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User's Manual

Vitamin D3 ELISA Kit

REF **DEIA4442A**

Σ **96T**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Vitamin D3 ELISA Kit is for the quantitative determination of Vitamin-D3 in dairy samples.

General Description

Vitamin D is a group of fat-soluble secosteroids, the two major physiologically relevant forms of which are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D without a sub-script refers to either D2 or D3 or both. Vitamin D3 is produced in the skin of vertebrates after exposure to ultraviolet B light from the sun or artificial sources, and occurs naturally in fish and a few other foods. In some countries, staple foods such as milk, flour and margarine are artificially fortified with vitamin D, and it is also available as a supplement in pill form. Light-exposed mushrooms may provide up to 100% of the recommended Daily Value of vitamin D.

Principles of Testing

The Vitamin D3 ELISA Kit is based on the principle of a competitive enzyme immunoassay. The assay system utilizes a fixed number of Vitamin D3 molecules immobilized on a solid phase. These molecules compete with an unknown number of Vitamin D3 molecules extracted from milk samples for a fixed number of binding sites on enzyme-labelled monoclonal antibodies directed against the Vitamin D3. As the number of Vitamin D3 molecules in the sample increases, the number of bound labelled antibody molecules to solid phase antigen decreases due to competition. The amount of enzyme-labelled antibodies bound to the solid phase Vitamin D3 is inversely proportional to the concentration of Vitamin D3 present in the sample.

Reagents And Materials Provided

1. Vitamin-D3 coated wells: 96 wells with Vitamin-D3 immobilized in the well, in a foil pouch with a dessicant.
2. Anti-Vitamin D3 conjugate with HRP: one vial containing 0.1 mL of concentrated Anti-Vitamin-D3 conjugate with HRP, in a stabilizer solution.
3. Vitamin-D3 Standard: Standards prepared with hexane: 0, 0.125, 0.25, 0.50, 0.75 IU/mL. Content is 0.5 mL per vial.
4. CONTROL 1: Control 0.6 IU/mL, 0.5 mL per vial.
5. CONTROL 2: Control 0.2 IU/mL, 0.5 mL per vial.
6. Reaction Buffer: one vial containing 7 mL of peptide based buffer with thimerosal as preservative.
7. Enzyme substrate: one vial containing 7 mL of TMB solution.
8. Conjugate Diluent: one vial containing 7 mL of carbohydrate based buffer with thimerosal as preservative.
9. H_2SO_4 Stopping solution: one vial containing 7 mL of 0.2 M sulfuric acid.

Materials Required But Not Supplied

1. Precision pipettes with disposable tips.

2. 8 channels pipette (100-200 µL) with disposable tips.
3. Plate shaker set at 180 ± 10 rpm.
4. Microplate reader with filter at 450 nm.
5. Microplate washer.
6. Deionized or distilled water.
7. Absorbent paper
8. Potassium hydroxide (KOH) pellets.
9. Hexane.
10. 10 mL screw capped glass tubes.
11. 1 or 2 mL screw capped amber glass vials.
12. Centrifuge.

Storage

Storage: 2 - 8°C.

Stability: refer to expiration date on reagent labels.

Specimen Collection And Preparation

Extraction Procedure (Fluid Milk Only)

Bring fluid milk container to room temperature. Rotate slowly at least 10 times without foaming. Extractions are slightly different based on the percentage of milk fat as described below and summarized in table below.

Table 1.

	Step	3.25% M.F.	2% M.F.	1% M.F.	Skim milk	Condition
Saponification and extraction	Fluid Milk	1 mL	1 mL	1 mL	1 mL	Warm milk to room temperature.
	KOH (g)	0.55	0.55	0.55	0.3	Gently mix for 2 minutes in the dark.
	Incubate for 4 minute, and shake vigorously for 2 minute in the dark. Repeat 4 minute incubation and 2 minute vigorous shaking 2 more times (totals 12 minutes incubation and 6 min. shaking)					
	Hexane	2 mL	2 mL	2 mL	2 mL	Shake vigorously for 2 minutes in the dark and centrifuge at 3500 rcf for 10 minutes.
	Ethanol 95%	-	-	-	20 µL	if needed to separate the layers. Wait for 5 minute (only for skim milk).
Transfer extraction	Upper organic phase	200 µL	200 µL	200 µL	200 µL	The Vitamin-D extract in screw capped amber coloured glass vial should be assayed immediately.

A. Milk with 3.25 %M.F., 2%M.F., and 1%M.F.

1. Label 10 mL screw capped glass tubes and pipette 1 mL of milk in corresponding tube. Add 0.55 g of KOH into each tube. Do not cap the tubes. Mix gently for 2 minutes in the dark.

2. Cap the tubes and incubate the milk samples at room temperature for 4 minutes in the dark. Shake vigorously for 2 minutes. Repeat 4 minute incubation and 2 minute vigorous shaking 2 more times (totals 12 minute incubation and 6 minute shaking).
3. Pipette 2 mL of hexane into above solutions. Cap and shake vigorously for another 2 minutes in the dark.
4. Centrifuge each tube at room temperature for 10 minutes at 3500 RCF using swing bucket rotor.
5. Label 1 or 2 mL screw capped amber glass viais. After centrifugation, handle tubes carefuly. The upper organic phase must be perfectly clear and well separated. Transfer 200 µL of vitamin-D3 extract in corresponding amber coloured glass vials. The amber coloured glass vials, which contain the Vitamin D3 extract, must be capped very well and should be assayed immediately.

B. Skim Milk

1. Label 10 mL screw capped glass tubes and pipette 1 mL of skim milk in corresponding tube. Add 0.3 g of KOH into each tube and gently mix for 2 minutes in the dark.
2. Cap and Incubate at room temperature for 4 minutes in the dark. Shake vigorously for 2 minute. Repeat 4 minute incubation and 2 minute vigorous shaking 2 more times (totals 12 minute incubation and 6 minute shaking).
3. Pipette 2 mL of hexane into above solutions. Cap and shake vigorously for another 2 minutes in the dark.
4. Centrifuge each tube at room temperature for 5 minutes at 2500 rpm. Add 20 µL of ethanol if needed to separate the upper hexane and lower aqueous layers, and wait for 5 minute. Label 1 or 2 mL screw capped amber coloured glass viais. The upper organic phase must be perfectly clear and well separated. Transfer 200 µL of vitamin-D extract in corresponding amber coloured glass vials. The amber coloured glass vials, which contain the Vitamin D3 extract, must be capped very well and should be assayed immediately.

Reagent Preparation

1. All reagents should be brought to room temperature before use (22 ± 2°C), except enzyme conjugate concentrate that should be at 2 - 8°C.
2. Enzyme conjugate concentrate should be diluted as indicated on the bottle label with conjugate diluent according to the number of wells used. Mix the enzyme conjugate concentrate by pipetting 2-3 times with a pipette tip before diluting with diluent. Add required amount of enzyme conjugate concentrate to the conjugate diluent and mix thoroughly before use.

Diluted Handling Notes:

1. Do not mix materials from different kit lots.
2. Bring all reagents except Anti-Vitamin D3 conjugate with HRP to room temperature before using.
3. Use a clean disposable pipette tip for addition of each different sample and reagent to avoid cross-contamination.
4. Only use glass vials for the extraction of vitamins.
5. Prepare a standard curve for each run. Do not use data from previous runs.
6. Cap all Vitamin-D3 calibrators and vitamin-D3 extracted specimens immediately after loading onto ELISA plate. This will allow the reference calibrators and extracts to be used more than once if desired.
7. Load all extracted specimens and reference calibrators within 5 minutes and accurately onto the ELISA strips to limit variations in evaporation time between the first and last well loaded.
8. Work all hexane steps under the hood.

Assay Procedure

Refer to the table below.

Note: Read the absorbances immediately after completing the assay.

Wells	Identification	Assay Volumer	Evaporate	Assay Buffer		Dil. Conjugate			Substrate		Stop. Sol.	
A_1,A_2	0 IU/mL	10 µL		60 µL	INCUBATE	60 µL	INCUBATE	WASH	60 µL	INCUBATE	60 µL	READ AT 450 nm
B_1,B_2	0.125											
C_1,C_2	0.25											
D_1,D_2	0.5											
E_1,E_2	0.75											
F_1,F_2	Control 1											
G_1,G_2	Control 2											
H_1,H_2	Sample extract											

Standards, specimens and controls shoud be assayed in duplicate. Secure the desired number of coated wells Vitamin-D3 coated wells in the holder.

1. Pipette 10 µL of calibrators Vitamin-D3 Standard 1-5, extracted specimens, and controls CONTROL 1 , CONTROL 2 into the corresponding wells.
2. Shake the wells 8 minutes on a plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C) to evaporate hexane.
3. Pipette 60 µL of Assay Buffer into each well. Mix gently for 30 seconds. Place opaque lid over the strips.
4. Incubate for 5 minutes in the dark on the plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C).
5. Pipette 60 µL of freshly diluted Anti-Vitamin-D conjugate-HRP in each well. Mix gently for 20 seconds. Place opaque lid over the strips.
6. Incubate for 10 minutes in the dark on the plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C).
7. Wash six times with distilled water using Microplate washer. Manual washing may also be used with wash bottle or using multi-channel pipette add 380 µl of distilled water in each well in each wash cycle. Care should be taken to avoid spillage of distilled water into adjacent wells. After the wash, decant completely the water by tapping the plate against absorbing paper until no trace of water is visible on the paper.
8. Pipette 60 µL of TMB (Substrate) into each well. Gently mix for 10 seconds.
9. Incubate for 5 minutes in the dark at room temperature (22 ± 2°C).
10. Add 60 µL of the stopping solution H_2SO_4. Gently mix for 10 seconds.
11. Measure the absorbance at 450 nm using a microplate reader.

Quality Control

Good laboratory practice requires that quality control specimens be run with each calibration curve to check the assay performance.

Calculation

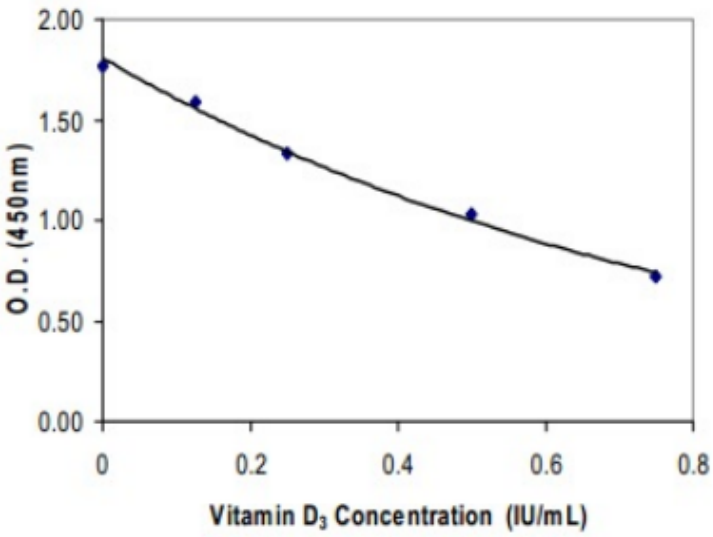
The standard curve is used to determine the amount of Vitamin D3 in unknown sample. The standard curve is generated by

plotting the average of O.D. (450 nm) obtained for each of the standard concentrations on the vertical (y) axis versus the

corresponding standard concentrations on the horizontal (X) axis.

Examine data for acceptance criteria with quality control guidelines.

40 I.U. of Vitamin D3 = 1 µg



Precision

The relative standard deviation for interassay and intrassay was determined to be 8% and 4% respectively.

Sensitivity

The range for this assay under the specified conditions is from 0.125 I.U./mL to 0.75 I.U./mL.

Specificity

The kit did not exihibit any cross reactivity with cholesterol and vitamin A.

Linearity

Linearity was determined to be 0.98 (Average of six independent assays) with %RSD of 1.3%

Precautions

1. Respect laboratory quality controls rules.
2. Reagents are matched in each kit and therefore, reagents from different lot numbers should not be mixed.
3. This kit should not be used after the expiration date.
4. Optimal results will be obtained by strict adherence to this protocol.
5. The stopping solution contains 0.2M sulfuric acid. This solution should be handled with caution, avoiding skin contact.
6. KOH pellets should be handled with caution, avoiding skin contact.
7. Prior to assay, bring all reagents except Anti-Vitamin D3 conjugate with HRP to ambient temperature by allowing them to stand at room temperature (22 ± 2°C). Gently mix all reagents.

Limitations

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with strict adherence to the procedure described within this package insert and good laboratory practice.
2. A maximal total pipetting time of 5 minutes for calibrators, controls and specimens is suggested.
3. Improper handling, and washing might result in O.D. of 0.0 vitamin D3 standard lower than the 0.125 I.U./mL vitamin D3 standard.