Monobind Inc.
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AccuLite
CLIA Microwells

25-OH Vitamin D Total (Vit D-Direct) Test System

Product Code: 9475-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of 25-OH Vitamin D Concentration in Human Serum by a Microplate Enzyme Immunoassay, Microplate, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

Vitamin D is a fat soluble secosteroid hormone that is important in the management of calcium and phosphorus concentrations required in the mineralization of bone. Vitamin D has two important forms: cholecalciferol (D_3) formed in the skin from ultraviolet light and ergocalciferol (D_2) found in dairy products. However, these forms do not have significant biological activity. The hormonal active form, 1, 25-dihydroxylcholecalciferol, is produced through transformations in the liver and kidney. The first step in this conversion is an enzymatic reaction of D_2 or D_3 into 25OH-D_2 or 25OH-D_3. These 25OH D forms are not freely circulating in blood, but are primarily bound to vitamin D binding protein (VDBP). The high binding affinity of the 25OH $D_{(2 \text{ or } 3)}$ compared to other derivatives of vitamin D leads to a long half-life in blood and its use as an accurate indicator of Vitamin D status. Vitamin D deficiency has been associated to diseases related to bone damage such as osteomalacia and rickets. Vitamin D can be dietarily supplemented through the use of Vitamin D_2 or vitamin D_3. The sum of the 25OH $D_{(2 \text{ and } 3)}$ in serum or plasma is referred to as total 25OH Vitamin D. The accurate measurement of total vitamin D is necessary in monitoring deficient vitamin D patients to achieve the optimum dosage and avoid excessive levels, which are considered toxic.

3.0 PRINCIPLE

Sequential Competitive Method (Type 6):
The essential reagents required for a solid phase sequential enzyme immunoassay include immobilized antibody, enzyme-antigen conjugate and native antigen. Upon mixing immobilized antibody, and a whole blood sample containing the native antigen, a binding reaction results between the native antigen for a limited number of insolubilized binding sites. The interaction is illustrated by the following equation:

$$Ag + Ab_{CW} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{CW}$$

Ab_{CW} = Monospecific Immobilized Antibody (Constant Quantity)
Ag = Native Antigen (Variable Quantity)
$AgAb_{CW}$ = Antigen-Antibody Complex
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation
$K = k_a / k_{-a}$ = Equilibrium Constant

After removing any unreacted native antigen by a wash step, the enzyme-conjugated antigen is introduced. The conjugate reacts with sites of the antibody unoccupied by the native antigen.

$$^{Enz}Ag + Ab_{CW} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} {}^{Enz}AgAb_{CW}$$

^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)
$^{Enz}AgAb_{CW}$ = Enzyme-antigen Conjugate-Antibody Complex

After a short second incubation, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is inversely proportional to the native antigen concentration. By utilizing several different calibrators of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

4.0 REAGENTS

Materials Provided:

A. Vit D Calibrators – 1ml/vial – Icons A-G
Seven (7) vials containing human serum albumin reference calibrators for 25-OH Vitamin D at approximate* concentrations of 0 (**A**), 5 (**B**), 10 (**C**), 25 (**D**), 46 (**E**), 85 (**F**), and 150 (**G**) in ng/ml. A preservative has been added. Store at 2-8°C.
* Exact levels are given on the labels on a lot specific basis
The calibrators can be expressed in molar concentrations (nM/L) by multiplying by 2.5. For example:
10ng/ml x 2.5 = 25nM/L

B. Vit D Controls – 1ml/vial – Icons M-N
Two (2) vials containing human serum reference controls at concentration established (exact value listed on label). A preservative has been added. Store at 2-8°C.

C. Vit D Releasing Agent – 12 ml/vial – Icon
One (1) vial containing vitamin D binding protein releasing agents. Store at 2-8°C.

D. Vit D Tracer Reagent – 12ml/vial – Icon
One (1) vial containing 25-OH Vitamin D_3 (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix. Store at 2-8°C.

E. Light Reaction Wells – 96 wells – Icon
One 96-well microplate coated with < 1.0 µg/ml anti-Vitamin D sheep IgG and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

F. Wash Solution Concentrate – 20 ml/vial – Icon
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.

G. Signal Reagent A – 7 ml/vial – Icon C^A
One (1) vial containing luminol in buffer. Store at 2-8°C (see Reagent Preparation section).

H. Signal Reagent B– 7 ml/vial – Icon C^B
One (1) vial containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C (see Reagent Preparation section).

I. Product Insert

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Avoid extended exposure to heat and light. **Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.**
Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided:
1. Pipette capable of delivering 0.025 & 0.100ml (50 & 100µl) with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.
3. Microplate washer or a squeeze bottle (optional).
4. Microplate Luminometer.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. 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 Publication No. (CDC) 88-8395.

Safe Disposal of kit components must be according to local regulatory and statutory requirements.

6.0 SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood, serum in type, and taken with the usual precautions in the collection of venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venipuncture tube(s) with no anti-coagulants. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum 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. When assayed in duplicate, 0.050ml (50µl) of the specimen is required.

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and high 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. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. 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 solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.
2. **Working Signal Reagent Solution –** Store at 2 - 8°C.
Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1ml of A and 1ml of B per two (2) eight well strips (A slight excess of solution is made). **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (20-27°C).
*****Test Procedure should be performed by a skilled individual or trained professional*****

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.025 ml (25 µL) of the appropriate extracted 25-OH Vitamin D calibrator, control or specimen into the assigned well.
3. Add 0.100 ml (100 µl) of the 25-OH Vitamin D Releasing Agent to all wells.
4. Mix **(Note 3)** the microplate for 20-30 seconds until homogeneous.
5. Cover and incubate for 30 minutes at room temperature
6. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
7. Add 0.350 ml (350 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 employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100 µl) of 25-OH Vitamin D Tracer Reagent to all wells.

DO NOT SHAKE THE PLATE AFTER ADDITION

9. Cover and incubate for 30 minutes at room temperature.
10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
11. Add 0.350 ml (350 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 employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
12. Add 0.100 ml (100 µl) of working signal reagent to all wells. **Always add reagents in the same order to minimize reaction time differences between wells.**

DO NOT SHAKE (MIX) THE PLATE AFTER SUBSTRATE ADDITION

13. Read the relative light units (RLUs) in each well for 0.2 – 1.0 seconds. **The results should be read within thirty (30) minutes of adding the signal reagent solution.**

Note1: **Do not use the working signal reagent solution if older than 36 hours.**
Note 2: **Do not use reagents that are contaminated or have bacteria growth.**
Note 3: **Cycle (start and stop) mixing (4 cycles) for 5-8 seconds/cycle is more efficient than one continuous (20-30 seconds) cycle to achieve homogeneity. A plate mixer can be used to perform the mixing cycles.**
Note 4: **It is extremely important to accurately dispense the correct volume with a calibrated pipette and by adding near the bottom of the microwells at an angle while touching the side of the well.**

10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of 25OH-vitamin D in unknown specimens.
1. Record the RLUs obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the RLUs for each duplicate calibrator versus the corresponding Vitamin D concentration in ng/ml on linear graph paper (do not average the duplicates of the calibrators before plotting).
3. Connect the points with a best-fit curve.
4. To determine the concentration of Vitamin D for an unknown, locate the average RLU 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). In the following example, the average RLU (39853) intersects the dose response curve at 20.3ng/ml Vitamin D concentration (See Figure 1).

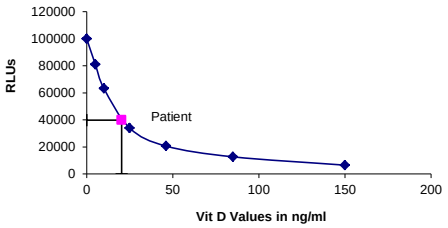
Note: Computer data reduction software designed for CLIA assay may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

Sample I.D.	Well Number	RLUs (A)	Mean RLUs (B)	Value (ng/ml)
Cal A	A1	99015	100000	0
	B1	100985		
Cal B	C1	81052	81109	5
	D1	81166		
Cal C	E1	65035	63266	10
	F1	61538		
Cal D	G1	33130	33944	25
	H1	34759		
Cal E	A2	19529	20708	46
	B2	21887		
Cal F	C2	12959	12715	85
	D2	12472		
Cal G	G2	6783	6567	150
	H2	6352		
Pat# 1	A3	38221	39853	20.3
	A4	41486		

* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLUs of the calibrators have been normalized to 100,000 RLUs for the F calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.

Figure 1



Note: Multiply the horizontal values by 2.5 to convert into nM/ml.

11.0 Q.C. PARAMETERS

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

1. The dose response curve should be within established parameters.
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 signal reagent solution initiates a kinetic reaction; therefore, the solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed, is essential. Any deviation from Monobind's IFU may yield inaccurate results.
9. 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.
10. 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.
11. 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 procedure 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 RANGES OF VALUES

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

TABLE 1
Expected Values for the Vit D-Direct CLIA

LEVEL	RANGE (ng/ml)
Very severe vitamin D deficiency	< 5
Severe vitamin D deficiency	5-10
Vitamin D deficiency	10-20
Suboptimal vitamin D provision	20-30
Optimal vitamin D level	30-50
Upper norm	50-70
Overdose, but not toxic	70-150
Vitamin D intoxication	> 150

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 AccuLite® CLIA 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	20	23.74	1.90	8.02
2	20	36.50	3.32	9.08
3	20	87.61	6.07	6.92

TABLE 3
Between Assay Precision

Serum	N	X	σ	%C.V.
1	33	24.28	2.88	11.87
2	33	38.58	5.14	13.31
3	33	90.38	7.68	8.5

14.2 Sensitivity

The sensitivity of the Vit-D Direct AccuLite® CLIA test system method was ascertained by determining the variability of the '0' calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose. The test system has an analytical sensitivity of 0.517 ng/ml of Vitamin D concentrations.

14.3 Specificity

The % cross-reactivity of the 25-OH Vitamin D antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of 25-OH Vitamin D needed to displace the same amount of labeled analog.

TABLE 5

Substance	Cross Reactivity
25-OH Vitamin D2	1.0000
25-OH Vitamin D3	1.0000
Vitamin D2	0.0076
Vitamin D3	0.0039
D2 Active 1,3,25-Hydroxy Vitamin D 2	1.9000
D3 Active 1,3,25-Hydroxy Vitamin D 3	1.1500

15.0 REFERENCES

1. Holick, MF. "Vitamin D Status: Measurement, Interpretation and Clinical Application". *Ann Epidemoil*. 2009, 19(2):73 - 78
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.

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MP9475 **Product Code: 9475-300**

Size		96(A)	192(B)	480(D)	960(E)
Reagent (fill)	A)	1ml set	1ml set	2ml set	2(2ml set)
	B)	1ml set	1ml set	2ml set	2(2ml set)
	C)	1 (12ml)	2 (12ml)	1 (60ml)	2 (60ml)
	D)	1 (12ml)	2 (12ml)	1 (60ml)	2 (60ml)
	E)	1 plate	2 plate	5 plate	10 plate
	F)	1 (20ml)	1 (20ml)	1 (60ml)	2 (60ml)
	G)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)
	H)	1 (7ml)	2 (7ml)	1 (30ml)	2 (30ml)

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Glossary of Symbols
(EN 980/ISO 15223)