Version 22-04/24

User's Manual

Vitamin B-12 ELISA Kit

REF **DEIA-H002**

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.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

The Quantitative Determination of Vitamin B-12 Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric.

General Description

Vitamin B-12 is one of the nine water soluble vitamins important for healthy body functioning. The most important roles Vitamin B-12 plays in the human body are in the formation of red blood cells and the formation of the myelin sheath around the nerves. Since the effects are seen in body systems with a large range of function, the symptoms of Vitamin B-12 deficiency can sometimes be very ambiguous. A deficiency may also take from months to years to manifest depending on the cause and severity. Two of the most common causes of Vitamin B-12 deficiency are diet and age. Because most sources of dietary Vitamin B-12 come from animals, vegans who do not efficiently supplement their diet are at risk. The elderly community is also at high risk because of their diet, as well as the less efficient functioning of their digestive system. Intake of Vitamin B-12 starts by ingestion and then digestion by saliva. Once reaching the gut, Vitamin B-12 bound to proteins in food are released by the acids present. The B12 can then bind the Intrinsic factor. Once bound to IF, Vitamin B-12 is stable enough to travel into the intestines where it can be absorbed into your body through of its association with IF. Two very useful tests to distinguish between Vitamin B-12 deficiency and folate deficiency are methylmalonyl CoA (MMA) and homocysteine (hcy). Both deficiencies are represented by similar symptoms; however, even though both show increased levels of homocysteine, only Vitamin B-12 deficiency causes an increase in methylmalonyl CoA. The increase in levels of methylmalonyl CoA and homocysteine is thought to be the root cause of any symptoms that accompany a Vitamin B-12 deficiency. High levels of these two analytes in the blood stream causes increased oxidative stress to cells therefore causing increased apoptosis. In turn, vascular disease results in the form of atherosclerosis, coronary heart disease and/or neurodegeneration.

Principles of Testing

Delayed Competitive Enzyme Immunoassay:

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated antibody with a serum containing the antigen, a reaction results between the antigen and the antibody. The interaction is illustrated by the following equation:

$$Ag + Ab_{Btn} \rightleftharpoons AgAb_{Btn}$$

Ab_{Btn} = Biotinylated antibody

Ag = Antigen (Variable Quantity) AgAbBtn = Immune Complex

After a short incubation, the enzyme conjugate is added (this delayed addition permits an increase in sensitivity for low concentration samples). Upon the addition of the enzyme conjugate, competition reaction results between the enzyme analog and the antigen in the sample for a limited number of antibody binging

sites (not consumed in the first incubation).

$$^{Enz}Ag + Ag + rAb_{Btn} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

^{Enz}Ag = Enzyme-antigen Conjugate (Constant Quantity)

$^{Enz}Ag\ Ab_{Btn}$ = Enzyme-antigen Conjugate -Antibody Complex

rAb_{Btn} = Biotinylated antibody not reacted in first incubation

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Disassociation

$K = k_a/k_{-a}$ = Equilibrium Constan

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration.

$$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin_{CW}} \Rightarrow \underline{immobilized\ complex}$$

$Streptavidin_{CW}$ = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Reagents And Materials Provided

1. Vitamin B12 Calibrators -1ml/vial

Six (6) vials containing human serum albumin reference calibrators for Vitamin B12 at concentrations of 0 (A), 100 (B), 200 (C), 400 (D), 1000 (E), and 2000 (F) in pg/ml. A preservative has been added. Store at 2-8°C. The calibrators can be expressed in molar concentrations (pM/L) by multiplying by 0.738. For example: 100pg/ml × 0.738= 73.8 pM/L

2. Vitamin B12 Enzyme Reagent - 7.0 ml/vial

One (1) vial containing Vitamin B12 (Analog)-horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix. Store at 2-8°C.

3. Vitamin B12 Biotin Reagent-7.0 ml/vial

One (1) vial containing anti-Vitamin B12 biotinylated purified rabbit lgG conjugate in buffer, dye and preservative. Store at 2-8°C

4. Streptavidin Coated Plate - 96 wells

One 96-well microplate coated with 1.0 µg/ml streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C

5. Wash Solution Concentrate -20.0 ml/vial

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C

6. Substrate Reagent - 12.0 ml/vial

One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer.Store at 2-8°C.

7. Stop Solution -8.0 ml/vial

One (1) vial containing a strong acid (H_2SO_4). Store at 2-8°C

8. Releasing Agent -14.0 ml/vial

One (1) vial containing a strong base (sodium hydroxide) and potassium cyanide. Store at 2-8°C.

9. Stabilizing Agent -0.7 ml/vial

One (1) vial containing tris 2-carboxyethyl)phosphine (TCEP) solution. Store at 2-8°C

10. Neutralizing Buffer-7.0 ml/vial

One (1) vial containing buffer with dye that reduces the pH of sample extraction. Store at 2-8°C.

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Avoid extended exposure to heat and light. Opened reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on the label.

Note 3: Above reagents are for a single 96-well microplate.

Materials Required But Not Supplied

1. Pipette capable of delivering 0.050 & 0.100ml (50 & 100µl) with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.
3. Adjustable volume (200-1000µl) dispenser(s) for conjugate.
4. Glass test tubes for serum reference, control, and patient sample preparation.
5. Microplate washer or a squeeze bottle (optional).
6. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
7. Absorbent Paper for blotting the microplate wells.
8. Plastic wrap or microplate cover for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.
11. Quality control materials.

Storage

Store the kit at 4°C upon receipt. For more detailed information, please download the following document on our website.

Specimen Collection And Preparation

The specimens shall be blood, serum in type, and taken with the usual precautions in the collection of

venipuncture samples. For accurate comparison to establish normal values, a fasting morning serum sample should be obtained. The blood should be collected in a redtop (with or without gel additives) venipuncture tube(s) with no anti-coagulants. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum from the cells.

In patients receiving therapy with high biotin doses (i.e. >5mg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml(100ul) of the specimen is required.

Reagent Preparation

1. Wash Buffer

Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at 2-30°C for up to 60 days.

2. EXTRACTION AGENT

Add an aliquot of the stabilizing agent in order to prepare a 1/40 (stabilizing agent / releasing agent) dilute solution. For example, to make 4ml(4000µl),add 0.100ml(100µl) stabilizing agent to 3.9ml(3900ul) releasing agent.

3. SAMPLE EXTRACTION(See Note 3)

Obtain enough test tubes for preparation of all patient samples, controls, and calibrators. Dispense 0.10ml(100ul) of all samples into individual test tubes. Pipette 0.050ml (50ul) of the prepared extraction agent to each test tube, shaking (see note 3) after each addition. Let the reaction proceed for 15 min. At end of the 15 min, dispense 0.050 ml (50ul) of the neutralizing buffer, vortex (see note 3).

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

Note 3: Use of multiple (3) touch vortex is recommended.

Note 4: It is extremely important to accurately dispense the correct volume with a calibrated pipette and by adding near the bottom of the glass tubes at an angle while touching the side of the tubes

Note 5: Samples with high protein concentration should be diluted 1:1 with a saline solution before performing the extraction.

FOLATE & VITAMIN B12 SAMPLE PREPARATION

Step 1: Add 100ul of sample (patient, calibrator or control) into glass tube. Hint touch side of tube with pipette while dispensing at an angle near the bottom of the tube. Hint prepare all samples. Hint patient samples with a high protein concentration should be diluted 1:1 with a saline solution (before proceeding to Step 2).

Step 2: Add 50ul of working Extraction Reagent agent (see reagent preparation) into glass tube. Hint touch side of tube with pipette while dispensing at an angle slightly above the sample (do not touch the sample to avoid contamination. Mix on vortex 1-2 seconds using multiple touch technique (3x 1-2 seconds),immediately after adding the stabilizing reagent. Hint mix before moving onto the next tube.

Step 3: Incubate 15 minutes

Step 4: Add 50ul of Neutralizing Buffer into glass tubes. Hint touch side of tube with pipette while dispensing at an angle slightly above the sample (do not touch the sample to avoid contamination). Mix on vortex 1-2 seconds using multiple touch technique (3 x 1-2 seconds), immediately after adding the neutralizing reagent. Hint mix before moving onto the next tube.

Step 5: Use prepared samples accordingly to procedure. Hint for Folate, use after the 5 min wait time according to procedure (no wait time is needed for Vit B12).

Assay Procedure

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (20-27°C).

*Test Procedure should be performed by a skilled individual or trained professional

1. Format the microplates' wells for each serum reference calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.
2. Pipette 0.050 ml (50 uL) of the appropriate extracted Vitamin B12 calibrator, control or specimen into the assigned well.
3. Add 0.050 ml (50 uL) of the Vitamin B12 Biotin Reagent to all wells
4. Mix the microplate gently for 20-30 seconds to mix.
5. Cover and incubate for 45 minutes at room temperature.
6. Add 0.050 ml(50 uL) of Vitamin B12 Enzyme Reagent to all wells. Add directly on top the reagents dispensed in the wells.
7. Mix the microplate gently for 20-30 seconds to mix.
8. Cover and incubate for 30 minutes at room temperature.
9. Discard the contents of the microplate by decantation or aspiration. If decanting. blot the plate dry with absorbent paper.
10. Add 0.350 ml (350 uL) of wash buffer (see Reagent Preparation Section), decant (lap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage.If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.
11. Add 0.100 ml (100 uL) of substrate reagent to all wells. Always add reagents in the same order to minimize reaction time differences between wells. **DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION**
12. Incubate at room temperature for twenty (20) minutes.
13. Add 0.050 ml (50 uL) of stop solution to each well and gently mix for 15-20 seconds. Always add reagents in the same order to minimize reaction time differences between wells.
14. Read the absorbance in each well at 450nm (using a reference wavelength of 620-63Onm. The results should be read within fifteen (15) minutes of adding the stop solution.

Note: Dilute the samples suspected of concentrations higher than 2000pg/ml 1:5 and 1:10 with Vitamin B12 '0' pg/ml calibrator and re-assay

Assay Notes:

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift
3. Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
5. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
6. Plate readers measure vertically. Do not touch the bottom of the wells.
7. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
8. Use components from the same lot. No intermixing of reagents from different batches.
9. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed, is essential.
10. All applicable national standards, regulations and laws. including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage
11. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

Quality Control

Each laboratory should assay controls at levels in the low, normal and high range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental'conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations

Calculation

A dose response curve is used to ascertain the concentration of Vitamin B12 in unknown specimens

1. Record the absorbance obtained from the printout of the microplate reader.
2. Plot the absorbance for each duplicate calibrator versus the corresponding Vitamin B12 concentration in pg/ml on linear graph paper (do not average the duplicates of the calibrators before plotting)
3. Connect the points with a best-fit curve.
4. To determine the concentration of Vitamin B12 for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in pg/ml) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated).

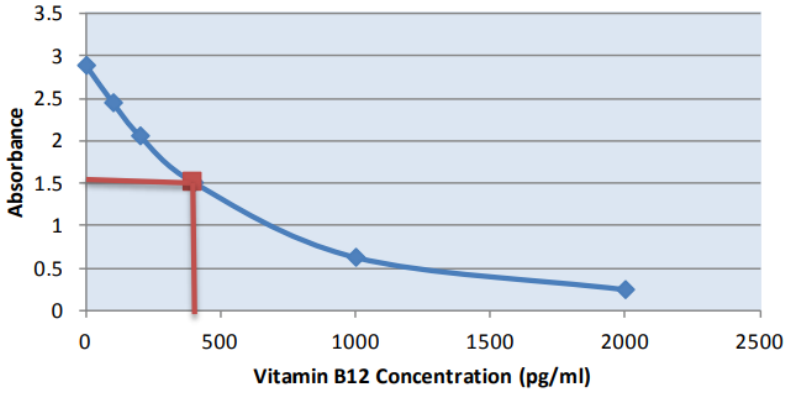
Note: Computer data reduction software designed for ELISA assay may also be used for the data

reduction. If such software is utilized. the validation of the software should be ascertained.

Typical Standard Curve

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (pg/ml)
Cal A	A1	2.898	2.89	0
	B1	2.891		
Cal B	C1	2.495	2.45	100
	D1	2.415		
Cal C	E1	2.107	2.06	200
	F1	2.023		
Cal D	G1	1.544	1.51	400
	H1	1.468		
Cal E	A2	0.662	0.63	1000
	B2	0.604		
Cal F	C2	0.263	0.25	2000
	D2	0.239		
Pat# 1	G2	1.479	1.53	391.4
	H2	1.573		

*The above data and table below is for example only. Do not use it for calculating your results



Note: Multiply the horizontal values by 0.738 to convert into pM/ml.

Evaluation

In order for the assay results to be considered valid the following criteria should be met:

1. The absorbance (OD) of calibrator 0 pg/ml should be > 1.3
2. Four out of six quality control pools should be within the established ranges.

Performance Characteristics

Accuracy:

The Vitamin B12 ELISA Test System was compared with a reference method. Biological specimens from low, normal and relatively high Vitamin B12 level populations were used (the values ranged from 156 pg/ml-1830

pg/ml). The total number of such specimens was 56. The least square regression equation and the correlation coefficient were computed for this Vitamin B12 test in comparison with the reference method. The data obtained is displayed in Table.

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (Y)	654.3	y= 1.0186x -48.82	0.9506
Reference (X)	690.2		

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the mean values. The least square regression equation and correlation coefficient indicates excellent method agreement.

Precision

The within and between assay precision of the Vitamin B12 ELISA Test System were determined by analyses on three different levels of pool control sera.The number, mean values, standard deviation and coefficient of variation for each of these control sera are presented in Tables.

Within Assay Precision (Values in pg/ml)

Sample	N	X	σ	C.V.
Low	20	334.8	24.3	7.3%
Normal	20	484.9	17.6	3.6%
High	20	925.3	28.3	3.1%

Between Assay Precision (Values in pg/ml)

Sample	N	X	σ	C.V.
Low	18	314.9	49.4	15.7%
Normal	18	441.3	46.7	10.6%
High	18	913.1	39.4	4.8%

*As measured in ten experiments in duplicate over a ten day period.

Sensitivity

The Vitamin B12 ELISA Test System has a sensitivity of 70.13 pg/ml. The sensitivity was ascertained by determining the variability of the 0 pg/ml serum calibrator and using the 2σ(95% certainty) statistic to calculate the minimum dose.

Specificity

The % cross reactivity of the Vitamin B12 antibody to selected substances was evaluated by adding the interfering substance to a serum matrix at various concentrations. The cross-reactivity was calculated by deriving a ratio between dose of interfering substance to dose of Vitamin B12 needed to displace the same amount of labeled analog.

Substance	Cross Reactivity
Bilirubin	0.0003
Rhematoid Factor	0.0008
Cobinamide	<0.0001
Lipemia	<0.0001
Hemoglobin	<0.0001

Precautions

All products that contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control/National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories, " 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395. Safe Disposal of kit components must be according to local regulatory and statutory requirements.

Limitations

1. Measurements and interpretation of results must be performed by a skilled individual or trained professional.
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. The reagents for the test system procedure have been formulated to eliminate maximal interference; however, potential interaction between rare serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems for all kinds of immunoassays. The results from this assay should be used in combination with clinical examination, patient history, and all other clinical findings.
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements
5. If test kits are altered, such as by mixing parts of different kits which could produce false test results, or if results are incorrectly interpreted, CD shall have no liability.
6. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall within 10% of the assigned concentrations.