# *VitaKit A™*

050406

## *FOR FLUID MILK SAMPLES*

**Cat. No.: KTSP-71051 96 tests**

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

**For *in vitro quantification* use only.**

### I. PROPRIETARY NAME

The *VitaKit A™* from SciMed, Cat. No. KTSP-71051 contains sufficient material to assay at least 96 tests.

### II. APPLICATION AND INTENDED USE

Dairy milk is fortified with vitamins A & D, 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 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 the 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 A™* provides materials for the quantitative measurement of vitamin-A in dairy products. This assay is intended for in vitro quantification only.

### III. PRINCIPLES OF THE METHOD

The vitamin A ELISA test is based on the principle of a sandwich enzyme-linked immunosorbent assay. The assay system utilises a solid phase immobilisation of a monoclonal antibody directed against a distinct antigenic determinant on the vitamin-A molecule (retinol palmitate). A second Anti-vitamin-A monoclonal antibody conjugated to horseradish peroxidase (HRP) is used as the detecting antibody in the assay mixture. The test sample is allowed to react sequentially with the two antibodies, resulting in the vitamin-A molecules being sandwiched between the solid phase and enzyme-linked antibodies. After the incubation, the wells are washed with distilled water to remove all unbound labelled antibodies. A solution of TMB is added as a substrate, resulting in the development of a blue color. The reaction is stopped with the addition of 0.2M $H_2SO_4$, changing the color to yellow. The concentration of vitamin-A is directly proportional to the color intensity of the test sample. Absorbency is measured spectrophotometrically at 450 nm.

### IV. REAGENTS SUPPLIED WITH KIT

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

1. SORB **Anti-Vitamin-A antibody coated wells :** REF **CW-71051:** 96 wells with Anti-Vitamin-A Mab immobilized in the well.

2. CONJ ENZ **Anti-Vitamin-A antibody conjugate with HRP** REF **EC-71051:** one (1) vial containing 0.2 mL of concentrated Anti-Vitamin-A conjugate with HRP, in a stabilizer solution.

3. CAL 1-5 **Vitamin-A Standard** REF **WSC-71051:** Standards prepared with hexane: 0.0, 0.055, 0.11, 0.22, 0.45 IU/mL. Content is 0.5 mL per vial.

4. CONTROL 1 **Control 0.3 IU/mL** REF **QC-71051:** 0.5 mL per vial.

5. CONTROL 2 **Control 0.1 IU/mL** REF **QC-71052:** 0.5 mL per vial.

6. BUF **Reaction Buffer** REF **RB:71051** one (1) vial containing 13 mL of protein based (milk) buffer with thimerosal as preservative.

7. SUBS TMB **Enzyme substrate** REF **ES-71051:** one (1) vial containing 11 mL of TMB solution.

8. CONJ DIL **Conjugate Diluent** REF **CD-71051:** one (1) vial containing 13 mL of glycine based Buffer.

9. $H_2SO_4$ **Stopping solution** REF **SS-71051:** one (1) vial containing 12 mL of 0.2 M sulfuric acid.

10. STAB **Stabilizer** REF **S-71051:** one (1) vial containing 100 uL of stabilizer.

### V. EQUIPMENT & MATERIAL REQUIRED BUT NOT PROVIDED

- ✓ Precision pipettes with disposable tips
- ✓ 8 channel 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
- ✓ Super mixer (vortex)

### VI. REAGENT PREPARATION

1. All reagents should be brought to room temperature before use (22 ± 2°C), except enzyme conjugate concentrate CONJ ENZ (EC-71051) that should be at 2 - 8°C

2. Enzyme conjugate concentrate (EC-71051) should be diluted as indicated on the bottle (label) with conjugate diluent (CD-71051) according to the number of wells used. Diluted conjugate can not be stored and should be prepared fresh in each assay 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-A calibrators and vitamin-A 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

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. Pipette 15 µL of ethanol into each tube, shake for 20 seconds in the dark.
2. Add 0.60 g of KOH into milk with 3.25% and 2% M.F., and 0.50g into 1% M.F. Gently mix for 2 minutes in the dark. 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 solution. Cap and shake vigorously for another 2 min in the dark.
4. Centrifuge each tube at room temperature for 5 minutes at 2500 rpm.
5. Label 1 or 2 mL screw capped amber coloured glass vials and add 300 µL of hexane into each vial for dilution of vitamin-A extract. After centrifugation, handle tubes carefuly. The upper organic phase must be perfectly clear and well separated. Transfer 100 µL of vitamin-A extract in corresponding amber coloured glass vials (100 µL of organic phase + 300 µL of hexane). The amber coloured glass vials, which contain 8-fold diluted vitamin A extract, must be capped very well and should be assayed immediately. If necessary dilute the extract with more hexane.

#### B. Skim Milk

1. Label 10 mL screw capped glass tubes and pipette 1 mL of milk into corresponding tube. Pipette 1mL of distilled water into one tube as a water control. Pipette 15 µL of ethanol into each tube and gently mix for 20 seconds in the dark.
2. Add 0.45 g of KOH into each tube and gently mix for 2 minutes in the dark. Add 5uL of stabilizer STAB. Cap and vortex tubes for 10 seconds. 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).
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.
5. Label 1 or 2 mL screw capped amber coloured glass vials and add 300 µL of hexane into each vial for dilution of extract. After centrifugation, handle tubes carefuly. The upper organic phase must be perfectly clear and well separated. Transfer 100 µL of upper extract in corresponding amber coloured glass vials (100 µL of organic phase + 300 µL of hexane). The amber coloured glass vials, which contain 8-fold diluted extract, must be capped very well and shoul be assayed immediately. If necessary, dilute the extract with more hexane.

Table I

| | Steps | 3.25 & 2%M.F. | 1% M.F. | Skim milk | Distilled water | Conditions |
|---|---|---|---|---|---|---|
| | Fluid Milk | 1 mL | 1 mL | 1 mL | 1 mL | Run water control only for skim milk. |
| Saponification and extraction | Ethanol 95% | 15 µL | 15 µL | 15 µL | 15 µL | Gently mix for 20 seconds. |
| | KOH (g) | 0.6 | 0.5 | 0.45 | 0.45 | Gently mix for 2 minutes in the dark. |
| | Stabilizer | - | - | 5 µL | 5 µL | Vortex for 10sec. (skim milk & water control only). |
| | Incubate 4 minute, and shake vigorously for 2 minute in the dark. Repeat 4 minute incubation and 2 minutes vigorous shaking two more times (totals 12 min. 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. |
| Extract dil. with hexane | Upper organic phase | 100 µL | 100 µL | 100 µL | 100 µL | The diluted extract in screw capped amber coloured glass vial should be assayed immediately. |
| | Hexane | 300 µL | 300 µL | 300 µL | 300 µL | |

### 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, diluted extracted specimens and controls CONTROL 1, CONTROL 2 into the corresponding wells.

2. Shake the wells 6 minutes on a plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C) to evaporate hexane

3. Pipette 120 µL of Reaction Buffer BUF into each well. Mix gently for 30 seconds. Place opaque lid or adhesive cover over the strips.

4. Incubate for 30 minutes in the dark on the plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C).

5. Wash four 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, completely decant the water by tapping the plate against absorbing paper until no trace of water is visible on the paper.

6. Pipette 100 µL of diluted Anti-Vitamin-A conjugate-HRP (**CONJ ENZ** diluted in **CONJ DIL**) in each well. Mix gently for 20 seconds. Place opaque lid or adhesive cover over the strips.

7. Incubate for 20 minutes in the dark at room temperature (22 ± 2°C).

8. Wash 6 times (refer to step no. 5).

9. Pipette 100 µL of TMB **SUBS TMB** (Substrate) into each well. Gently mix for 10 seconds.

10. Incubate upto 10 minutes in the dark at room temperature (22 ± 2°C).

11. Add 100 µL of the stopping solution **$H_2SO_4$**. Gently mix for 10 seconds.

12. 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 | Reaction Buffer | | | Dil. Conjugate | | | Substrate | | Stop. Sol. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $A_1,A_2$ | 0 IU/mL | 10 µL | | 120 µL | INCUBATE | DECANT & WASH | 100 µL | INCUBATE | WASH | 100 µL | INCUBATE | 100 µL | READ AT 450 nm |
| $B_1,B_2$ | 0.055 IU/mL | | | | | | | | | | | | |
| $C_1,C_2$ | 0.11 IU/mL | | | | | | | | | | | | |
| $D_1,D_2$ | 0.22 IU/mL | | | | | | | | | | | | |
| $E_1,E_2$ | 0.45 IU/mL | | | | | | | | | | | | |
| $F_1,F_2$ | Sample extract | | | | | | | | | | | | |
| $G_1,G_2$ | Sample extract | | | | | | | | | | | | |
| $H_1,H_2$ | Sample extract | | 1 | | 2 | 3 | | 4 | 5 | | 6 | | |
| etc… | etc… | | | | | | | | | | | | |

1 6 minutes on a plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C)
2 Incubate 30 minutes in the dark on a plate shaker (180 ± 10 rpm) at room temperature (22 ± 2°C)
3 Wash 4 times with distilled or deionized water
4 Incubate 20 minutes in the dark at room temperature (22 ± 2°C)
5 Wash 6 times with distilled or deionized water
6 Incubate upto 10 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.

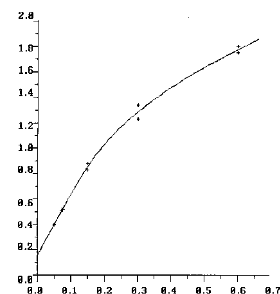
- The absorbance value of the standard zero should not exceed 0.5. This is an indication of improper washing and the assay must be repeated.
- For each standard, control and unknown sample, the optical density values are averaged (if there are duplicates). Subtract the means of the absorbance values of the zero standard from the mean absorbance values of other standards, controls and samples with 1.0 - 3.25% M.F.
  For skim milk only, subtract the means of the absorbance values of the water control from the mean absorbance values of skim milk sample.
- 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 8 as per the stipulated assay conditions.
- 3.33 I.U. of Vitamin A = 1 µg

**TABLE III**

| WELLS | OPTICAL DENSITY at 450 nm | CONCENTRATION (IU/mL) |
|---|---|---|
| 0 IU/mL | 0.0 | -- |
| 0.055 IU/mL | 0.289 | -- |
| 0.11 IU/mL | 0.486 | -- |
| 0.22 IU/mL | 0.852 | -- |
| 0.45 IU/mL | 1.595 | -- |
| Control 1 | 1.020 | 0.270 |
| Control 2 | 0.394 | 0.092 |
| etc… | … | … |

EXAMPLE OF Vitamin-A STANDARD CURVE

O.D.



Vitamin-A CONCENTRATION (IU/ML)

## X. NORMAL RANGE

The range for this assay under the specified conditions is from 0.055 I.U./mL to 0.45 I.U./mL.

## XI. SPECIFIC PERFORMANCE CHARACTERISTICS

1. **Sensitivity:** Sensitivity is defined as the minimum concentration of Vitamin-A which can be statistically distinguished from standard 0. This value is 0.03 IU/mL.

2. **Precision & reproducibility:**

a) **Intra-assay variation:** the precision of the assays was verified by assaying twelve (12) replicates of three (3) different extractions. The results were :

| Parameters | Samples 1 | Samples 2 | Samples 3 |
|---|---|---|---|
| Number of determinations (N) | 12 | 12 | 12 |
| Mean (IU/mL) | 1.744 | 1.198 | 2.028 |
| Standard deviation | 0.249 | 0.149 | 0.344 |
| Coefficient of variation (%) | 14 | 12 | 16 |

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

| Parameters | Samples 1 | Samples 2 | Samples 3 |
|---|---|---|---|
| Number of determinations (N) | 12 | 12 | 12 |
| Mean (IU/mL) | 1.548 | 0.774 | 2.518 |
| Standard deviation | 0.09 | 0.057 | 0.228 |
| Coefficient of variation (%) | 6 | 7 | 9 |

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

| Samples | Dilution factor | Theoretical value (IU/ML) | Experimental value (IU/mL) |
|---|---|---|---|
| 1 | 1:4 | 0.277 | 0.277 |
| | 1:8 | 0.139 | 0.132 |
| | 1:16 | 0.069 | 0.058 |
| | 1:32 | 0.035 | 0.037 |
| 2 | 1:4 | 0.216 | 0.216 |
| | 1:8 | 0.108 | 0.136 |
| | 1:16 | 0.054 | 0.051 |
| | 1:32 | 0.027 | 0.036 |

4. **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.

## 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* quantitative tests 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 sulfuric acid. This solution should be handled with caution, avoiding skin contact.
7. 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-71051 |
|---|---|---|
| CW-71051 | Anti-Vitamin-A coated wells | 96 wells |
| EC-71051 | Anti-Vitamin-A conjugate HRP | 1 |
| WSC-71051 | CAL 1-5 | 1 |
| QC-71051 | Control 1 | 1 |
| QC-71052 | Control 2 | 1 |
| CD-71051 | Conjugate Diluent | 1 |
| RB-71051 | Reaction Buffer | 1 |
| ES-71051 | Enzyme Substrate (TMB) | 1 |
| SS-71051 | Stopping Solution | 1 |
| S-71051 | Stabilizer | 1 |
| | English Protocol | 1 |


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## XVII. SYMBOLS ON REAGENTS LABEL

Toxic (T) substances

Use by

Consult instructions for use

Temperature limitation

Manufacturer

Sufficient for "n" tests

QC Manager :______________________________