HUMAN VITAMIN D BINDING PROTEIN ELISA Assay Kit

Immunoperoxidase Assay for Determination of Vitamin D Binding Protein in Human Samples

VBP31-K01

DIRECTIONS FOR USE

For Research Use Only, NOT for Diagnostic Purposes

Please Read this Package Insert Completely Before Using This Product

INTENDED USE

The Human Vitamin D Binding Protein ELISA Assay Kit is a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring VDBP in human biological samples.

PRINCIPLE OF THE ASSAY

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the VDBP present in samples reacts with the anti-VDBP antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-VDBP antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound VDBP. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of VDBP in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of VDBP in the test sample. The quantity of VDBP in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

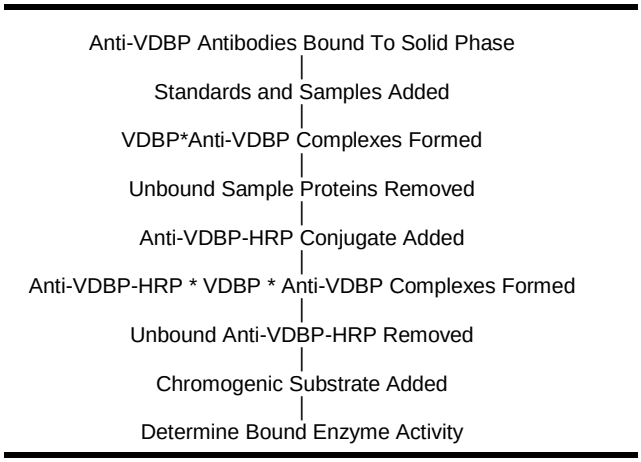

Figure 1.

REAGENTS (Quantities sufficient for 96 determinations)

1. DILUENT CONCENTRATE (Running Buffer)
One bottle containing 50 ml of a 5X concentrated diluent running buffer.

2. WASH SOLUTION CONCENTRATE
One bottle containing 50 ml of a 20X concentrated wash solution.

3. ENZYME-ANTIBODY CONJUGATE 100X
One vial containing 150 µL of affinity purified anti-Human VDBP antibody conjugated with horseradish peroxidase in a stabilizing buffer.

4. CHROMOGEN-SUBSTRATE SOLUTION
One vial containing 12 mL of 3,3',5,5'-tetramethybenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. STOP SOLUTION
One vial containing 12 ml 0.3 M sulfuric acid.

WARNING: Avoid contact with skin.

6. ANTI-HUMAN VDBP ELISA MICRO PLATE
Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-Human VDBP.

7. HUMAN VDBP CALIBRATOR
One vial containing a lyophilized Human VDBP calibrator.

FOR RESEARCH USE ONLY

REAGENT PREPARATION

1. DILUENT CONCENTRATE
The Diluent Solution supplied is a 5X Concentrate and must be diluted 1/5 with distilled or deionized water (1 part buffer concentrate, 4 parts dH2O).

2. WASH SOLUTION CONCENTRATE
The Wash Solution supplied is a 20X Concentrate and must be diluted 1/20 with distilled or deionized water (1 part buffer concentrate, 19 parts dH2O). Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.

3. ENZYME-ANTIBODY CONJUGATE
Calculate the required amount of working conjugate solution for each microtitre plate test strip by adding 10 µL Enzyme-Antibody Conjugate to 990 µL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.

4. CHROMOGEN-SUBSTRATE SOLUTION
Ready to use as supplied.

5. STOP SOLUTION
Ready to use as supplied.

6. ANTI-HUMAN VDBP ELISA MICRO PLATE
Ready to use as supplied. Unseal Microtiter Pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal along with desiccant.

7. HUMAN VDBP CALIBRATOR
Add 1.0 ml of distilled or de-ionized water to the Human VDBP calibrator and mix gently until dissolved. The calibrator is now at a concentration of 33.1 µg/ml **(the reconstituted calibrator should be aliquoted and frozen if future use is intended). Human VDBP standards need to be prepared immediately prior to use (see chart below).** Mix well between each step. Avoid foaming.

Standard	ng/ml	Volume added to 1x Diluent →	Volume of 1x Diluent
6	200	4 µl of Human VDBP Calibrator	658 µl
5	100	250 µl standard 6	250 µl
4	50	250 µl standard 5	250 µl
3	25	250 µl standard 4	250 µl
2	12.5	250 µl standard 3	250 µl
1	6.25	250 µl standard 2	250 µl
0	0		500 µl

STORAGE AND STABILITY

The expiration date for the package is stated on the box label.

1. DILUENT
The 5X Diluent Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4-8°C.

2. WASH SOLUTION
The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C) or at 4-8°C.

3. ENZYME-ANTIBODY CONJUGATE
Undiluted horseradish peroxidase anti-VDBP conjugate should be stored at 4-8°C and **diluted immediately prior to use**. The working conjugate solution is stable for up to 1 hour when stored in the dark.

4. CHROMOGEN-SUBSTRATE SOLUTION
The Substrate Solution should be stored at 4-8°C and is stable until the expiration date.

5. STOP SOLUTION
The Stop Solution should be stored at 4-8°C and is stable until the expiration date.

6. ANTI-HUMAN VDBP ELISA MICRO PLATE
Anti-Human VDBP coated wells are stable until the expiration date, and should be stored at 4-8°C in sealed foil pouch with desiccant pack.

7. HUMAN VDBP CALIBRATOR
The lyophilized Human VDBP calibrator should be stored at 4C or frozen until reconstituted. The reconstituted calibrator should be aliquoted out and stored frozen (Avoid multiple freeze-thaw cycles). The working standard solutions should be prepared immediately prior to use and are stable for up to 8 hours.

INDICATIONS OF INSTABILITY
If the test is performing correctly, the results observed with the standard solutions should be within 20 % of the expected values.

SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture. The serum should be separated from the cells after clot formation by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize

hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freeze-thaw cycles.

1. Precautions

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

2. Additives and Preservatives

No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

MATERIAL PROVIDED
See "REAGENTS"

MATERIALS REQUIRED
BUT NOT PROVIDED

- Precision pipette (2 µL to 200 µL) for making and dispensing dilutions
- Test tubes
- Microtitre washer/aspirator
- Distilled or Deionized H_2O
- Microtitre Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer

ASSAY PROTOCOL

DILUTION OF SAMPLES

The assay for quantification of VDBP in samples requires that each test sample be diluted before use. For a single step determination a dilution of 1/40,000 is appropriate for most serum/plasma samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.**

1. To prepare a 1/40,000 dilution of sample, transfer 5 µL of sample to 495µL of 1X diluent. Next, dilute the 1/100 samples by transferring 2 µL, to 798 µL of 1X diluent. You now have a 1/40,000 dilution of your sample. Mix thoroughly at each stage.

PROCEDURE

1. **Bring all reagents to room temperature before use.**

2. Pipette 100 µL of

Standard 0 (0.0 ng/ml) in duplicate
Standard 1 (6.25 ng/ml) in duplicate
Standard 2 (12.5 ng/ml) in duplicate
Standard 3 (25 ng/ml) in duplicate
Standard 4 (50 ng/ml) in duplicate
Standard 5 (100 ng/ml) in duplicate
Standard 6 (200 ng/ml) in duplicate

3. Pipette 100 µL of sample (in duplicate) into pre designated wells.

4. Incubate the micro titer plate at room temperature for thirty (30 ± 2) minutes. Keep plate covered and level during incubation.

5. Following incubation, aspirate the contents of the wells.

6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill wells with wash buffer, invert the plate then pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

7. Pipette 100 µL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at room temperature for thirty (30 ± 2) minutes. Keep plate covered in the dark and level during incubation.

8. Wash and blot the wells as described in Steps 5/6.

9. Pipette 100 µL of TMB Substrate Solution into each well.

10. Incubate in the dark at room temperature for precisely ten (10) minutes.

11. After ten minutes, add 100 µL of Stop Solution to each well.

12. Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to manufacture's specifications.

STABILITY OF THE FINAL REACTION MIXTURE

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

RESULTS

1. Subtract the average background value from the test values for each sample.

2. Using the results observed for the standards construct a Standard Curve. The appropriate curve fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.

3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at the VDBP concentration in original samples.

SENSITIVITY

1. The detection limit was defined as B_0 + 2SD and determined to be 3 ng/mL.

LINEARITY

Two samples were diluted. The results are shown below.

Sample	Dilution	Expected (µg/mL)	Measured (µg/mL)
A	1/10,000	320	317.78
A	1/20,000	160	249.83
A	1/40,000	80	288.29
A	1/80,00	40	226.15
B	1/10,000	320	321.73
B	1/20,000	160	272.25
B	1/40,000	80	281.43
B	1/80,00	40	278.06

3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at the VDBP concentration in original samples.

LIMITATION OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.

2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

3. Do not mix or substitute reagents with those from other lots or sources.

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