



Vitamin B12 (Vit B12) Test System Product Code: 7675-300

1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Vitamin B12 Concentration in Human Serum by a Microplate Enzyme Immunoassay, Chemiluminescence

2.0 SUMMARY AND EXPLANATION OF THE TEST

Vitamin B12 is one of the nine water soluble vitamins important for healthy body functioning. The most important roles Vitamin B12 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 B12 deficiency can sometimes be very ambiguous. A deficiency may also take from months to years to manifest depending on the cause and severity.^{1,2,3}

Two of the most common causes of Vitamin B12 deficiency are diet and age. Because most sources of dietary Vitamin B12 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.^{1,3,4}

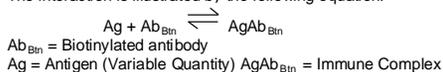
Intake of Vitamin B12 starts by ingestion and then digestion by saliva. Once reaching the gut, Vitamin B12 bound to proteins in food are released by the acids present. The B12 can then bind the intrinsic factor. Once bound to IF, Vitamin B12 is stable enough to travel into the intestines where it can be absorbed into your body through of its association with IF.^{1,5,6,7}

Two very useful tests to distinguish between Vitamin B12 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 B12 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 B12 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 (ex. Parkinson's Disease).^{1,8,9}

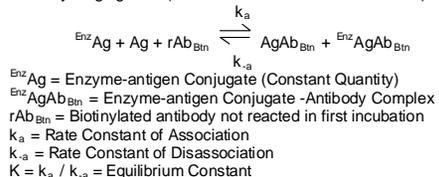
3.0 PRINCIPLE

Delayed Competitive Enzyme Immunoassay (TYPE 9):

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:



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 binding sites (not consumed in the first incubation).



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_{B₁₂} + EnzAgAb_{B₁₂} + Streptavidin_{CW} ⇒ 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.

4.0 REAGENTS

Materials Provided:

- Vitamin B12 Calibrators – 1ml/vial – Icons A-F**
Six (6) vials containing human serum albumin reference 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 (nM/L) by multiplying by 0.738. For example:
100pg/ml x 0.738= 73.8 pM/L
- Vitamin B12 Tracer Reagent – 7.0ml/vial – Icon ☒**
One (1) vial containing Vitamin B12 (Analog)-horseradish peroxidase (HRP) conjugate in a protein-stabilizing matrix red with dye. Store at 2-8°C.
- Vitamin B12 Biotin Reagent – 7.0ml/vial – Icon ▽**
One (1) vial containing anti-Vitamin B12 biotinylated purified rabbit IgG conjugate in buffer, dye and preservative. Store at 2-8°C.
- Light Reaction Wells – 96 wells – Icon ↓**
One 96-well white microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.
- Wash Solution Concentrate – 20.0 ml/vial – Icon ☉**
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.
- Signal Reagent A – 7.0 ml/vial – Icon CA**
One (1) vial containing luminol in buffer. Store at 2-8°C (see Reagent Preparation section).
- Signal Reagent B – 7.0 ml/vial – Icon CB**
One (1) vial containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C (see Reagent Preparation section).
- Releasing Agent – 14.0 ml/vial – Icon ☒**
One (1) vial containing a strong base (sodium hydroxide) and potassium cyanide. Store at 2-8°C.
- Stabilizing Agent – 0.7 ml/vial – Icon ☐**
One (1) vial containing tris 2-carboxyethyl)phosphine (TCEP) solution. Store at 2-8°C.
- Neutralizing Buffer – 7.0 ml/vial – Icon NZ**
One (1) vial containing buffer with dye that reduces the pH of sample extraction. Store at 2-8°C.
- 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°C. Kit and component stability are identified on the label.**

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

4.1 Required But Not Provided:

- Pipette capable of delivering 0.050 & 0.100ml (50 & 100µl) with a precision of better than 1.5%.
- Dispenser(s) for repetitive deliveries of 0.100 & 0.350ml (100 & 350µl) volumes with a precision of better than 1.5%.
- Adjustable volume (200-1000µl) dispenser(s) for conjugate.
- Glass test tubes for calibrator, control, and patient sample preparation.
- Microplate washer or a squeeze bottle (optional).
- Microplate Luminometer.
- Absorbent Paper for blotting the microplate wells.
- Plastic wrap or microplate cover for incubation steps.
- Vacuum aspirator (optional) for wash steps.
- Timer.
- Quality control materials.

5.0 PRECAUTIONS

**For In Vitro Diagnostic Use
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 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.

6.0 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) without anticoagulants. 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, 0.100 ml (100µl) of the specimen is required.

7.0 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.

8.0 REAGENT PREPARATION

- 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. Working Signal Reagent Solution - Store at 2 - 8°C.

Determine the amount of reagent needed and prepare by mixing equal portions of Signal Reagent A and Signal Reagent B in a clean container. For example, add 1ml of A and 1ml of B per two (2) eight well strips. (A slight excess of solution is made.) **Discard the unused portion if not used within 36 hours after mixing.** If complete utilization of the reagents is anticipated, within the above time constraint, pour the contents of Signal Reagent B into Signal Reagent A and label accordingly.

3. 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 4ml (4000µl), add 0.100ml (100µl) stabilizing agent to 3.9ml (3900µl) releasing agent.

4. SAMPLE EXTRACTION

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).

Note 1: Do not use reagents that are contaminated or have bacteria growth.

Note 2: Use of multiple (3) touch vortex is recommended.

Note 3: 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 4: Samples with high protein concentration should be diluted 1:1 with a saline solution before performing the extraction.

Note 5: See www.monobind.com/education-center for Step-by-Step Guide on Sample Extraction for Vitamin B12 (& Folate) in Lab Tips

9.0 TEST PROCEDURE

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

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

- 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.**
- Pipette 0.050 ml (50 µl) of the appropriate extracted Vitamin B12 calibrator, control or specimen into the assigned well.
- Add 0.050 ml (50 µl) of the Biotin Reagent to all wells.
- Swirl the microplate gently for 20-30 seconds to mix.
- Cover and incubate for 45 minutes at room temperature.
- Add 0.050 ml (50 µl) of Vitamin B12 Tracer Reagent to all wells.
- Add directly on top the reagents dispensed in the wells.**
- Swirl the microplate gently for 20-30 seconds to mix.
- Cover and incubate for 30 minutes at room temperature.
- Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
- Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) 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 four (4) additional times.**
- Add 0.100 ml (100 µl) of working signal reagent solution to all wells (see Reagent Preparation section). **Always add reagents in the same order to minimize reaction time differences between wells. DO NOT SHAKE THE PLATE AFTER SIGNAL ADDITION**
- Incubate at room temperature for five (5) minutes.
- Read the relative light units (RLUs) in each well for 0.2 – 1.0 seconds. **The results should be read within thirty (30) minutes of adding the signal reagent 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.

10.0 CALCULATION OF RESULTS

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

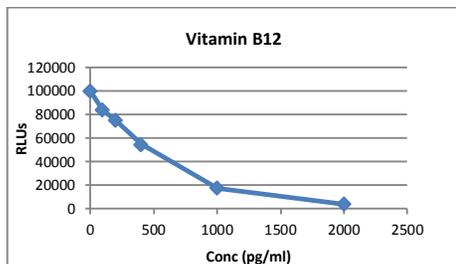
- Record the RLU's obtained from the printout of the microplate reader as outlined in Example 1.
- Plot the RLU's for each duplicate calibrator versus the corresponding Vitamin B12 concentration in pg/ml on linear graph paper (do not average the duplicates of the calibrators before plotting).
- Connect the points with a best-fit curve.
- To determine the concentration of Vitamin B12 for an unknown, locate the average RLU 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 RLU (42160) intersects the dose response curve at 601.3 pg/ml Vitamin B12 concentration (See Figure 1).

Note: Computer data reduction software designed for chemiluminescence assay may also be used for the data reduction. **If such software is utilized, the validation of the software should be ascertained.**

EXAMPLE 1

Sample I.D.	Well Number	RLU (A)	Mean RLU (B)	Value (pg/ml)
Cal A	A1	102903	100000	0
	B1	97097		
Cal B	C1	84051	83689	100
	D1	83328		
Cal C	E1	75125	74866	200
	F1	74607		
Cal D	G1	52757	54706	400
	H1	56656		
Cal E	A2	17699	17304	1000
	B2	16910		
Cal F	C2	4602	4091	2000
	D2	3580		
Pat# 1	G2	41954	42160	601.3
	H2	42365		

* The data presented in Example 1 and Figure 1 is for illustration only and **should not** be used in lieu of a dose response curve prepared with each assay. In addition, the RLU's of the calibrators have been normalized to 100,000 RLU's for the A calibrator (greatest light output). This conversion minimizes differences caused by efficiency of the various instruments that can be used to measure light output.



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

11.0 Q.C. PARAMETERS

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

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

12.0 RISK ANALYSIS

The MSDS and Risk Analysis Form for this product are available on request from Monobind Inc.

12.1 Assay Performance

- It is important that the time of reaction in each well is held constant to achieve reproducible results.
- Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift.
- Highly lipemic, hemolyzed or grossly contaminated specimen(s) should not be used.
- If more than one (1) plate is used, it is recommended to repeat the dose response curve.
- The addition of signal reagent solution initiates a kinetic reaction; therefore, the solution should be added in the same sequence to eliminate any time-deviation during reaction.
- Plate readers measure vertically. Do not touch the bottom of the wells.
- Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
- Use components from the same lot. No intermixing of reagents from different batches.
- Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from Monobind IFU may yield inaccurate results.
- 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.
- 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.
- Risk Analysis- as required by CE Mark IVD Directive 98/79/EC - for this and other devices, made by Monobind, can be requested via email from Monobind@monobind.com.

12.2 Interpretation

- Measurements and interpretation of results must be performed by a skilled individual or trained professional.**
- 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 (*Boscato LM Stuart MC. 'Heterophilic antibodies: a problem for all immunoassays' Clin.Chem. 1988;34:27-33*). For diagnostic purposes, the results from this assay should be used in combination with clinical examination, patient history, and all other clinical findings.
- For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
- 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, **Monobind shall have no liability.**
- 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.

13.0 EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a "normal" population the expected ranges for the Vitamin B12 AccuLite® CLIA Test System are detailed in Table 1.

TABLE 1
Expected Values for the Vitamin B12 Test System¹²

Population	pg/ml	pmol/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.

14.0 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precision of the Vitamin B12 AccuLite® CLIA 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.

Sample	N	X	σ	C.V.
Low	20	352.6	19.4	5.5%
Normal	20	517.2	14.3	2.8%
High	20	986.0	23.3	2.4%

Sample	N	X	σ	C.V.
Low	12	337.2	40.6	12.0%
Normal	12	500.6	65.3	13.0%
High	12	927.5	58.2	6.3%

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

14.2 Sensitivity

The Vitamin B12 AccuLite® CLIA Test System has a sensitivity of 37.92 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.

14.3 Accuracy

The Vitamin B12 AccuLite® CLIA Test System was compared with a reference method. Biological specimens from low, normal and relatively high Vitamin B12 level populations were used (values ranged from 273.9 pg/ml - 1865.0 pg/ml). The total number of such specimens was 57. The least square regression equation and the correlation coefficient were computed for this test system in comparison with the reference method. The data obtained is displayed in Table 4.

Method	Mean (x)	Least Square Regression Analysis	Correlation Coefficient
This Method (Y)	721.5	$y = 1.1263x - 72.462$	0.9516
Reference (X)	708.2		

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.

14.4 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
Rheumatoid Factor	0.0008
Cobinamide	<0.0001
Lipemia	<0.0001
Hemoglobin	<0.0001

15.0 REFERENCES

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MP7675 Product Code: 7675-300

Reagent (fill)	Size	96(A)
	A)	1ml set
B)	1 (7ml)	
C)	1 (7ml)	
D)	1 plate	
E)	1 (20ml)	
F)	1 (7ml)	
G)	1 (7ml)	
H)	1 (14ml)	
I)	1 (0.7ml)	
J)	1 (7ml)	

For Orders and Inquires, please contact

Monobind Inc.
100 North Pointe Drive
Lake Forest, CA 92630 USA

Tel: +1 949.951.2665 Mail: info@monobind.com
Fax: +1 949.951.3539 Fax: www.monobind.com



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Glossary of Symbols (EN 980/ISO 15223)

