Diagnostic Automation / Cortez Diagnostics, Inc.
IMMUNO DIAGNOSTICS

AccuDiag™ Vitamin B12 ELISA Kit

Cat# 3125-15

IVD See external Label 2°C 8°C Σ 96 Tests

Test	Vitamin B12
Method	Delayed Linked Immunosorbent ELISA
Principle	Delayed Competitive ELISA
Sample	50 µl serum or plasma
Total Time	~ 95 min.
Shelf Life	12-14 Months
Sensitivity	70.13 pg/ml

INTENDED USE

The Diagnostic Automation, Inc. Vitamin B12 ELISA Kit is an enzyme immunoassay (ELISA) for the quantitative detection of Vitamin B12 concentration in human serum. The Vitamin B12 ELISA test is for in-vitro diagnostic use only.

SUMMARY AND EXPLANATION

Vitamin B12 plays an important role in the functioning of a healthy body. It is essential to the production of red blood cells that carry oxygen through the body's tissue. This in turn helps in the development of myelination for proper functioning of the nervous system. The effects of Vitamin B12 deficiency can range from fatigue, weakness, depression, and memory problems to more severe symptoms such as anemia. Vitamin B12 deficiencies progress slowly, and thus it may take several years for initial signs to develop.

Two groups of individuals that are most prone to Vitamin B12 deficiency are the elderly and vegans. As a person ages, the ability to absorb and digest water soluble vitamins (like Vitamin B12) decreases, and if there is not an efficient source of Vitamin B12 in the diet, they are at risk for a deficiency. Unless vegans take a regular supplement of Vitamin B12, they are prone to deficiency because their diet is void of one of the major sources of Vitamin B12 - animal meat.

The absorption of Vitamin B12 occurs through saliva at the ingestion stage, and then during digestion, the B12 from food proteins is released with the help of acids in the system. The absorption of B12 depends on a protein called intrinsic factor (IF). Once combined with IF, Vitamin B12 can be absorbed in the intestines.

Vitamin B12 and Folate deficiencies have similar symptoms, so it is important to have accurate tests that can detect the differences. Even though the symptoms of both deficiencies show increased levels of homocysteine, a Vitamin B12 deficiency is the only one of the two that causes an additional increase in methylmalonyl CoA. Severe diseases that affect the vascular system, like Parkinson's Disease, atherosclerosis, or coronary heart disease, are caused by increased levels of methylmalonyl CoA and homocysteines. This increase also causes stress at the cellular level, eventually leading to apoptosis.

TEST PRINCIPLE

Delayed Competitive Enzyme Immunoassay (TYPE 9): The essential reagents required for a 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:

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

AbBtn = Biotinylated antibody

Ag = Antigen (Variable Quantity) $AgAb_{Btn}$ = Immune Complex

After a short incubation, the enzyme conjugate is added (This delayed addition permits and 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 Constant

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.

$$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + \underline{Strept_{C.W.}} \Rightarrow \underline{\text{immobilized complex}}$$

$\underline{Strep_{C.W.}}$ = 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.

MATERIALS AND COMPONENTS

Materials provided with the test kits

1. **Vitamin B-12 Calibrators – 1ml/vial – Icons A-F**
   Six (6) vials of human serum albumin reference for Vitamin B-12 at concentrations of 0 (A), 100 (B), 200 (C), 400 (D), 1000 (E), and 2000 (F) in pg/ml. Store at 2 – 8 ºC. A preservative has been added. The calibrators can be expressed in molar concentrations (pM/L) by multiplying by 0.738. For example: 100 pg/ml x 0.738 = 73.8 pM/L.
2. **Vitamin B -12 Enzyme Reagent – 6.0 ml/vial**
   One (1) vial of Vitamin B-12 (Analog) – horseradish peroxides (HRP) conjugate in a protein-stabilizing matrix. Store at 2 – 8 ºC.
3. **Vitamin B-12 Biotin Reagent – 6.0**

One (1) bottle of reagent contains anti-Vitamin B-12 biotinylated purified rabbit IgG conjugate in buffer, blue dye and preservative. Store at 2 – 8 ºC.

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 ºC.
5. **Wash Solution Concentrate – 20ml**
   One (1) vial contains a surfactant in buffered saline. A preservative has been added. Store at 2 – 8 ºC.
6. **Substrate Reagent – 12 ml/vial**
   One (1) bottle contains tetramethylbenzidine (TMB) and hydrogen peroxide (H2O2) in buffer. Store at 2 – 8 ºC.
7. **Stop Solution (8 ml / vial**
   One (1) vial contains a strong acid (H2 SO 4). Store at 2-30 ºC.
8. **Releasing Agent – 12ml/vial**
   One (1) vial contains a strong base (sodium hydroxide) and potassium cyanide.
9. **Stabilizing Agent – 0.5 ml / vial**
   One (1) vial contains dithiothreitol (DTT) solution.
10. **Neutralizing Buffer – 7 ml / vial**
    One (1) contains buffer that reduces the pH of sample extraction.
11. **Product Insert**

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 ºC. Kit and component stability are identified on the label.**
Note: 3: Above reagents are for a single 96- well microplate.

Materials required but not provided

1. Pipette capable of delivering 50 µl and 100 µl with a precision of better than 1.5 %.
2. Dispenser (s) for repetitive deliveries of 0.100 ml and 0.350 ml volumes with a precision of better than 1.5%.
3. Adjustable volume (200 -1000 µl) 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.

REAGENT PREPARATION

1. **Wash Buffer**
   Dilute contents of wash solution to 1000 ml with distilled or deionized water in a suitable storage container. Diluted buffer can be stored at room temperature (20-27 ºC) for up to 60 days.
2. **RELEASING 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 4000 µl (4ml), and 100 µl stabilizing agent to 3900 µl releasing agent.
3. **SAMPLE EXTRACTION**
   Obtain enough test tubes for preparation of all patient samples, controls, and serum references. Dispense 0.10ml (100µl) of each sample into individual test tubes. Pipette 0.050 ml (50 µL) of the prepared releasing agent to each test tube, shaking after each addition. Let the reaction proceed for 15 min. At end of the 15 min, dispense 0.050 ml (50 µL) of the neutralizing buffer, shaking after each addition, to finish the extraction.

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.

SPECIMEN COLLECTION AND PREPARATION

- The specimens shall be blood; serum or heparanised plasma 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 tubes (s) or for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for serum samples. Centrifuge the specimen to separate the serum or plasma from the cells.

- Samples may be refrigerated at 2-8 º 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 ºC for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050 ml of the specimen is required.

ASSAY PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 -27 ºC)
**** Test procedure should be run by a skilled individual or trained professional**

1. Format the microplates' wells for each serum reference, 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 ºC.
2. Pipette 0.050 ml (50 µl) of the appropriate extracted vitamin B12 calibrator, control or specimen into the assigned well.
3. Add 0.050 ml (50 µl) of the Vitamin B-12 Biotin Reagent to all wells.
4. Swirl 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 µl) of Vitamin B-12 Enzyme Reagent to all wells.

   Add directly on top the reagent dispensed in the wells.

7. Swirl 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 350 µl of wash buffer (see Reagent Preparation Section) , decant (tap 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 (avoid air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.
11. Add 0.100 ml (100 µl) 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 0.050ml (50 µl) 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 450 nm (using a reference wavelength of 620 – 630 nm. **The results should be read within thirty (30) minutes of adding the stop solution.**

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

ASSAY PERFORMANCE

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 are essential. Any deviation from Diagnostic Automation, Inc. IFU may yield inaccurate results.
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 performed routine preventative maintenance.

Interpretation

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. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. 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, **Diagnostic Automation, Inc. shall have no liability.**
5. 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.

RESULTS

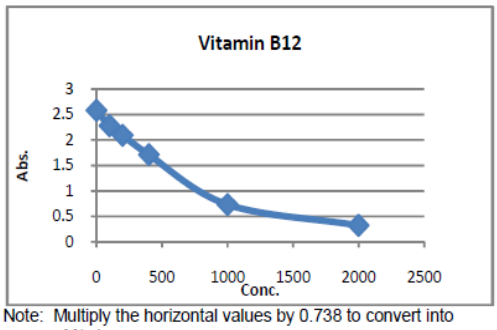
A dose response curve is used to ascertain the concentration of Vitamin B12 in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding Vitamin B12 concentration in pg / ml on linear graph paper (do not average the duplicates of the serum references 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 duplicates for each unknown on the vertical axis of the graph, find the intersecting point 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). In the following example, the average absorbance (1.202) intersects the dose response curve at (160 pg / ml) Vitamin B12 concentration (See Figure 1).

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.**

Sample I.D.	Well Number	Abs (A)	Mean Abs (B)	Value (pg/ml)
Cal A	A1	2.636	2.58	0
	B1	2.523		
Cal B	C1	2.245	2.28	100
	D1	2.306		
Cal C	E1	2.093	2.09	200
	F1	2.089		
Cal D	G1	1.657	1.71	400
	H1	1.760		
Cal E	A2	0.741	0.73	1000
	B2	0.718		
Cal F	C2	0.336	0.32	2000
	D2	0.308		
Patient 1	G2	1.396	1.466	536.5
	H2	1.496		

*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.

Q.C. PARAMETERS

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 quantity control pools should be within the established ranges.

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.

EXPECTED VALUES

In agreement with established reference intervals for a "normal" population the expected ranges for the Vitamin B-12 ELISA Test system are detailed in Table 1.

Table 1
Expected Values for the Vitamin B-12 Test System [12]

Population	Pg/ml	PmoI/L
Newborn	160 - 1300	118 - 959
Adult	200 - 835	148 - 616
Adult > 60 y	110 - 800	81 - 590

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal" persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.

PERFORMANCE CHARACTERISTICS

Precision

The within and between assay precision of the Vitamin B12 Diagnostic Automation, Inc. Microplate 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 Table 2 and Table 3.

TABLE 2
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%

TABLE 3
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 Microplate 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 2 σ (95 % certainty) statistic to calculate the minimum dose.

Accuracy

The Vitamin B 12 Microplate ELISA Test system was compared with a chemilum-inescence immunoassay method. Biological specimens from low, normal and relatively high vitamin B12 level populations were used (The values ranged from 100 pg/ml – 1300 pg/ml). The total number of such specimens was 65. The least square regression equation and the correlation coefficient were computed for this Vitamin B12 ELISA in comparison with the reference method. The data obtained is displayed in Table 4.

TABLE 4

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (Y)	139	Y=10+0.985*(X)	0.979
Reference (X)	148.		

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.

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

PRECAUTION

1. For In Vitro Diagnostic Use.
2. Not for Internal or External Use in Humans or Animals.

- 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 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.**

ISO 13485
ISO 9001

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Date Adopted	Cat # 3125-15
2011-11-11	AccuDiag™- Vitamin B12 ELISA -2012
EC REP	CEpartner4U , Esdoornlaan 13, 3951DB Maarn. The Netherlands. www.cepartner4u.eu
Revision Date: 10-01-2013	