Version 19-04/24

User's Manual

Vitamin B12 ELISA Kit

REF **DEIA280**

Σ **96T**

RUO

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

Enzyme Immunoassay for the Quantitative Determination of Vitamin B12 in Food.

General Description

Vitamin B12 as a trace element belongs to the biologically important chelate formers. The basic unit consists of a corrin ring with cobalt as a central atom. Cobalt is sixfold coordinated by four nitrogen atoms, one cyanide and a dimethylbenzimidazol group. Vitamin B12 forms a stable complex, which is absorbed in the lower part of the small intestine, with the so-called intrinsic factor present in the gastric juice. A lack of vitamin B12 can lead among other things to pernicious anemia. This disease is not generated by an insufficient supply of vitamin B12, but by the absence of intrinsic factor. A pernicious anemia can be treated by a high dosage of vitamin B12. The existing detection procedures are mainly microbiological methods, but also HPLC and thin-layer chromatography, all of which are associated with a high amount of time and instrumentation. With the present test kit it is possible, to determine vitamin B12 quantitatively in vitaminated food in a significantly faster way (2.5 to 4 hours inclusive sample pretreatment) compared with a conventional microbiological assay (24 to 48 hours).

Principles of Testing

The Vitamin B12 quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against vitamin B12 is bound on the surface of a microtiter plate. Vitamin B12 containing samples or standards and a vitamin B12 -peroxidase conjugate are given into the wells of the microtiter plate. Enzyme labeled and free vitamin B12 compete for the antibody binding sites. After one hour incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A substrate solution is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is inhibited by the addition of a stop solution, and the color turns yellow. The yellow color is measured photometrically at 450 nm. The concentration of vitamin B12 is indirectly proportional to the color intensity of the test sample.

Reagents And Materials Provided

The kit contains reagents for 96 determinations. They have to be stored at 2-8°C. Expiry data are found on the labels of the bottles and the outer package.

1. Microtiter plate consisting of 12 strips with 8 breakable wells each, coated with anti-vitamin B12.
2. Vitamin B12 Standards (0; 0.4; 1; 4; 10; 40 ng/mL): 6 vials with 0.5 mL each, dyed red, ready-to-use.
3. Conjugate (Vitamin B12-Peroxidase): 6 mL, dyed red, ready-to-use.
4. Substrate Solution (TMB): 15 mL; ready-to-use.
5. Stop Solution (0.5 M H_2SO_4): 15 mL; ready-to-use.
6. Sample Diluent (PBS): 2 × 60 mL; dyed red, readyto-use.
7. Washing Solution (PBS + Tween 20): 30 mL as 10x concentrate, dyed blue. Dilute 1+9 with distilled water. If

during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.

8. Two plastic foils to cover the strips during the incubation.
9. Plastic bag to store unused microtiter strips.
10. Instruction Manual.

Materials Required But Not Supplied

Instrumentation

1. 10 - 1000 µL - micropipets
2. Volumetric flask
3. Mortar, mixer
4. Centrifuge
5. ELISA reader (450 nm)

Reagents

1. Potassiumhexacyanoferrate(II)-3-hydrate (150 g/L; Carrez I)
2. Zincsulfate-7-hydrate (300 g/L; Carrez II)
3. Double-distilled water
4. 1 M caustic soda solution
5. 1 M hydrochloric acid
6. PBS (8.77 g/L NaCl, 0.70 g/L $NaH_2PO_4 \times 2H_2O$, 2.90 g/L $Na_2HPO_4 \times 2H_2O$)

Storage

Store at 2 - 8°C. Do not use the kit beyond the expiration date.

Specimen Collection And Preparation

The vitamin is extracted from the sample by double-distilled water. After the dissolution, the pH is adjusted by 1 M caustic soda solution or 1 M hydrochloric acid to 6- 7. Afterwards potential turbid matter is precipitated by Carrez I (150 g/L Potassiumhexacyanoferrate(II)-3-hydrate) and Carrez II (300 g/L Zincsulfate-7-hydrate). The extract is filled up to a defined volume and is centrifuged. Samples which are difficult to dissolve in cold water can be brought in solution by gentle warming. After the centrifugation, the samples are further diluted by the supplied sample diluent. To exclude interfering matrix or pH effects, a minimal dilution of 1 in 5 should be followed. We recommend a dilution to 1-10 ng/mL, in order to obtain an optimal accuracy during the measurement. Grain products normally contain low concentrations of vitamin B12. In order to avoid high dilutions, the sample can be extracted directly by sample diluent instead of double-distilled water.

1. **Multivitamin Tablets and Capsules**

   The tablets and capsules are dissolved in double-distilled water, and the pH value is adjusted to 6-7. Then 0.5 mL each of Carrez I and Carrez II are added, and the solution is filled up to a defined volume by doubledistilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent. To dissolve the capsules, heating to 30-40°C is recommended.

2. **Multivitamin Juices**

   The juice is adjusted to pH 6-7, 0.5 mL each of Carrez I and Carrez II are added, and the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent.

3. **Multivitamin Jam**

   The jam is homogenised in a mixer, and approximately 8 grams are extracted by double-distilled water, the pH is adjusted to 6-7 and 0.5 mL each of Carrez I and Carrez II are added. Afterwards the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent.

4. **Grain Products (Corn Flakes and Muesli)**

   3-5 grams of sample are homogenised by a mortar or a mixer, extracted by double-distilled water, the pH is adjusted to 6-7, and 0.5 mL each of Carrez I and Carrez II are added. Afterwards the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent. Grain products normally contain low concentrations of vitamin B12. In order to avoid high dilutions, the sample can be extracted directly by sample diluent instead of double-distilled water.

5. **Multivitamin Sweets**

   The sweets are dissolved by gentle heating (if necessary) in double-distilled water, the pH is adjusted to 6- 7, and 0.5 mL each of Carrez I and Carrez II are added. Afterwards the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent.

6. **Milk**

   5 mL of a fresh milk sample (full-cream milk or skim milk) are pipetted into a test tube and refrigerated for 30 minutes at 2-8°C. Afterwards the sample is centrifuged for 10 min at 3000 g. The upper fat layer is aspirated and discarded. The remaining aqueous layer is diluted 1:5 in sample diluent.

7. **Dry Milk Instant Formula**

   10 g of dry milk instant formula are suspended in 25 mL PBS and filled up to 50 mL. The mixture is vortexed intensely for 10 min and heated for 3 min in boiling water afterwards. After cooling to 20-25°C it is centrifuged for 10 min at 3000 g. The upper fat layer is aspirated and discarded. The remaining aqueous layer is diluted 1:5 in sample diluent.

Assay Procedure

1. Prepare samples as described above.
2. Pipet 50 µL standards or prepared samples in duplicate into the appropriate wells of the microtiter plate. Immediately add 50 µL folic acid antibody into each well.
3. Cover the microtiter plate with a plastic foil and incubate for 60 minutes at room temperature on a microtiter plate shaker (or 90 minutes without shaker).
4. Wash the plate three times as follows: Discard the contents of the wells (dump or aspirate). Pipet 300 µL of diluted washing solution into each well. After the third repetition empty the wells again and remove residual liquid by striking the plate against a paper towel. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbencies.

5. Pipet 100 µL of substrate solution into each well.
6. Allow the reaction to develop in the dark (e.g. cupboard or drawer; the chromogen is lightsensitive) for 20 minutes at room temperature.
7. Stop enzyme reaction by adding 100 µL of stop solution (0.5 M H_2SO_4) into each well. The blue color will turn yellow upon addition.
8. After thorough mixing, measure absorbance at 450 nm (reference wavelength 620 nm), using an ELISA reader. The color is stable for 30 minutes.

Calculation

1. Calculate the average optical density (OD 450 nm) for each set of reference standards or samples.
2. Construct a standard curve by plotting the mean optical density obtained for each reference standard against its concentration in ng/mL on semi-log graph paper with the optical density on the vertical (y) axis and the concentration on the horizontal (x) axis.
3. Using the mean optical density value for each sample, determine the corresponding concentration of vitamin B12 in ng/mL from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

The diluted samples must be further converted by the appropriate dilution factor. The factor is dependent on the sample preparation procedure employed. Applying the procedures for milk and dry milk instant formula the dilution factors are 5 or 25 respectively.

Typical Standard Curve

The following table contains an example for a typical standard curve. The binding is calculated as percent of the absorption of the 0 ng/mL standard. These values are only an example and should not be used instead of the standard curve which has to be measured in every new test.

Vitamin B_{12} (ng/mL)	(% binding of 0 ng/mL)
0	100
0.4	86
1.0	70
4.0	24
10	10
40	4

Performance Characteristics

The intra-assay variation of the vitamin B12 test was determined to 3%

Sensitivity

0.3 ng/mL

Specificity

Cross-reactivity relative to

vitamin B12 = 100%

Hydroxycobalamine = 29%

Recovery

0.98

Precautions

Full compliance of the following good laboratory practices (GLP) will determine the reliability of the results:

1. Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).
2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3. Once the assay has been started, all subsequent steps should be completed without interruption and within the recommended time limits.
4. Replace caps in all the reagents immediately after use. Do not interchange vial stoppers.
5. Use a separate disposable tip for each specimen to prevent cross-contamination.
6. All specimens and standards should be run at the same time, so that all conditions of testing are the same.
7. Do not mix components from different batches.
8. Do not use reagents after expiration date.
9. Check both precision and accuracy of the laboratory equipment used during the procedure (micropipets, ELISA reader etc.).