# VitaKit D™

091130

# FOR FLUID MILK SAMPLES

**Cat. No.: KTSP-72051 96 tests**

**Enzyme immunoassay kit for the quantitative determination of Vitamin-$D_3$ 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 96 tests.

## II. APPLICATION AND INTENDED USE

Dairy milk is fortified with vitamins A & $D_3$, 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 $D_3$ 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-$D_3$ 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 $D_3$ molecules immobilized on a solid phase. These molecules compete with an unknown number of Vitamin $D_3$ molecules extracted from milk samples for a fixed number of binding sites on enzyme-labelled monoclonal antibodies directed against the Vitamin $D_3$. As the number of Vitamin $D_3$ 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 $D_3$ 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

1. **SORB Vitamin-$D_3$ coated wells : REF CW-72051:** 96 wells with Vitamin-$D_3$ immobilized in the well, in a foil pouch with a dessicant.
2. **CONJ ENZ Anti-Vitamin $D_3$ conjugate with HRP REF EC-72051:** one (1) vial containing 0.1 mL of concentrated Anti-Vitamin-$D_3$ conjugate with HRP, in a stabilizer solution.
3. **CAL 1-5 Vitamin-$D_3$ 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.
4. **CONTROL 1 Control 0.6 IU/mL REF QC-72051:** 0.5 mL per vial.
5. **CONTROL 2 Control 0.2 IU/mL REF QC-72052:** 0.5 mLper vial.
6. **BUF Reaction Buffer REF RB:72051** one (1) vial containing 7 mL of peptide based buffer with thimerosal as preservative.
7. **SUBS TMB Enzyme substrate REF ES-71051:** one (1) vial containing 7 mL of TMB solution.
8. **CONJ DIL Conjugate Diluent REF CD-72051:** one (1) vial containing 7 mL of carbohydrate based buffer with thimerosal as preservative.
9. **$H_2SO_4$ Stopping solution REF SS-71051:** one (1) vial containing 7 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
- ✓ Potassium hydroxide (KOH) pellets
- ✓ Hexane
- ✓ 10 mL screw capped glass tubes
- ✓ 1 or 2 mL screw capped amber glass vials
- ✓ Centrifuge

## VI. REAGENT PREPARATION

1. 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.
2. 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 except Anti-Vitamin $D_3$ CONJ ENZ 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-$D_3$ calibrators and vitamin-$D_3$ 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 within 5 minutes 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 I.

### 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-$D_3$ extract in corresponding amber coloured glass vials. The amber coloured glass vials, which contain the Vitamin $D_3$ 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 $D_3$ 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 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. |

## VIII. ASSAY PROCEDURE

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

1. Pipette 10 µL of calibrators **CAL 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 **BUF** 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 (**CONJ ENZ** diluted with **CONJ DIL**) 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 **SUBS TMB** (Substrate) into each well. Gently mix for 10 seconds.
9. Incubate for upto 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.

**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 | | 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 | | | | | | | | | | | |

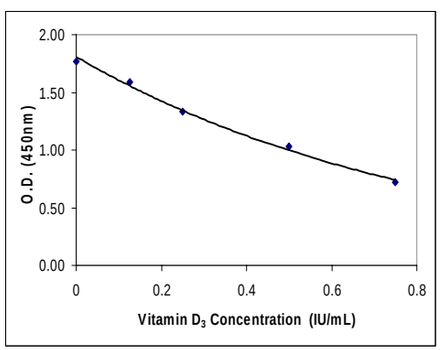
## IX. CALCULATIONS

The standard curve is used to determine the amount of Vitamin $D_3$ 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 $D_3$ = 1 µg

EXAMPLE OF Vitamin-$D_3$ STANDARD CURVE




## X. PERFORMANCE CHARACTERISTICS

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

**Precision & reproducibility:** The relative standard deviation for interassay and intrassay was determined to be 8% and 4% respectively.

**Cross Reactivity and Specificity:** The kit did not exihibit any cross reactivity with cholesterol and vitamin A.

**Standard curve Linearity:** Linearity was determined to be 0.98 (Average of six independent assays) with %RSD of 1.3%.

## XI. Limitations of the procedure:

a) 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.

b) A maximal total pipetting time of 5 minutes for calibrators, controls and specimens is suggested.

c) Improper handling, and washing might result in O.D. of 0.0 vitamin $D_3$ standard lower than the 0.125 I.U./mL vitamin $D_3$ 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

1. All materials in this kit may be used only for *in vitro* quatification not involving internal or external administration of the material to humans or animals.
2. Respect laboratory quality controls rules.
3. Reagents are matched in each kit and therefore, reagents from different lot numbers should not be mixed.
4. This kit should not be used after the expiration date.
5. Optimal results will be obtained by strict adherence to this protocol.
6. The stopping solution contains 0.2M sulfuric acid. This solution should be handled with caution, avoiding skin contact.
7. Prior to assay, bring all reagents except **CONJ** **ENZ** 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. 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- CAL 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 |

## XVI. SYMBOLS ON REAGENTS LABEL