Human Vitamin D binding protein

ELISA

Catalog # LF-EK0141 (1 kit)
Catalog # LF-EK0142 (4 kits bundle)
Catalog # LF-EK0143 (10 kits bundle)
Catalog # LF-EK0144 (20 kits bundle)

Sandwich Enzyme-Linked Immunosorbent Assay for Quantitative Detection of human Vitamin D binding Protein (VDBP)

For research use only
Not for diagnostic or therapeutic procedures
1. Introduction

Vitamin D-binding protein (DBP, VDBP), also called group-specific component (Gc) and macrophage-activating factor (GcMAF/DBP-MAF), is a 52 to 58kDa plasma glycoprotein with many functions, such as transport of vitamin D metabolites, control of bone development, binding of fatty acids, sequestration of actin, and modulating immune and inflammatory responses. DBP is synthesized predominantly by hepatic parenchymal cells and detected in plasma, cerebrospinal fluid, seminal fluid, saliva and breast milk. The exploitation of the unique properties of DBP could enable the development of important therapeutic agents such as vitamin D-associated conditions, actin sequestering in trauma and inflammation, chronic obstructive pulmonary disease, osteopetrosis, cancer therapy and immune modulation by macrophage activation. The DBP molecule is therefore an ideal candidate molecule for further investigation by biotechnology-based companies seeking a platform from which to pursue new therapeutic options.

2. Principles of Method

The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to human Fibrinogen. Samples are pipetted into these wells. Nonbound fibrinogen and other components of the sample should be removed by washing, then biotin-conjugated monoclonal antibody specific to fibrinogen added. In order to quantitatively determine the amount of fibrinogen present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) should be added to each microplate well. The final step, a TMB-substrate solution added to each well. Finally, a sulfuric acid solution is added and the resulting yellow colored product is measured at 450nm. Since the increases in absorbency is directly proportional to the amount of captured VDBP.
3. Intended Use

The Abfrontier human VDBP ELISA kit is to be used for the in vitro quantitative determination of human VDBP in human serum, human plasma, cell lysate and buffered solution. The assay will recognize native human VDBP.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

4. Storage and Stability

All kit components of this kit are stable at 2 to 8°C. Any unused reconstituted standard should be discarded or frozen at -70°C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

5. Chemical Hazard

- Stop solution: This reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. In the event of contact with eyes or skin, wash immediately with plenty of water.
- Standard protein and 2nd Antibody containing Sodium Azide as a preservative.
6. Kit Contents

<table>
<thead>
<tr>
<th>Contents</th>
<th>Number</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 Well Plate</td>
<td>1</td>
<td>(in aluminum foil bag with desiccant)</td>
</tr>
<tr>
<td>Incubation Buffer</td>
<td>1</td>
<td>30 ml</td>
</tr>
<tr>
<td>Washing Buffer</td>
<td>2</td>
<td>(20X) 25 ml</td>
</tr>
<tr>
<td>Standard Protein</td>
<td>1</td>
<td>Glass vial (lyophilized)</td>
</tr>
<tr>
<td>Standard/Sample Dilution Buffer</td>
<td>1</td>
<td>25 ml</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td>1</td>
<td>Glass vial (lyophilized)</td>
</tr>
<tr>
<td>AV-HRP</td>
<td>1</td>
<td>150 µl</td>
</tr>
<tr>
<td>Secondary Antibody/AV-HRP</td>
<td>1</td>
<td>25 ml</td>
</tr>
<tr>
<td>Dilution Buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate (TMB)</td>
<td>1</td>
<td>15 ml</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>1</td>
<td>15 ml</td>
</tr>
<tr>
<td>Protocol booklet</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Plate sealers</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

① 96 Well Plate
   : Human VDBP microtiter plate, one plate of 96 wells (8well strip x 12
   A plate using break-apart strips coated with a mouse monoclonal antibody specific to human fibrinogen.

② Standard Protein
   : Native human VDBP.

③ Secondary Antibody
   : Biotinylated anti human VDBP antibody.

④ AV-HRP
   : Avidin linked Horseradish Peroxidase (HRP, enzyme)

⑤ Substrate (Stabilized chromogen)
   : Tetramethylbenzidine (TMB) solution

⑥ Stop Solution
   : 1N solution of sulfuric acid (H₂SO₄)

⑦ Plate sealer
   : Adhesive sheet.

- Do not mix or interchange different reagents from various kit lots.
7. Materials Required But Not Provided

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated, adjustable precision pipettes, preferably with disposable plastic tips (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water
4. Data analysis and graphing software
5. Vortex mixer
6. Polypropylene tubes for diluting and aliquoting standard
7. Absorbent paper towels
8. Calibrated beakers and graduated cylinders of various sizes

8. Reagent Preparation

1) Human VDBP standard

1. Reconstitute the lyophilized Human VDBP standard by adding 1 ml of Standard/Sample Dilution Buffer to make the 1 μg/ml standard stock solution. Allow solution to sit at RT for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting.

2. Prepare 1 ml of 50 ng/ml top standard by adding 50 μl of the above stock solution in 950 μl of Standard/Sample Dilution Buffer. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (0.78 ng/ml ~ 50 ng/ml) as below. Standard/Sample Dilution Buffer serves as the zero standard(0 ng/ml).

<table>
<thead>
<tr>
<th>Standard (ng/ml)</th>
<th>Add</th>
<th>Into</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50 μl of the std.(1.0 μg/ml)</td>
<td>950.0 μl of the Standard/Sample Dilution Buffer</td>
</tr>
<tr>
<td>25</td>
<td>500 μl of the std.(50 ng/ml)</td>
<td>500.0 μl of the Standard/Sample Dilution Buffer</td>
</tr>
<tr>
<td>12.5</td>
<td>500 μl of the std.(25 ng/ml)</td>
<td>500.0 μl of the Standard/Sample Dilution Buffer</td>
</tr>
<tr>
<td>6.25</td>
<td>500 μl of the std.(12.5 ng/ml)</td>
<td>500.0 μl of the Standard/Sample Dilution Buffer</td>
</tr>
<tr>
<td>3.125</td>
<td>500 μl of the std.(6.25 ng/ml)</td>
<td>500.0 μl of the Standard/Sample Dilution Buffer</td>
</tr>
<tr>
<td>1.56</td>
<td>500 μl of the std.(3.13 ng/ml)</td>
<td>500.0 μl of the Standard/Sample Dilution Buffer</td>
</tr>
<tr>
<td>0.78</td>
<td>500 μl of the std.(1.56 ng/ml)</td>
<td>500.0 μl of the Standard/Sample Dilution Buffer</td>
</tr>
<tr>
<td>0 ng/ml</td>
<td>1.0 ml of the Standard/Sample Dilution Buffer</td>
<td></td>
</tr>
</tbody>
</table>

2) Secondary Antibody

100X secondary antibody solution can be made by adding 150 μl secondary antibody/AV-HRP dilution buffer in the vial.

1. Equilibrate to room temperature, mix gently.
2. Mix 20 µl Secondary Antibody concentrated solution (100X) + 1.98 ml Secondary Antibody/AV-HRP dilution buffer. (Sufficient for two 8-well strip, prepare more if necessary)

   Label as “Working Secondary antibody Solution”.

3. Return the unused Secondary Antibody concentrated solution to the refrigerator.

3) AV-HRP

1. Equilibrate to room temperature, mix gently.

2. Mix 20 µl AV-HRP concentrated solution (100X) + 1.98 ml Secondary Antibody/AV-HRP dilution buffer. (Sufficient for two 8-well strip, prepare more if needed)

   Label as “Working AV-HRP Solution”.

3. Return the unused AV-HRP concentrated solution to the refrigerator.

4) Washing buffer

1. Equilibrate to room temperature, mix to re-dissolve any precipitated salt.

2. Mix 0.5 volume Wash buffer concentrate solution (20X) + 9.5 volumes of deionized water. Label as “Working Washing Solution”.

3. Store both the concentrated and the Working Washing Solution in the refrigerator.

* Directions for washing

1. Fill the wells with 300 µl of “Working Washing Buffer”.

   Let soak for 1 to 3 minutes and then all residual wash-liquid must be drained from the wells by aspiration (taking care not to scratch the inside of the well) or decantation, followed by forceful tapping of the plate on absorbent paper. Never insert absorbent paper directly into the wells.

   If using an automated washer, the operating instructions for washing equipment should be carefully followed.

2. Incomplete washing will adversary affects the assay and renders false results.

3. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing to avoid strips coming free of the frame.

5) Sample preparation

   Blood should be collected by veinpuncture. For plasma samples, blood may be drawn into tubes containing sodium citrate or heparin, EDTA. The serum or plasma should be separated from the coagulated or packed cells by centrifugation. Specimens may be shipped at room temperature and then stored refrigerated at 2-8°C if testing is to take place within one week after collection. If testing is to take place later than one week, specimens should be stored at -20°C. Avoid repeated freeze/thawing.
9. Assay Procedure

- Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
- All standards, controls and samples should be run in duplicate for confirmation of reproducibility.
- A standard curve must be run with each assay.
- If particulate matter is present in the analyte, centrifuge or filter prior to analysis.
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.

1) Determine the number of 16-well strips needed for assay. Insert these in the flame(s) for current use (Re-bag extra strips and frame. Refrigerate for further use).
2) Add 300 µl of Incubation buffer to all wells and incubate the plate for 5 minutes at room temperature.
3) Thoroughly aspirate or decant the solution from the wells. Wash wells 2 times (See “Directions for washing”).
4) For the standard curve, add 100 µl of the standard to the appropriate microtiter wells. Add 100 µl of the Standard/Sample Dilution Buffer to zero wells.
5) Serum and plasma require at least 4000 fold dilution in the Standard/Sample Dilution Buffer. And add 100 µl of samples to each wells.
6) Cover the plate with the plate cover and incubate for 2 hours at room temperature.
7) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
8) Pipette 100 µl of “Working Secondary Antibody Solution” into each well.
9) Cover the plate with the plate cover and incubate for 1 hour at room temperature.
10) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
11) Add 100 µl “Working AV-HRP Solution” to each well.
12) Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
13) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
14) Add 100 µl of Substrate to each well. The liquid in the wells should begin to turn blue.
15) Incubate the plate at room temperature.

- Do not cover the plate with aluminum foil, or color may develop.

The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450 nm
can only be read after the Stop Solution has been added to each well.

- Because the Substrate is light sensitive, avoid the remained Substrate solution prolonged exposure to light.
- Typically, reaction is stopped 5~10 minutes after treatment of Substrate, but this time can be adjusted as the user desires.

16) Add 100 µl of Stop Solution to each well. The solution in the wells should change from blue to yellow.

17) Read the absorbance of each well at 450nm. Read the plate within 20 minutes of adding the Stop Solution.

18) Plot on graph paper the absorbance of the standard against the standard concentration (Optimally, the background absorbance can be subtracted from all data points, including standards, unknowns and controls, prior to plotting.). Draw a smooth curve through these points to construct the standard curve.

19) Read the human fibrinogen concentrations for the unknown samples and controls from the standard curve plotted in step 18. Multiply value(s) obtained for the unknown sample by the dilution factor (Samples producing signals greater than that of the highest standard should be further diluted in the Standard/Sample Dilution Buffer).

10. Characteristics

1) Typical result
The standard curve below is for illustration only and should not be used to calculate results in your assay.

A standard curve must be run with each assay.

<table>
<thead>
<tr>
<th>Standard human VDBP (ng/ml)</th>
<th>Optical Density (at 450nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.116</td>
</tr>
<tr>
<td>0.78</td>
<td>0.129</td>
</tr>
<tr>
<td>1.56</td>
<td>0.131</td>
</tr>
<tr>
<td>3.125</td>
<td>0.155</td>
</tr>
<tr>
<td>6.25</td>
<td>0.226</td>
</tr>
<tr>
<td>12.5</td>
<td>0.405</td>
</tr>
<tr>
<td>25</td>
<td>0.853</td>
</tr>
<tr>
<td>50</td>
<td>1.798</td>
</tr>
</tbody>
</table>
<Limitations>
- Do not extrapolate the standard curve beyond the 50 ng/ml standard point.
- Other buffers and matrices have not been investigated.
- The rate of degradation of native human VDBP in various matrices has not been investigated.

(TMB reaction incubate at room temperature for 5min)

2) Sensitivity
The minimal detectable dose of human VDBP was calculated to be 2.8 ng/ml, by subtracting two standard deviations from the mean of 12 zero standard replicates (ELISA buffer, S0) and intersecting this value with the standard curve obtained in the same calculation.

<table>
<thead>
<tr>
<th>N</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZERO</td>
<td>0.126</td>
<td>0.123</td>
<td>0.08</td>
<td>0.124</td>
<td>0.116</td>
<td>0.13</td>
<td>0.126</td>
<td>0.123</td>
<td>0.08</td>
<td>0.124</td>
<td>0.116</td>
<td>0.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average</th>
<th>SD</th>
<th>LLD</th>
<th>LLD mean(ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1165</td>
<td>0.0176</td>
<td>0.1517</td>
<td>2.7994</td>
</tr>
</tbody>
</table>
3) Specificity

The following substances were tested and found to have no cross-reactivity: human serum albumin, transferrin, IgG, alpha-fetoprotein (AFP), fibrinogen, plasminogen, Hemoglobin.

4) Precision

① Within-Run (Intra-Assay)

<table>
<thead>
<tr>
<th>Mean (ng/ml)</th>
<th>SD (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.99</td>
<td>10.96</td>
<td>5.63</td>
</tr>
<tr>
<td>10.26</td>
<td>19.63</td>
<td>7.94</td>
</tr>
<tr>
<td>23.48</td>
<td>30.01</td>
<td>5.73</td>
</tr>
<tr>
<td>51.36</td>
<td>28.77</td>
<td>4.07</td>
</tr>
</tbody>
</table>

② Between-Run (Inter-Assay)

<table>
<thead>
<tr>
<th>Mean (ng/ml)</th>
<th>SD (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.95</td>
<td>0.34</td>
<td>6.83</td>
</tr>
<tr>
<td>10.56</td>
<td>0.33</td>
<td>3.15</td>
</tr>
<tr>
<td>23.38</td>
<td>1.56</td>
<td>6.66</td>
</tr>
<tr>
<td>51.45</td>
<td>3.11</td>
<td>6.04</td>
</tr>
</tbody>
</table>

5) Recovery

Recovery on addition is 98.74~105.85% (mean 101.3%)

(VDBP in Serum A: 154.718 µg/ml, VDBP in Serum B: 126.836 µg/ml)

<table>
<thead>
<tr>
<th>Analyte added (ng/ml)</th>
<th>Added analyte+1/256000 diluted Serum A (ng/ml)</th>
<th>Recovery (%)</th>
<th>Added analyte+1/256000 diluted Serum B (ng/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>6.854</td>
<td>99.59</td>
<td>6.745</td>
<td>103.18</td>
</tr>
<tr>
<td>12.5</td>
<td>13.104</td>
<td>104.26</td>
<td>12.995</td>
<td>98.74</td>
</tr>
<tr>
<td>25</td>
<td>25.604</td>
<td>99.43</td>
<td>25.495</td>
<td>99.26</td>
</tr>
<tr>
<td>50</td>
<td>50.604</td>
<td>99.74</td>
<td>50.495</td>
<td>105.85</td>
</tr>
<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>----------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| High signal and background in all wells | • Insufficient washing  
• Too much AV-HRP  
• Incubation time too long  
• Development time too long | • Increase number of washes  
• Increase time of soaking between in wash  
• Check dilution, titration  
• Reduce incubation time  
• Decrease the incubation time before the stop solution is added |
| No signal | • Reagent added in incorrect order, or incorrectly prepared  
• Standard has gone bad (If there is a signal in the sample wells)  
• Assay was conducted from an incorrect starting point | • Review protocol  
• Check the condition of stored standard  
• Reagents allows to come to 20~30℃ before performing assay |
| Too much signal – whole plate turned uniformly blue | • Insufficient washing  
– unbound AV-HRP remaining  
• Too much AV-HRP  
• Plate sealer or reservoir reused, resulting in presence of residual AV-HRP | • Increase number of washes carefully  
• Check dilution  
• Use fresh plate sealer and reagent reservoir for each step |
| Standard curve achieved but poor discrimination between point | • Plate not developed long enough  
• Improper calculation of standard curve dilution | • Increase substrate solution incubation time  
• Check dilution, make new standard curve |
| No signal when a signal is expected, but standard curve looks fine | • Sample matrix is masking detection | • More diluted sample recommended |
| Samples are reading too high, but standard curve is fine | • Samples contain protein levels above assay range | • Dilute samples and run again |
| Edge effect | • Uneven temperature around work surface | • Avoid incubating plate in areas where environmental conditions vary  
• Use plate sealer |
12. Reference


2) Svasti, J. et al., Biochemistry (1979) vol.18: pp.1611-1617

 Ordering Information

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