



VitaKit D™

050420

FOR FLUID MILK SAMPLES

Cat. No.: KTSP-72051 96 tests

Enzyme immunoassay kit for the quantitative determination of Vitamin-D₃ in dairy samples.

For in vitro quantification use only.

I. PROPRIETARY NAME

The VitaKit D™ from SciMed, Cat. No. KTSP-72051 contains sufficient material to assay at least 96 tests.

II. APPLICATION AND INTENDED USE

Dairy milk is fortified with vitamins A & D₃, as milk has become the major source of these vitamins for human beings. Regulatory agencies have set standards specifying the amount of vitamins A and D₃ to be added to milk products. The methodology has been designed to extract the vitamins from milk fat, and to directly quantify the amount of vitamins in an ELISA based assay. Other methods that can detect vitamins in dairy milk are time consuming and require specialised laboratory equipment and trained personnel. The VitaKit D™ provides materials for the quantitative measurement of vitamin-D₃ in dairy products. This assay is intended for in vitro quantification only.

III. PRINCIPLES OF THE METHOD

The Vitamin D3 ELISA test 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.

IV. REAGENTS SUPPLIED WITH KIT

Storage : 2 - 8°C
Stability : refer to expiration date on reagent labels

- SORB** Vitamin-D₃ coated wells : **REF CW-72051**: 96 wells with Vitamin-D₃ immobilized in the well, in a foil pouch with a desiccant.
- CONJ ENZ** Anti-Vitamin-D₃ conjugate with HRP **REF EC-72051**: one (1) vial containing 0.2 mL of concentrated Anti-Vitamin-D₃ conjugate with HRP, in a stabilizer solution.
- CAL 1-5** Vitamin-D₃ Standard **REF WSC-72051**: Standards prepared with hexane: 0, 0.125, 0.25, 0.50, 0.75 IU/mL . Content is 0.5 mL per vial.
- CONTROL 1** Control 0.6 IU/mL **REF QC-72051**: 0.5 mL per vial.
- CONTROL 2** Control 0.2 IU/mL **REF QC-72052**: 0.5 mL per vial.
- BUF** Reaction Buffer **REF RB:72051** one (1) vial containing 7 mL of peptide based buffer with thimerosal as preservative.
- SUBS TMB** Enzyme substrate **REF ES-71051**: one (1) vial containing 11 mL of TMB solution.
- CONJ DIL** Conjugate Diluent **REF CD-72051**: one (1) vial containing 7 mL of carbohydrate based buffer with thimerosal as preservative.
- H₂SO₄** Stopping solution **REF SS-71051**: one (1) vial containing 12 mL of 0.2 M sulfuric acid.

V. EQUIPMENT & MATERIAL REQUIRED BUT NOT PROVIDED

- ✓ Precision pipettes with disposable tips
- ✓ 8 channels pipette (100-200 µL) with disposable tips
- ✓ Plate shaker set at 180 ± 10 rpm
- ✓ Microplate reader with filter at 450 nm
- ✓ Microplate washer
- ✓ Deionized or distilled water
- ✓ Absorbent paper
- ✓ 95% ethanol
- ✓ Potassium hydroxide (KOH) pellets
- ✓ Hexane
- ✓ 10 mL screw capped glass tubes
- ✓ 1 or 2 mL screw capped amber coloured glass vials
- ✓ Centrifuge

VI. REAGENT PREPARATION

- All reagents should be brought to room temperature before use (22 ± 2°C), except enzyme conjugate concentrate **CONJ ENZ** (EC-72051) that should be at 2 - 8°C.
- Enzyme conjugate concentrate **CONJ ENZ** (EC-72051) should be diluted as indicated on the bottle (label) with conjugate diluent **CONJ DIL** (CD-72051) according to the number of wells used. Diluted conjugate cannot be stored and should be prepared fresh in each run.

Handling notes:

Do not mix materials from different kit lots. Bring all reagents to room temperature before using. Use a clean disposable pipette tip for addition of each different sample and reagent to avoid cross-contamination. Only use glass vials for the extraction of vitamins. Prepare a standard curve for each run. Do not use data from previous runs.

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.

Load all extracted specimens and reference calibrators quickly and accurately onto the ELISA strips to limit variations in evaporation time between the first and last well loaded.

Work all hexane steps under the hood.

VII. 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

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

- 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 and mix gently for 2 minutes in the dark.
- Incubate 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).
- Pipette 2 mL of hexane into above solutions. Cap and shake vigorously for another 2 minutes in the dark.
- Centrifuge each tube at room temperature for 5 minutes at 2500 rpm.
- Label 1 or 2 mL screw capped amber coloured glass vials. After centrifugation, handle tubes carefully. 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

- 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.
- 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).
- Pipette 2 mL of hexane into above solutions. Cap and shake vigorously for another 2 minutes in the dark.
- 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 vials. 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.

Table I

	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 2500 rpm for 5 minutes.
Transfer extraction	Ethanol 95%	-	-	-	20 µL	if needed to separate the layers. Wait for 5 minute (only for skim milk).
	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.

VIII. ASSAY PROCEDURE

Refer to the assay procedure, Table II. Standards, specimens and controls should be assayed in duplicate. Secure the desired number of coated wells **SORB** in the holder.

- Pipette 10 µL of calibrators **CAL 1-5**, extracted specimens, and controls **CONTROL 1**, **CONTROL 2** into the corresponding wells.
- Shake the wells 8 minutes on a plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C) to evaporate hexane
- Pipette 60 µL of Assay Buffer **BUF** into each well. Mix gently for 30 seconds. Place opaque lid or adhesive cover over the strips.
- Incubate for 3 minutes in the dark on the plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C).
- Pipette 60 µL of diluted Anti-Vitamin-D conjugate-HRP **CONJ ENZ** diluted with **CONJ DIL** in each well. Mix gently for 20 seconds. Place opaque lid or adhesive cover over the strips.
- Incubate for 20 minutes in the dark on the plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C).
- 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.
- Pipette 60 µL of TMB **SUBS TMB** (Substrate) into each well. Gently mix for 10 seconds.
- Incubate for upto 5 minutes in the dark at room temperature (22 ± 2°C).
- Add 60 µL of the stopping solution **H₂SO₄**. Gently mix for 10 seconds.
- Measure the absorbance at 450 nm using a microplate reader.

NOTE: READ THE ABSORBANCES IMMEDIATELY AFTER COMPLETING THE ASSAY.

TABLE II

Wells	Identification	Assay Volumer	Evaporate	Assay Buffer	Dil. Conjugate	Substrate	Stop. Sol.
A ₁ ,A ₂	0 IU/mL	10 µL	1	60 µL	INCUBATE	WASH	60 µL
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	Sample extract						
G ₁ ,G ₂	Sample extract						
H ₁ ,H ₂	etc...						

- 8 minutes on a plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C)
- Incubate 3 minutes in the dark on a plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C)
- Incubate 20 minutes in the dark at (180 ± 10 rpm) at room temperature (22 ± 2°C)
- Wash 6 times with distilled or deionized water
- Incubate upto 5 minutes in the dark at room temperature (22 ± 2°C)

IX. CALCULATIONS

DO NOT ATTEMPT TO SUBSTITUTE ANY PART OF THESE SAMPLE DATA FOR YOUR OWN.

Examine data for acceptance consistent with quality control guidelines. Aberrant values should be rejected.

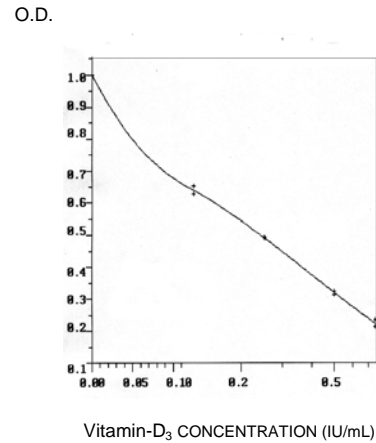
Refer to the sample data and calculations, Table II and graphic.

- For each standard, control and unknown sample, the optical density values are averaged (from duplicate).
- On millimeter paper using the ordinate for the optical density and the abscissa for the standard concentrations (IU/mL), a smooth standard curve is plotted.
- The values of the controls and of unknown samples are read directly from the standard curve. For unknown samples, multiply the values by a factor of 2 as per the stipulated assay conditions.
- 40 I.U. of Vitamin D₃ = 1 µg

TABLE III

WELLS	AVERAGE OPTICAL DENSITY at 450 nm	CONCENTRATION (IU/mL)
0 IU/mL	2.225	--
0.125 IU/mL	1.425	--
0.25 IU/mL	1.094	--
0.50 IU/mL	0.870	--
0.75 IU/mL	0.497	--
Control	0.659	0.59
Control	1.190	0.20
Sample extract	1.409	0.13
etc...

EXAMPLE OF Vitamin-D₃ STANDARD CURVE



X. NORMAL RANGE

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

XI. SPECIFIC PERFORMANCE CHARACTERISTICS

- Sensitivity:** Sensitivity is defined as the minimum concentration of Vitamin-D₃ which can be statistically distinguished from standard 0. This value is 0.05 IU/mL.
- Precision & reproducibility:**
 - Intra-assay variation:** the precision of the assays was verified by assaying twelve (12) replicates of three (3) different extracts. The results were :

Parameters	Samples		
	1	2	3
Number of determinations (N)	12	12	12
Mean (IU/mL)	1.337	0.437	0.284
Standard deviation	0.256	0.038	0.033
Coefficient of variation (%)	19	8	11

- Inter-assay variation:** reproducibility of the protocol was established by assaying three different extracts in 12 replicates in successive runs. The results were :

Parameters	Samples		
	1	2	3
Number of determinations (N)	12	12	12
Mean (IU/mL)	1.350	0.562	0.334
Standard deviation	0.172	0.092	0.053
Coefficient of variation (%)	12	16	15

- Linearity:** or dilution study; two (2) extracts were diluted and run in the VitaKit D™ kit. The results are as follows :

Samples	Dilution factor	Theoretical value (IU/mL)	Experimental value (IU/mL)
1	undiluted	0.927	0.927
	1:2	0.464	0.447
	1:4	0.232	0.209
	1:8	0.116	0.087
2	Undiluted	0.539	0.539
	1:2	0.269	0.219
	1:4	0.135	0.159
	1:8	0.067	0.044

4. Limitations of the procedure:

- 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.
- A maximal total pipetting time of 5 minutes for calibrators, controls and specimens is suggested.
- Improper handling, and washing might result result in O.D. of 0.0 vitamin D₃ standard lower than the 0.125 I.U./mL vitamin D₃ standard.

XII. QUALITY CONTROL

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

XIII. SAFETY MEASURES

- All materials in this kit may be used only for *in vitro* quantification not involving internal or external administration of the material to humans or animals.
- Respect laboratory quality controls rules.
- Reagents are matched in each kit and therefore, reagents from different lot numbers should not be mixed.
- This kit should not be used after the expiration date.
- Optimal results will be obtained by strict adherence to this protocol.
- The stopping solution contains sulfuric acid. This solution should be handled with caution, avoiding skin contact. Prior to assay, warm all reagents to ambient temperature by allowing them to stand at room temperature (22 ± 2°C). Gently mix all reagents.

XIV. MANUFACTURER & CUSTOMER SERVICE

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XV. REVISION DATE: 2005 Aug

XVI. LIST OF REAGENTS SUPPLIED WITH KIT

Cat #	Description	KTSP-72051
CW-72051	Vitamin-D ₃ coated wells	96 wells
EC-72051	Anti-Vitamin-D ₃ conjugate-HRP	1
WSC-72051	CAL 1-5	1
QC-72051	Control 1	1
QC-72052	Control 2	1
CD-72051	Conjugate Diluent	1
RB-72051	Reaction Buffer	1
ES-71051	Enzyme Substrate (TMB)	1
SS-71051	Stopping solution	1
	English Protocol	1

XVII. SYMBOLS ON REAGENTS LABEL



SciMed Laboratories Inc. does not offer a warranty of any kind, neither express nor implied, other than that this product meets all the quality control standards set by the SciMed Laboratories and will, at its discretion, replace any product which proves to be defective in terms of workmanship or material. SciMed Laboratories is not liable in any way for loss of profits or any damage, direct or otherwise, to persons or property, arising from the use of this product or these procedures.

QC Manager : _____