# Vitamin $B_{12}$ Assay Kit

(Product No. VT3102)

GFAD[01]10.23

## 1. Introduction

The test kit uses the specificity of *Lactobacillus leichmannii* ATCC 7830 for Vitamin $B_{12}$ to determine the amount of Vitamin $B_{12}$ through the turbidity formed by bacterial growth in samples containing Vitamin $B_{12}$.

## 2. Principle of the Method

The growth intensity (turbidity) of *Lactobacillus leichmannii* is linearly related to the amount of Vitamin $B_{12}$ in the medium containing all nutrients except Vitamin $B_{12}$. The medium, *Lactobacillus leichmannii* and the prepared sample extracts (or standards) were added to the 96-well microplate and *Lactobacillus leichmannii* will grow until Vitamin $B_{12}$ is depleted. A standard curve is plotted using the turbidity of the bacteria after incubation in standards against the different concentrations of the standards, and the amount of Vitamin $B_{12}$ in the sample is obtained by measuring the turbidity of *Lactobacillus leichmannii* in the samples on the standard curve.

## 3. Product properties

Procedure time: operation time: 1h; incubation time: 44-48h

Range: 0.02-0.16 μg/100 g(mL)

Recovery: 80-120%

Intra-batch variation: <10%

Inter-batch variation: <10%

Storage condition: 1 year shelf life under 2-8℃ storage

## 4. Reagents provided

| | |
|---|---|
| Vitamin $B_{12}$ Standards | 3 vials |
| Vitamin $B_{12}$ Test Bacterial Ball | 3 vials |
| Vitamin $B_{12}$ Medium | 3 vials |
| 1X Vitamin $B_{12}$ Buffer (50 mL/vial) | 3 vials |
| Vitamin $B_{12}$ Protectant | 3 vials |
| Sterile Water (10mL/vial) | 3 vials |
| Sterile 96-well Microplate individually | 3 plates |

packed

| | |
|---|---|
| Sealing Film | 3 pieces |

## 5. Materials required but not provided

| | |
|---|---|
| 5.1 Ultra-clean workstation | 5.7 Pipette and sterile tips, 20-200 μL, 100-1000 μL |
| 5.2 Microplate reader (550 or 630 nm) | 5.8 15mL and 50mL sterile centrifuge tubes with screw cap and 1.5 mL or 2 mL sterile centrifuge tubes |
| 5.3 Constant temperature incubator, 36℃±1℃ | 5.9 Sterile syringes and 0.22 μm sterile filter membrane |
| 5.4 Autoclave | 5.10 Distilled water |
| 5.5 Water bath, 95℃ | 5.11 Graduated cylinder and 300 mL conical flask |
| 5.6 Vortex mixer | |

## 6. Reagent preparation

**6.1 Vitamin $B_{12}$ Protective Solution:** In ultra-clean workstation, take 1 vial of Vitamin $B_{12}$ Protectant, add 10 mL of 1X Vitamin $B_{12}$ Buffer and dissolve thoroughly to make Vitamin $B_{12}$ Protective Solution (Prepare it fresh when needed).

Note: Vitamin $B_{12}$ Protectant can also be dissolved in 10 mL of distilled water.

**6.2 Sample Preparation Solution (20 mL/sample):** According to the number of samples to be tested, measure the required volume of distilled water, and dilute Vitamin $B_{12}$ Protective Solution in 6.1 with distilled water at the ratio of Vitamin $B_{12}$ Protective Solution: distilled water=1:50 to create **Sample Preparation Solution** (Prepare it fresh when needed).

**Note:** Calculate the volume of Sample Preparation Solution required based on the number of samples to be tested. Each 96-well plate can generally accommodate the test of 8 samples and it requires 160 mL of Sample Preparation Solution, so measure 200 mL of distilled water and add 4 mL of Vitamin $B_{12}$ Protective Solution to obtain the needed Sample Preparation Solution.

**6.3 Standard/sample Diluent**: In the ultra-clean workstation, remove bacteria by filtering the Vitamin $B_{12}$ Protective Solution in 6.1 through a 0.22 μm filter membrane and then add the filtered Vitamin $B_{12}$ Protective Solution to 1X Vitamin $B_{12}$ Buffer at a ratio of filtered Vitamin $B_{12}$ Protective Solution : 1X Vitamin $B_{12}$ Buffer = 1:50.

## 7. Sample preparation of milk powder

**7.1** Weigh 1 g (to the accuracy of 0. 001 g) of milk powder in a 50 mL centrifuge tube and add **20 mL of Sample Preparation Solution, corresponding to an extraction dilution factor of 20, which is already included in the standard curve.** Mix it well and put the tube in a water bath at 95°C for 30 min, during which mixing it periodically on a vortex (at least 5 times), then quickly cool it to below 30°C in ice water.

**The following operations need to be carried out in the ultra-clean workstation:**

**7.2** Filter the cooled extract through a 0.22 μm filter membrane into a 2 mL sterile centrifuge tube to create a sterile sample solution.

**7.3** Dilute the sterile sample extract with Standard/sample diluent in 6.3 to Vitamin $B_{12}$ concentrations of approximately 0.06, 0.08 and 0.10 μg/100 g (mL).

### Example of sample preparation

For example, to test Vitamin $B_{12}$ concentration in an infant milk powder sample labeled with 2 μg/100 g (mL) Vitamin $B_{12}$ inside, weight 1 g of sample in a 50 mL centrifuge tube and add 20 mL Sample Preparation Solution in 6.2. Put the tube in water bath at 95°C for 30 min for extraction, and then quickly cool it down to below 30°C. Filter the cooled extract through a 0.22 μm filter membrane to remove bacteria. Dilute the filtered sample extract 33.3 times, 25 times and 20 times to obtain the final concentrations of Vitamin $B_{12}$ approximately at 0.06 μg/100 g (mL), 0.08 μg/100 g (mL) and 0.10 μg/100 g (mL) respectively. For operational convenience, samples can be diluted 35X, 25X and 20X respectively. The dilution method is listed as follows:

| Sample dilution times | Dilution protocol |
|---|---|
| ①5 times | 400 μL **Standard/sample Diluent** +100 μL **sample extract** |
| ②20 times | 300 μL **Standard/sample Diluent** +100 μL **① solution** |
| ③25 times | 400 μL **Standard/sample Diluent** +100 μL **① solution** |
| ④35 times | 600 μL **of Standard/sample Diluent** +100 μL **① solution** |

Note: Samples should be fully mixed after each dilution step and sample extracts must be used on the same day and stored in dark place. In case of unknown samples, two Vitamin $B_{12}$ concentrations should be assumed and processed separately as above, with the two assumed values being adjacent orders of magnitude, following the steps above.

## 8. Method procedure

**8.1 Vitamin $B_{12}$ Standards preparation (to be carried out in an ultra-clean workstation)**

Take 1 vial of lyophilized Vitamin $B_{12}$ Standard and add 5 mL of Standard/sample Diluent to prepare a standard solution of Vitamin $B_{12}$. Take 8 sterile 1.5 mL centrifuge tubes and prepare a series of standard solutions from 0.02 to 0.16 μg/100 g (mL) according to the table below:

| μg/100 g(mL) | Volume of standard solution (μL) | | Volume of **Standard/sample Diluent** (μL) | | Total volume (μL) |
|---|---|---|---|---|---|
| Standard 1: 0.02 | 100 | + | 900 | = | 1000 |
| Standard 2: 0.04 | 200 | + | 800 | = | 1000 |
| Standard 3: 0.06 | 300 | + | 700 | = | 1000 |
| Standard 4: 0.08 | 400 | + | 600 | = | 1000 |
| Standard 5: 0.10 | 500 | + | 500 | = | 1000 |
| Standard 6: 0.12 | 600 | + | 400 | = | 1000 |
| Standard 7: 0.14 | 700 | + | 300 | = | 1000 |
| Standard 8: 0.16 | 800 | + | 200 | = | 1000 |

Note: Standard solutions should be prepared fresh when needed and they cannot be stored.

### 8.2 Preparation of Vitamin $B_{12}$ Medium Solution

1) Pour one bottle of sterile water provided in the kit into one vial of Vitamin $B_{12}$ Medium, and then tighten the cap and shake it until dissolved (sterile water provided in the kit must be used).
2) Put the Vitamin $B_{12}$ medium solution vial in water bath at 95°C for 5 min and shake it 2-3 times during this time, and then quickly cool it in ice water to below 30°C.
3) In the ultra-clean workstation, filter the Vitamin $B_{12}$ Medium Solution through a sterile 0.22 μm filter membrane into a 15 mL sterile centrifuge tube. Each vial of Vitamin $B_{12}$ Medium is sufficient for 1 microplate of 96-well.

**8.3 and 8.4 below need to be carried out in the ultra-clean workstation:**

### 8.3 Preparation of Vitamin $B_{12}$ Test Bacterial Solution

Dissolve 1 vial of Vitamin $B_{12}$ Bacterial Ball in the filtered Vitamin $B_{12}$ Medium Solution in 8.2, and then tighten the cap and shake it until fully mixed.

### 8.4 Assay procedure

1) Determine the number of microwell strips required to test the desired number of samples plus the number of wells needed for standards, considering that each sample and standard

need be tested in triplet. Insert the appropriate number of strips in the holder, and record the position of the wells to create a layout. Immediately reseal the unused strips in the bag together with the desiccant bag provided and store in 2-8℃.

2) Add 100 μL of **Vitamin $B_{12}$ Test Bacterial Solution in 8.3** in each well.

3) Add 100 μL of Standard 1 to wells A1, A2 and A3; add 100 μL of each standard solution (Standard 2-8) to wells B1, B2 and B3, C1, C2 and C3 -- H1, H2 and H3 as shown below. The concentrations of Standard 1-8 are 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16 μg/100 g(mL)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | St1 | St1 | St1 | | | | | | | | | |
| B | St2 | St2 | St2 | | | | | | | | | |
| C | St3 | St3 | St3 | | | | | | | | | |
| D | St4 | St4 | St4 | | | | | | | | | |
| E | St5 | St5 | St5 | | | | | | | | | |
| F | St6 | St6 | St6 | | | | | | | | | |
| G | St7 | St7 | St7 | | | | | | | | | |
| H | St8 | St8 | St8 | | | | | | | | | |

4) Add 100 μL of each prepared sample to the remaining microtiter wells.

5) Seal the wells on the strip with a sealing film and press the film to ensure that all wells are adequately sealed.

8.5 Incubate at 36°C ± 1°C for 44-48 h in an incubator, avoiding light.

**8.6 Measurement**

1) Take out the plate from the incubator and press the sealing film again to ensure that all wells are adequately sealed. Shake the plate upside down repeatedly to mix the microorganisms well.

2) Remove the sealing film diagonally and puncture the air bubbles on the surface of each well with a needle.

3) Measure the absorbance at 550 or 630 nm. 550nm is recommended.

Note: If the absorbance cannot be measured in time after incubation, keep the plate at 2-8°C for no more than 48 h.

## 9. Data analysis

### 9.1 Determination of validity of test results:

OD values of low concentration standards < OD values of high concentration standards

### 9.2 Select the optimally diluted sample to calculate the results:

For each sample, which is diluted to three different levels of concentration, select the one(s) whose OD value locates at the middle of the standard curve. In case two or more are in the middle, calculate the average result.

Use the 4-Parameter calculation formula in professional ELISA statistical analysis software to calculate the concentration of Vitamin $B_{12}$ in the samples. (Note: when multiple the dilution factors of samples, do NOT consider the 20X dilution during extraction.)

**Note: The consumables required for the experiment must be sterile; waste must be disposed of after the experiment in accordance with the relevant regulations.**

**Note:**

For infant and young children's complementary foods samples like baby rice cereal or baby noodles, sample preparation protocol for milk powder can also be applied. However, due to the high starch content of these products, a colloidal state is easily formed during the extraction process, which results in the incapability to apply membrane filtration directly after the extraction. In this case, the sample extract can be diluted first and then filtered to remove bacteria and debris. Use the filtrate for assay.

*For laboratory use in industry or R&D purpose. Not for drug, household or other uses.*

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