Monobind Inc.
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AccuBind
ELISA Microwells

25(OH) Vitamin D (Vit D) Test System

Product Code: 7725-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of 25-OH Vitamin D in human serum or plasma by a Microplate Enzyme Immunoassay, Colorimetric.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Vitamin D is a steroid hormone involved in the active intestinal absorption of calcium and in the regulation of its homeostasis. Vitamin D has two isomers: Vitamin D2 and Vitamin D3. Vitamin D2 is obtained from dairy products whereas Vitamin D3 is produced in the skin after exposure to ultraviolet light. In the liver, Vitamin D is hydroxylated at its carbon 25 to form 25-OH Vitamin D. This metabolite is the predominant circulating form of Vitamin D and is considered to be an accurate indicator of the general Vitamin D status of an individual. Vitamin D deficiency has been linked to many diseases including osteoporosis, rickets, osteomalacia, cancers, and cardiovascular diseases. Both dietary supplements of Vitamin D that are currently available in the market (Vitamin D2 and Vitamin D3) are converted to 25-OH Vitamin D in the liver. The sum of the concentrations of 25-OH Vitamin D2 and 25-OH Vitamin D3, in serum or plasma, is referred to as "Total 25-OH Vitamin D". Accurate monitoring of total 25-OH Vitamin D level is critical in clinical settings. Vitamin D deficient patients who are prescribed a daily Vitamin D supplement should regularly monitor their serum or plasma Vitamin D levels in order to reach an optimal level and prevent their 25-OH Vitamin D concentrations from reaching excessive levels that are considered toxic.[1-5]

3.0 PRINCIPLE

The kit is a solid phase enzyme-linked immunoassay (ELISA), based on the principal of competitive binding. Anti-Vitamin D antibody coated wells are incubated with Vitamin D standards, controls, samples, and Vitamin D-Biotin conjugate at room temperature for 90 minutes. During the incubation, a fixed amount of biotin-labeled vitamin D competes with the endogenous Vitamin D in the sample, standard, or quality control serum for a fixed number of binding sites on the anti Vitamin D antibody. Following a wash step, bound Vitamin D-Biotin is detected with Streptavidin-HRP (SA-HRP). SA-HRP conjugate immunologically bound to the well progressively decreases as the concentration of Vitamin D in the specimen increases. Unbound SA-HRP conjugate is then removed and the wells are washed. Next, a solution of TMB Reagent is added and incubated at room temperature for 30 minutes, resulting in the development of blue color. The color development is stopped with the addition of stop solution, and the absorbance is measured spectrophotometrically at 450 nm. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The color intensity will be inversely proportional to the amount of 25-OH Vitamin D in the sample. The assay measures both the 25-OH Vitamin D2 and D3. The total assay procedure run time is 2.5 hours.

4.0 REAGENTS

Materials Provided:

A. Vitamin D Calibrators – 0.5 ml/vial– [1 – 7]
Seven (7) vials of references for 25(OH) Vitamin D at approximate* concentrations of 0(**1**), 2.5(**2**), 5.0(**3**), 15(**4**), 35(**5**), 70(**6**) and 150(**7**) ng/ml. Store protected from light at 2-8°C.
*Exact levels are given on the labels on a lot specific basis.

Note: To convert to nmol/L, multiply results by 2.5.
Example 10ng/ml = 25 nmol/L

B. Vitamin D Controls – 0.5 ml/vial – [1 – 2]
Two (2) vials of controls for 25(OH) Vitamin D at approximate concentrations with levels given on the labels on a lot specific basis.

C. Biotinylated 25(OH)Vitamin D Reagent (51X) – 0.5 ml/vial
One (1) vial containing 25(OH) Vitamin D Biotin conjugate in 51X concentrated solution. See 'Reagent Preparation' section 8.0. Store protected from light at 2-8°C.

D. Assay Diluent – 24 ml/vial
See 'Reagent Preparation' section 8.0. Store protected from light at 2-8°C.

E. Streptavidin-HRP – 23 ml/vial
Store protected from light at 2-8°C.

F. Anti-Vitamin D Coated Plate – 96 wells
One 96-well microplate coated with anti-Vitamin D. Store protected from light at 2-8°C.

G. Wash Solution Concentrate (20X) – 25 ml/vial
See 'Reagent Preparation' section 8.0. Store at 2-8°C.

H. TMB Substrate – 24ml/vial
One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

I. Stop Solution – 12 ml/vial
One (1) vial containing a strong acid (1N HCl). Store at 2-8°C

J. Microplate Sealing film – 2 sheets

K. Product Instructions.

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. Kit and component stability are identified on the label.
Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:

1. Pipette(s) capable of delivering 0.050 & 0.100 ml (50 & 100 µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 & 0.300 ml (100 & 300 µl) volumes with a precision of better than 1.5% (optional).
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Reader with 450nm wavelength absorbance capability
5. Flat-head Vortex Mixer
6. Plate Shaker
7. Absorbent Paper for blotting the microplate wells.
8. Vacuum aspirator (optional) for wash steps.
9. Timer.
10. Storage container for storage of wash buffer.
11. Distilled or deionized water.
12. Quality Control Materials.

5.0 PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use in Humans or Animals

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface antigen, HIV 1&2 and HCV antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS.

Safe disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum, heparinized plasma, or EDTA plasma samples in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8.0 REAGENT PREPARATION

1. **Wash Buffer**
   Dilute contents of wash concentrate to 500 ml with distilled or deionized water (25ml, 20X to 475ml, water) in a suitable storage container. Store diluted buffer at room temperature.
2. **Working Biotin Solution**
   Immediately before use, prepare 1X working solution of 1:51 with assay diluents (e.g. add 0.1ml of the 51X Vitamin D-Biotin conjugate concentrate to 5ml of assay diluent). *Remaining 'Assay Diluent' must be stored at 2-8°C in dark and tightly capped.*

Note 1: Do not use the working substrate if it looks blue.
Note 2: Do not use reagents that are contaminated or have bacteria growth.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27°C).
*****Test procedure should be performed by a skilled individual or traned professional.*****
Once the procedure has started, all steps should be completed without interruption.

1. Format the microplates' wells for calibrator, control and patient specimen to be assayed in duplicate. **Store any unused microwell strips back into the container and store protected from light at 2-8°C.**
2. Pipette 0.010 ml (10 µl) of the appropriate calibrators, controls and samples into the assigned wells.
3. Add 0.200 ml (200 µl) of the Working Biotin Solution into each well.
4. Carefully mix the contents in the wells for 20 seconds using a plate shaker at 200-400 rpm (or equivalent motion). Remove from shaker and cover the plate with the adhesive plate seal making sure there is complete seal over each well.
5. Incubate sealed plate for ninety (90) minutes at room temperature (20-27°C).
6. Carefully remove the plate seal.
7. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
8. Add 0.300 ml (300 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is used, fill each well to the top by squeezing the container. Avoiding air bubbles. Decant the wash and repeat two (2) additional times.**
9. Add 0.200 ml (200µl) of Enzyme Conjugate (Streptavidin-HRP) to all wells.
10. Incubate at room temperature for thirty (30) minutes.
11. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
12. Add 0.300 ml (300 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is used, fill each well to the top by squeezing the container. Avoiding air bubbles. Decant the wash and repeat two (2) additional times.**
13. Add 0.200 ml (200 µl) of TMB substrate into each well (using a multi-channel pipette).
14. Incubate at room temperature for thirty (30) minutes.
15. Add 0.050 ml (50 µl) of stop solution to each well and mix gently for 20-30 seconds. **Always add reagents in the same order to minimize reaction time differences between wells.**
16. Read the absorbance in each well at 450nm in a microplate reader. **The results should be read within ten (10) minutes of adding the stop solution.**

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Vit D in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding Vit D concentration in ng/ml on linear graph paper (do not average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of Vit D for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

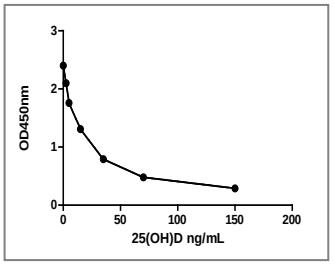
Note: Computer data reduction software designed for IEMA (ELISA) assays may also be used for the data reduction. If such softare is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample ID	Abs	Conc. (ng/ml)
Cal 1	2.40	0
Cal 2	2.10	2.5
Cal 3	1.76	5
Cal 4	1.31	15
Cal 5	0.79	35
Cal 6	0.48	70
Cal 7	0.29	150

*The data presented in Example 1 and Figure 1 are for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay.

FIGURE 1



11.0 QC PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrators 0 ng/ml should be ≥ 1.3.
2. Four out of six quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind's IFU may yield inaccurate results.
10. All applicable national standards, regulations and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.
12. Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

1. ***Measurements and interpretation of results must be performed by a skilled individual or trained professional.***
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system procedures have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays (Boscato LM, Stuart MC. "Heterophilic antibodies: a problem for all immunoassays" *Clin.Chem.* 1988:3427-33). For diagnostic purposes, the results from this assay should be used in combination with clinical examination, patient history and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, Monobind shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.

13.0 EXPECTED VALUES

Based on the published literature the following ranges have been assigned. **These ranges should be used as guidelines only:**

LEVEL	RANGE
Deficient	< 10 ng/ml
Insufficient	10 – 30 ng/ml
Sufficient	30 – 100 ng/ml
Intoxication	> 100 ng/ml

It is important to keep in mind that establishment of a range of values, which can be expected to be found by a given method for a population of "normal" persons, is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons, each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precision of the 25-OH Vitamin D AccuBind® ELISA Test System were determined by analyses on three different levels of pool control sera. The number, mean value, standard deviation and coefficient of variation for each of these control sera are presented in Table 2 and Table 3.

TABLE 2
Within Assay Precision

Serum	N	X	σ	%C.V.
1	16	8.1	0.315	3.87
2	16	25.3	1.608	6.36
3	16	35.9	1.661	4.62

TABLE 3
Between Assay Precision

Serum	N	X	σ	%C.V.
1	10	7.9	0.36	4.55
2	10	23.4	1.63	6.95
3	10	37.6	2.02	5.38

14.2 Sensitivity

The sensitivity of the 25-OH Vitamin D AccuBind® ELISA Test System is 0.67ng/ml. The sensitivity was determined by calculating the mean plus 2SD of the standard zero point tested 20 times in the same run.

15.0 REFERENCES

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2. Morris H. A. Vitamin D: A Hormone for All Seasons-How Much is enough? Clin. Biochem. Rev., 2005, 26, 21-32.
3. Bikle D. D. Vitamin D and the skin. J. Bone Miner. Metab., 2010, 28, 117-30.
4. Zerwekh J. E. Blood biomarkers of vitamin D status. Am. J. Clin. Nutr. 2008, 87, 1087S-91S.
5. Moyad M. A. Vitamin D: a rapid review. Dermatol Nurs., 2009, 21, 25-30.

Revision: 0 **Date: 2015-JAN-16** **DCO: N/A**

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