUIRED BUT NOT PROVIDED**

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and pipette tips to deliver 2 µl to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1-liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. Computer and software for ELISA data analysis.
8. Tubes to prepare standard or sample dilutions.

### **HEALTH AND SAFETY PRECAUTIONS**

1. Reagents provided in this kit may be harmful if ingested, inhaled, or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.
2. Stop Solution contains 2 N Sulfuric Acid (H<sub>2</sub>SO<sub>4</sub>) and is an extremely corrosive agent. Please wear proper eye, hand, and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution before disposing of the plate.
3. Standard protein and Detection Antibody containing Sodium Azide as a preservative.

## REAGENT PREPARATION

### 1. Sample Preparation

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

**Cell culture supernates:** Remove particulates by centrifugation, assay immediately or aliquot, and store samples at -20°C.

**Serum:** Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 minutes. Analyze the serum immediately or aliquot and store samples at -20°C.

**Plasma:** Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1500 X g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C.

**Cell Lysates:** Collect cells and rinse cells with PBS. Homogenize and lysate cells thoroughly in lysate solution. Centrifuge cell lysates at approximately 10000 X g for 5 minutes to remove debris. Aliquots of the cell lysates were removed and assayed.

**Bone Tissue:** Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors. Dissolve the final sample in 2 M Guanidine-HCl.

**Tissue Homogenates:** The preparation of tissue homogenates will vary depending upon tissue type. Rinse tissue with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS, and stored overnight at ≤ -20 °C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. The supernate was removed immediately and assayed. Alternatively, aliquot and store samples at ≤ -20 °C.

**Note:** Some lysis buffers, such as RIPA can not be used. Some components will affect the binding.

**Urine:** Urinary samples should be cleared by centrifugation and then can be used directly without dilution. Storage at -20°C.

### 2. Human Vitamin D-binding protein Standard Preparation

Reconstitute the lyophilized Human Vitamin D-binding protein Standard by adding 0.5 ml of Standard/Sample Diluent to make the 6000 pg/ml standard stock solution. Allow the solution to sit at room temperature for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting. Two tubes of the standard (3 ng per tube) are included in each kit. Use one tube for each experiment. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (93.75 pg/ml - 6000 pg/ml) as below. Standard/Sample Dilution Buffer serves as the zero standard (0 pg/ml).

Standard	Add	Into
6000 pg/ml		
3000 pg/ml	250 µl of the Standard (6000 pg/ml)	250 µl of the Standard/Sample Diluent
1500 pg/ml	250 µl of the Standard (3000 pg/ml)	250 µl of the Standard/Sample Diluent
750 pg/ml	250 µl of the Standard (1500 pg/ml)	250 µl of the Standard/Sample Diluent
375 pg/ml	250 µl of the Standard (750 pg/ml)	250 µl of the Standard/Sample Diluent
187.5 pg/ml	250 µl of the Standard (375 pg/ml)	250 µl of the Standard/Sample Diluent
93.75 pg/ml	250 µl of the Standard (187.5 pg/ml)	250 µl of the Standard/Sample Diluent
0 pg/ml	1 ml of the Standard/Sample Diluent	

**Note:** The standard solutions are best used within 2 hours. The 6000 pg/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

### 3. Biotin-Labeled Detection Antibody Working Solution Preparation

The Biotin-Labeled Detection Antibody should be diluted in 1: 100 with the Detection Antibody Diluent and mixed thoroughly. The solution should be prepared no more than 2 hours prior to the experiment.

### 4. Streptavidin-HRP Working Solution Preparation

The Streptavidin-HRP should be diluted in 1: 100 with the Streptavidin-HRP Diluent and mixed thoroughly. The solution should be prepared no more than 1 hour prior to the experiment.

### 5. Wash Buffer Working Solution Preparation

Pour entire contents (30 ml) of the Wash Buffer Concentrate into a clean 1, 000 ml graduated cylinder. Bring the final volume to 600 ml with glass-distilled or deionized water (1: 20).

## ASSAY PROCEDURE

The Streptavidin-HRP Working Solution and TMB Substrate Solution must be kept warm at 37°C for 30 minutes before use. When diluting samples and reagents, they must be mixed completely and evenly. A standard detection curve should be prepared for each experiment. The user will decide on sample dilution fold by crude estimation of protein amount in samples.

1. Add 100 µl of each standard and sample into appropriate wells.
2. Cover well and incubate for 90 minutes at room temperature or overnight at 4°C with gentle shaking.
3. Remove the cover, discard the solution, and wash the plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 100 µl of Biotin-Labeled Detection Antibody Working Solution into each well and incubate the plate at 37°C for 60 minutes.
5. Wash the plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Discard the Wash Buffer Working Solution and blot the plate onto paper towels or other absorbent material.
6. Add 100 µl of Streptavidin-HRP Working Solution into each well and incubate the plate at 37°C for 45 minutes.
7. Wash the plate 5 times with Wash Buffer Working Solution, and each time let the wash buffer stay in the wells for 1 - 2 minutes. Discard the wash buffer and blot the plate onto paper towels or other absorbent material.
8. Add 100 µl of TMB Substrate Solution into each well and incubate the plate at 37°C in the dark for 10-20 minutes.
9. Add 100 µl of Stop Solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 minutes after adding the Stop Solution.

For calculation,  $(\text{the relative O.D.450}) = (\text{the O.D.450 of each well}) - (\text{the O.D.450 of Zero well})$ . The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The concentration of the samples can be interpolated from the standard curve.

**Note:** If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution.

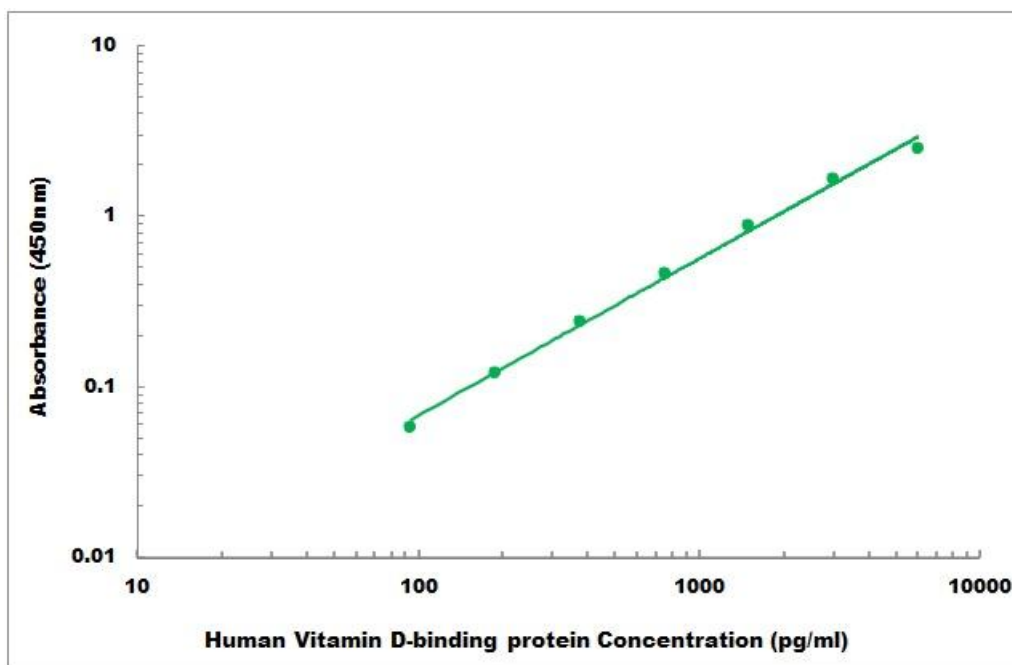
## ASSAY PROCEDURE SUMMARY

- Prepare all reagents, samples and standards
- Add 100  $\mu$ l Standard or Sample
- Wash plate 3 times with Wash Buffer Working Solution
- Add 100  $\mu$ l Biotin-Labeled Detection Antibody Working Solution
- Wash plate 3 times with Wash Buffer Working Solution
- Add 100  $\mu$ l Streptavidin-HRP Working Solution
- Wash plate 5 times with Wash Buffer Working Solution
- Add 100  $\mu$ l TMB Substrate Solution
- Add 100  $\mu$ l Stop Solution
- Read the plate at 450nm



## TYPICAL DATA

The standard curve is for demonstration only. A standard curve must be run with each assay.



## SENSITIVITY

The minimum detectable dose of Human Vitamin D-binding protein ELISA Kit is typically less than 50 pg/ml.

## SPECIFICITY

The Human Vitamin D-binding protein allows for the detection and quantification of endogenous levels of natural and/or recombinant Human Vitamin D-binding protein within the range of 93.75 pg/ml - 6000 pg/ml.

## CROSS REACTIVITY

No detectable cross-reactivity with other relevant proteins.

## TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase the time of soaking between in-wash
	• Too much Streptavidin-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents are allowed to come to 20 - 30 °C before performing the assay
Too much signal-whole plate turned uniformly blue	• Insufficient washing-unbound Streptavidin-HRP remaining	• Increase the number of washes carefully
	• Too much Streptavidin-HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in the presence of residual Streptavidin-HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make a new standard curve
No signal when a signal is expected, but the standard curve looks fine	• Sample matrix is masking detection	• More diluted sample Recommended
Samples are reading too high, but the standard curve is fine	• Samples contain protein levels above the assay range	• Dilute samples and run Again
Edge effect	• Uneven temperature around the work surface	• Avoid incubating plates in areas where environmental conditions vary • Use plate sealer