The data obtained is displayed below. 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.

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

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.

Bilirubin	**0.0003**
Rhematoid Factor	**0.0008**
Cobinamide	**<0.0001**
Lipemia	**<0.0001**
Hemoglobin	**<0.0001**

Species Reactivity

This kit has been optimized for human serum samples. Vitamin B12 is the same in all species. Therefore, this kit can be used to measure vitamin B12 in other species. We recommend using the same general method as described in this manual.

References: Snow CF (1999) Arch. Int. Med. 159, 1289-1298; Nijist TQ (1990) J. Neuurol. Neurosurg. Psychiat. 53, 951-954; Steele TJ (2010) Clin. Rev. 20, 16-19; Antony AC (2003) 78, 3-6; Liu YK ()1972) Blood 39, 428-432; Ubbink JB (1995) Clin. Chem. 41, 1033-1037

ELISA kits available from ADI (see details at the web site)

#0010	Human Leptin				
#200-120-AGH	Human globular Adiponectin (gAcrp30)				
#0700	Human Sex Hormone Binding Glob (SHBG)				
#0900	Human IGF-Binding Protein 1 (IGFBP1)				
#1000	Human C-Reactive Protein (CRP)				
#1190	Human Serum Albumin		#1200	Human Albumin (Urinary)	
#1750	Human IgG (total)		#1760	Human IgM	
#1800	Human IgE	#1810	Human Ferritin		
#1210	Human Transferrin (Tf)		#0020	Beta-2 microglobulin	
#1600	Human Growth Hormone (GH)				
#0030	Human Insulin	#0040	Human C-peptide		
#0100	Human Luteinizing Hormone (LH)				
#0200	Human Follicle Stimulating Hormone (FSH)				
#0300	Human Prolactin (PRL)				
#0400	Human Chorionic Gonadotropin (HCG)		#0410	HCG-free beta	
#0600	Human Thyroid Stimulating Hormone (TSH)				
#1100	Human Total Thyroxine (T4)	#1110	Human Free T4 (fT4)		
#1650	Human free triiodothyronine (fT3)	#1700	Human T3 (total)		
#1850	Human Cortisol		#1860	Human Progesterone	
#1865	Human Pregnolone		#1875	Human Aldosterone	
#1880	Human Testosterone		#1885	Human free Testosterone	
#1910	Human Androstenedione		#1920	Human Estradiol	
#1925	Human Estrone		#1940	Dihydrotestosterone (DHT)	

Instruction Manual No. M-0340-B12

Vitamin B12 ELISA KIT

Cat. # 0340-B12, 96 Tests

For Quantitative determination of vitamin B12 (VB12) in Human Serum

For In Vitro Research Use Only (RUO)

ALPHA DIAGNOSTIC INTERNATIONAL

6203 Woodlake Center Drive • San Antonio• Texas 78244 • USA.
Phone (210) 561-9515 • Fax (210) 561-9544
Toll Free (800) 786-5777

Email: service@4adi.com
Web Site: www.4adi.com

DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

Vitamin B12 ELISA Kit Cat. # 0340-B12

Kit Components	96 tests
Streptavidin coated strip plate (96 wells), #340B12-1	1 plate
Vitamin B12 Standards (A-F), #0340B12-2A-F (0, 100, 200, 400, 1000 & 2000 pg/ml), 1 ml/vial	6 Vials
Vitamin B12 **Enzyme Reagent**, 7 ml, #0340B12-3	1 Bottle
Vitamin B12 **Biotin Reagent** 7 ml, #0340B12-4	1 Vial
Wash buffer concn. (50X) , #0340B12-WB, 20 ml	1 Bottle
TMB substrate, # 0340B12-TMB, 12 ml	1 Bottle
Stop solution, 8 ml #0340B12-ST	1 Bottle
Releasing Agent, 14 ml, #0340B12-5	1 Bottle
Stabilizing Agent, 0.7 ml, #0340B12-6	1 Bottle
Neutralizing Buffer, 7 ml, #0340B12-7	1 Bottle
Instruction Manual, M-0340-B12	1

Intended Use

Vitamin B12 ELISA kit is used for the quantitative detection of vitamin B12 in human serum. For in vitro research use only (RUO).

Introduction

Vitamin B12, vitamin B12 or vitamin B-12, also called cobalamin, is a water-soluble vitamin with a key role in the normal functioning of the brain and nervous system, and for the formation of blood. It is one of the eight B vitamins. It is normally involved in the metabolism of every cell of the human body, especially affecting DNA synthesis and regulation, but also fatty acid metabolism and amino acid metabolism. Neither fungi, plants, nor animals are capable of producing vitamin B12. Only bacteria and archaea have the enzymes required for its synthesis, although many foods are a natural source of B12 because of bacterial symbiosis. The vitamin is the largest and most structurally complicated vitamin and can be produced industrially only through bacterial fermentation-synthesis.

Vitamin B12 was discovered from its relationship to disease pernicious anemia, which is an autoimmune disease in which parietal cells of the stomach responsible for secreting intrinsic factor are destroyed (these cells are also responsible for secreting acid in the stomach). Because intrinsic factor is crucial for the normal absorption of B12, its lack in pernicious anemia causes a vitamin B12 deficiency. Many other subtler kinds of vitamin B12 deficiency and their biochemical effects have since been elucidated. High blood levels of the amino acid homocysteine may be a risk factor for heart disease. Taking vitamin B6 supplements with other B vitamins (folic acid and vitamin B12) has been shown to be effective for lowering homocysteine levels. Vitamin B6 has been studied for the treatment of many conditions, including anemia (low amounts of healthy red blood cells), vitamin B6 deficiency, certain seizures in newborns, and side effects of the drug cycloserine. High vitamin B12 level in elderly individuals may protect against brain atrophy or shrinkage associated with Alzheimer's disease and impaired cognitive function. High-dose administration of Vitamin B12 has been additionally validated to stimulate the activity of the body's TH1 suppressor T-Cells, which then down-regulates the over-production of the allergen antibody IgE in allergic individuals.

For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.

6. 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, ADI shall have no liability.
7. 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.

EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" population the expected ranges for the Vitamin B12 ELISA Test System are detailed below.

	pg/ml	pmol/L
Newborn	160 - 1300	118-959
Adult	200 – 835	148 – 616
Adult > 60 y	110 – 800	81 – 590

PERFORMANCE CHARACTERISTICS

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 below.

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.

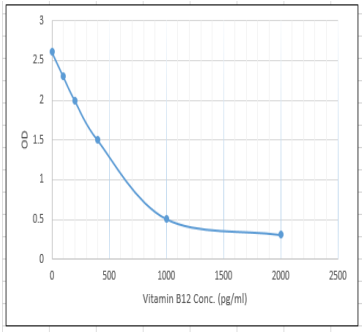
Accuracy

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.

WORKSHEET OF TYPICAL ASSAY

Wells	Stds/samples	Mean A450nm
A1, A2	Std. A (0 pg/ml)	2.89
B1, B2	Std. B (100 pg/ml)	2.45
C1, C2	Std. C(200 pg/ml)	2.06
D1, D2	Std. D (400 pg/ml)	1.51
E1, E2	Std. E (1000 pg/ml)	0.63
F1, F2	Std. F (2000 pg/ml)	0.25
G1, G2	Sample 1	1.53

NOTE: These data are for demonstration purpose only. A complete standard curve must be run in every assay to determine sample values. Each laboratory should determine their own normal reference values.



7_ADI-ELISA-graph

Calculation of results:

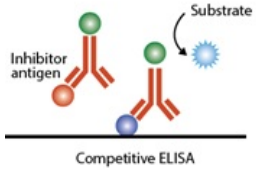
A dose response curve is used to ascertain the concentration of Vitamin B12 in unknown specimens.

1. Record the A450 obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the A450 for mean duplicate calibrator versus the Vitamin B12 concentration in pg/ml on linear graph paper. Connect the points with a best-fit curve.
3. To determine the concentration of Vitamin B12 for an unknown, locate the average A450 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). In the following example, the average absorbance (1.53) intersects the dose response curve at 391.4 pg/ml Vitamin B12 concentration (See Figure 1).

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.
   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. For diagnostic purposes, the results from this assay should be used in combination with clinical examination, patient history, and all other clinical findings.

PRINCIPLE OF THE TEST



Vitamin B12 ELISA kit uses Competitive-ELISA as the method. The microtiter plate provided in this kit has been pre-coated with VB16. During the reaction, VB12 in the sample or standard competes with a fixed amount of VB12 on the solid phase supporter for sites on the Biotinylated Detection Ab specific to VB12. Excess conjugate and unbound sample or standard are washed from the plate, and biotinylated conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of diluted sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of VB12 in the samples is then determined by comparing the OD of the samples to the standard curve.

MATERIALS AND EQUIPMENT REQUIRED

Adjustable micropipet (50-200 µl) and multichannel pipet with disposable plastic tips. Reagent troughs, plate shaker (orbital shaker), plate washer (recommended) and ELISA plates Reader.

SPECIMEN COLLECTION AND STORAGE

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. Samples may be refrigerated at 2-8oC 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 -20oC for up to 30 days. Avoid use of contaminated devices. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.100ml (100µl) of the specimen is required.

Notes:

1. Samples should be used within 7 days when stored at 2-8℃, otherwise samples must be divided and stored at -20℃ (≤1month) or -80℃ (≤6months) to avoid the loss of bioactivity and contamination. Avoid repeated freeze-thaw cycles.
2. Please take the samples to room temperature (18-25℃) without extra heating before performing the assay.
3. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

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, is essential. Any deviation from ADI's 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 perform routine preventative maintenance.

REAGENTS PREPARATION FOR THE ASSAY:

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 (3900µl) releasing agent.

SAMPLE EXTRACTION (See Note 3) Obtain enough test tubes for preparation of all patient samples, controls, and calibrators. Dispense 0.10ml (100µl) of all samples into individual test tubes. Pipette 0.050ml (50µl) 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 (50µl) of the neutralizing buffer, vortex (see note 3). After the neutralization buffer is added and mixed, let the reaction go to completion by waiting an additional 5 min before dispensing into the microwells.

Note1: 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.

Wash Buffer (50X): Dilute the wash buffer with distilled water (dissolve content of 1 bottle (20 ml) into 980 ml water. Some buffer components may crystallize in wash concentrate. These redissolve at room temperature. Store diluted wash buffer at 2-8oC.

STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{o}C until the expiration date printed on the label. The whole kit stability is usually 6 months from the date of shipping under appropriate storage conditions. Do not freeze and thaw.

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.

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 quality control pools should be within the established ranges.

TEST PROCEDURE *(ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE, 25-28oC, BEFORE USE). Prepare working solutions of biotin-antibody, HRP conjugate and wash buffer (see page 3). Bring all reagents and solutions to room temp. (25-28oC).*

1. Organize the microplates' wells for each serum reference standard, 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 **50 µL of Vitamin B12 standard, control or specimen** into the assigned well.
3. Add **50 µl of the Vitamin B12 Biotin Reagent** to all wells.
4. Mix the microplate gently for 20-30 seconds by gentle tapping against the palm. Cover and **incubate for 45 minutes** at room temp.
5. Add **50 µl of Vitamin B12 Enzyme Reagent** to all wells. Add directly on top the reagents dispensed in the wells. Mix the microplate gently for 20-30 seconds by tapping against the palm of your hand..
6. Cover and **incubate for 30 minutes** at room temp.
7. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
8. Add **350 µl of 1X 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 (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.
9. Add **100 µl of TMB substrate** reagent to all wells. Note: Always add reagents in the same order to minimize reaction time differences between wells. DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION.
10. **Incubate at room temperature for twenty (20) minutes**.
11. Add **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.
12. **Read the absorbance in each well at 450nm** (using a reference wavelength of 620-630nm. 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 standard and re-assay.