25-OH VITAMIN D TOTAL ENZYME IMMUNOASSAY TEST KIT
Catalog Number: OKBA00028

Enzyme Immunoassay for the Quantitative Determination of 25-OH Vitamin D in Human Serum

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

PRINCIPLE OF THE ASSAY

Sequential Competitive Method:

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 serum 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:

$$Ag + Abcw \underset{K\text{-}a}{\overset{Ka}{\rightleftharpoons}} AgAbcw$$

Abcw = Monospecific Immobilized Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

AgAbcw = Antigen-Antibody Complex

Ka = Rate Constant of Association

K-a = Rate Constant of Disassociation

K = Ka / 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.

$$^{Enz}Ag + Abcw \underset{K\text{-}a}{\overset{Ka}{\rightleftharpoons}} {}^{Enz}AgAbcw$$

EnzAg = Enzyme-antigen Conjugate (Constant Quantity)

EnzAgAbcw = 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.

MATERIALS PROVIDED

1. **Vit D Calibrators - 1ml/vial**

   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 2-8oC.

   * Exact levels are given on the labels on a lot specific basis

   The calibrators can be expressed in molar concentrations (nM/L) by multiplying 2.5. For example: 10 ng/ml x 2.5 = 25 nM/L

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

3. **Vit D Assay Buffer – 12 ml/vial**
   One (1) vial containing Vitamin D binding protein releasing agents. Store at 2-8°C

4. **Vit D Conjugate Reagent – 12 ml/vial**
   One (1) vial containing 25-OH Vitamin D3 (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix. Store at 2-8°C

5. **Vit D Antibody Coated Plate – 96 wells**
   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

6. **Wash Buffer Concentrate (50X)– 20 ml/vial**
   One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C

7. **TMB Reagent – 12 ml/vial**
   One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C

8. **Stop Solution – 8 ml/vial**
   One (1) vial containing a strong acid (H_2SO_4). Store at 2-8°C

9. **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 60 days when stored at 2-8ºC. Kit and component stability are identified on label.
Note 3: Above reagents are for a single 96-well microplate.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Pipette capable of delivering 25 & 100 µ L with a precision of better than 1.5%
2. Dispenser(s) for repetitive deliveries of 100 & 350 µ L volumes with a precision of better than 1.5%

3. A microtiter plate calibrated reader (450 ± 10 nm) and (620 ± 10 nm)
4. Absorbent paper
5. Distilled or deionized water
6. Timer

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 redstop (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 2-8ºC for a maximum period of 5 days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at -20ºC for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing the thawing. When assayed in duplicate, 50 µ L of the specimen is required.

REAGENT PREPARATION

Wash Buffer

Dilute contents of wash solution to 1000 ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30ºC for up to 60 days.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (18-25ºC).

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 aluminium bag, seal and store at 2-8ºC.*

2. Pipette 25 µ L of the appropriate extracted 25-OH Vitamin D calibrator, control or specimen into the assigned well.

3. Add 100 µ L of the Vitamin D Assay Buffer to all wells.

4. Mix (Note 3) the microplate for 20-30 seconds until homogenous.

5. Cover and incubator 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 350 µ L 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 manufacture's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubble) to dispense the wash. Decant the wash and repeat two (2) additional times.*

8. Add 100 µ L Vitamin D Conjugate 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 350 µ L 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 manufacture's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubble) to dispense the wash. Decant the wash and repeat two (2) additional times.*

12. Add 100 µ L of TMB 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. Incubate at room temperature for 20 minutes.

14. Add 50 µ L of stop solution to each well and gently mix for 15-20 seconds. *Always add reagents in the same order to minimize reaction time differences between wells.*

15. Read the absorbance in each well at 450nm. The result should be read within 15 minutes of adding the stop solution.

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.

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.

CALCULATION OF RESULTS

1. Calculate the mean absorbance value (OD_{450}) for each set of reference standards, controls and samples.

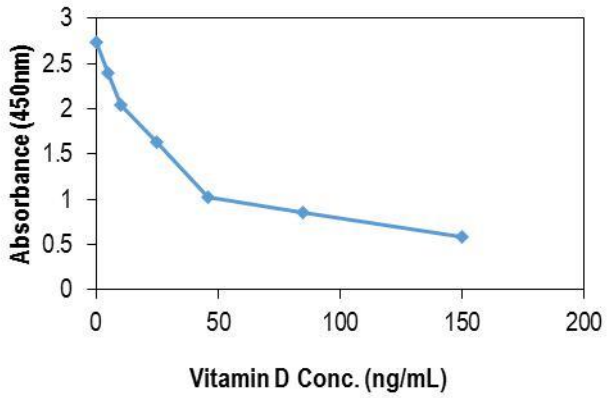
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on ***linear-linear graph paper***, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of 25-OH Vitamin D ng/ml from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

4. Any values obtained for diluted samples must be further converted by applying the appropriate dilution factor in the calculations.

EXAMPLE OF TYPICAL STANDARD CURVE

Results of a typical standard run with absorbency readings at 450nm shown on the Y axis against 25-OH Vitamin D concentrations shown on the X axis. **NOTE:** This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each laboratory must provide its own data and standard curve in each experiment.

Vitamin D (ng/mL)	Absorbance (450 nm)
0	2.744
5	2.391
10	2.038
25	1.627
46	1.027
85	0.855
150	0.583



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