1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Vitamin B12 and Folate Concentration in Human Serum and Plasma by a Monoclonal Enzyme Immunoassay, Chemiluminescence.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Folate plays an important role in brain development and is therefore vital during growth. The most common defects resulting from folate deficiencies occur in the central nervous system. With a vital role in nucleic acid synthesis, folate supplementation has been found to be beneficial during these times of rapid tissue growth. Folate also plays a vital role in maintaining proper balance of homocysteine, a contributing factor in occurrences of occlusive vascular diseases and strokes. Individuals with susceptibility to heart disease and several forms of cancer may also benefit from supplementation.1,2

Major sources of folate include green leafy vegetables, legumes, beans and fortified cereals. Foods fortified with folate are actually fortified with folic acid because of the higher bioavailability for absorption by the body. In circulation, folate is present in several different forms, some of which are more stable than others. Folic acid and N-methyltetrahydrofolate are two common forms, the latter being more stable and found in higher concentrations in serum. Due to the stability of the molecule, methyltetrahydrofolate is very often used as the form focused on during methods of analysis.3,4

Folate binding proteins are responsible for folate metabolism. Two types exist in circulation: one type aids in binding to the cell surface and the other soluble form exists in circulation. These folate binding proteins also have the capability of binding to several different folate derivatives including folic acid and N-methyltetrahydrofolate. Current assays on the market require an extraction step to release the folate derivatives from the folate binding protein.5,6

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 in 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.4,7,8

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.3

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 their elevated levels in the blood stream. In patients receiving therapy with high biotin doses (i.e. >500µg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.9

7.0 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated range for monitoring assay performance. Each laboratory should assay controls at levels in the low, normal and elevated range for monitoring assay performance. In patients receiving therapy with high biotin doses (i.e. >500µg/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) can not 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 extended exposure to heat and light. Avoid extended exposure to heat and light.

8.0 REAGENT PREPARATION

B. Folate Tracer Reagent – 7.0 ml/vial – Icon
One (1) vial containing Folate (Analog)-horseradish peroxidase (HRP) conjugate protein-stabilizing matrix with dye. Store at 2-8°C.

C. Folate Biotin Reagent – 7.0 ml/vial – Icon
One (1) vial containing biotinylated purified folate binding protein conjugate in buffer, dye and preservative. Store at 2-8°C.

D. Vitamin B12 Tracer Reagent – 7.0 ml/vial – Icon
One (1) vial containing Vitamin B12 (Analog)-horseradish peroxidase (HRP) conjugate in a protein-stabilizing matrix. Store at 2-8°C.

E. Vitamin B12 Biotin Reagent – 7.0 ml/vial – Icon
One (1) vial containing Vitamin B12 biotinylated purified rabbit IgG conjugate with buffer, dye and preservative. Store at 2-8°C.

F. Wash Solution Concentrate – 20.0 ml/vial – Icon
One (1) vial containing surfactant in buffered saline. A preservative has been added. Store at 2-8°C for up to 60 days.

9.0 LIGHT REACTION WELLs – 96 wells – Icon
One (1) well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

H. Signal Reagent A – 7.0 ml/vial – Icon
One (1) vial containing luminol in buffer. (See ‘Reagent Preparation’ section). Store at 2-8°C.

I. Signal Reagent B – 7.0 ml/vial – Icon
One (1) vial containing hydrogen peroxide (H2O2) in buffer. (See ‘Reagent Preparation’ section). Store at 2-8°C.

J. Releasing Agent – 14.0 ml/vial – Icon
One (1) vial containing biotinase (2-carboxyethyl) phosphine (TCEP) solution. Store at 2-8°C.

K. Neutralizing Buffer – 7.0 ml/vial – Icon
One (1) vial containing buffer with dye that reduces the pH of the sample extract. Store at 2-8°C.

M. Product Insert
Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Complete kit stability is indicated by the duration of reagents are stable for sixty (60) days when stored at 2-8°C. Kit and component stability are identified on label.
Note 3: Above reagents are for a single 96-well microplate.

4.1 Required But Not Provided
1. Pipette capable of delivering 0.050 ml (50 µl) and 0.100 ml (100 µl) with a precision of better than 1.5%.
2. Dispensers for repetitive deliveries of 0.100ml (100 µl) and 0.350ml (350µl) volumes with a precision of better than 1.5%.
3. Adjustable volume (200-1000µl) dispenser(s) for conjugate.
5. Microplate washer or a squeeze bottle (optional).
6. Microplate Luminometer
7. Absorbent Paper for lidding the microplate wells.
8. Plastic wrap or microplate covers for incubation steps.
9. Vacuum aspirator (optional) for wash steps.
10. Timer.

5.0 PRECAUTIONS

Safe Disposal of kit components must be according to local regulatory and statutory requirement.

6.0 SPECIMENT COLLECTION AND PREPARATION

The specimens shall be blood, serum or plasma in type, and the usual precautions in the collection of venipuncture samples should be followed. Specimens should be stored at ambient temperature until the specimen can be placed in the centrifuge. The centrifuge should be capable of separating normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain red top vial containing sodium or heparin for use in hematocrits (for serum) or evacuated tube(s) containing EDTA or heparin. Allow the blood clot for serum samples. Centrifuge the specimen to separate the serum from the plasma cells.

In patients receiving therapy with high doses of biotin (>500µg/day), no sample should be taken until at least 8 hours after the last biotin administration, preferably overnight to ensure fasting sample.
1. Prepare all samples according to the "Sample Extraction" by-Step Guide on Sample Extraction for Vit B12 ( & Folate) in Lab Tips.

2. Pipette 0.050 ml (50 µL) of the appropriate extracted Vitamin B12:
   - For Vitamin B12:
   - Add 0.050 ml (50 µl) of the Vitamin B12 Biotin Reagent to all wells.

3. Pipette 0.050 ml (50 µL) of the appropriate extracted Folate:
   - For Folate:
   - After the neutralization buffer is added and mixed, let the reaction go to completion by waiting an additional 5 min before dispense into the microwells. Wait time is not needed for Vitamin B12.

4. Wash Testing Signal Reagents: 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 1 ml of A and 1 ml 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. Complete utilization of the reagents is anticipated within the above time frame, pour the contents of Signal Reagent B into Signal Reagent A and use accordingly.

Note 1: Do not 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 wash procedure.


9.0 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, reference calibrators and controls to room temperature (20 - 22°C).

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

For Folate:
1. Prepare all samples according to the "Sample Extraction" procedure in section 4.2.0 "Reagent Preparation". It is important to wait 5 min before proceeding to allow the neutralization reaction to go to completion (see above).
2. Format the microplates wells for each 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.
3. Pipette 0.050 ml (50 µL) of the appropriate extracted Folate calibrator, control or specimen into the assigned well.
4. Add 0.050 ml (50 µl) of Folate Tracer Reagent to all wells.
5. Mix the microplate gently for 20-30 seconds.
6. Add 0.050 ml (50 µl) of Enzyme Biotin Reagent to all wells.
7. Mix the microplate gently for 20-30 seconds.
8. Cover and incubate for 45 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 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.
11. Add the final-filmit for the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to disperse the wash. Decant the wash and repeat four (4) additional times.
12. Add 0.100 ml (100 µl) of signal reagent to all wells. Always add reagents in the same order to minimize reaction time differences between wells.

DO NOT SHAKE THE PLATE AFTER SIGNAL ADDITION

12. Incubate at room temperature for five (5) minutes.
13. Read the relative light units (RLU) on each well for 0 - 1.0 seconds. The results should be read within thirty (30) minutes of adding the signal reagent solution.

Note 3: Dilute the samples suspected of concentrations higher than 25 ng/ml 1:5 with Folate '0' ng/ml calibrator and re-assay.

11.0 Q.C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met:
1. The Dose Response Curve (DRC) should be within established parameters.
2. 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
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 exceed beyond ten (10) minutes to avoid assay drift.
3. High hemolyzed or grossly contaminated specimen(s) should not be used.
4. If more than one (1) plate is used, it is recommended to repeat dose response curve.
5. The addition of signal solution initiates a kinetic reaction; therefore, the substrate and stop solution should be added as close to the sequence to eliminate any time-deviation during reaction.
6. Plate reader measures 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 Monobind’s IFU may yield inaccurate results.
10. All applicable national regulations, standards 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.
12. 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
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 interactions between serum specimens and test reagents can cause erroneous results. Heterophilic antibodies often cause these interactions and have been known to be problems in all kinds of immunoprecipitation (Boscato LM Stuart MC. 'Heterophilic antibodies: a problem for 'immunofluorescence'." Clin. Chem. 1988; 34(7):327-33).
4. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
5. If controls are altered, as 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.
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.

13.0 EXPECTED VALUES

In agreement with established reference intervals for a “normal” population the expected ranges for the Folate/Vit B12 VAST AccuLite® CLIA Test System are detailed in Table 1 and 2.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Expected Values - Folate</th>
<th>Normal Adult Population &gt; 3.0 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample I.D.</td>
<td>Well</td>
<td>RLU (A)</td>
</tr>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>101285</td>
</tr>
<tr>
<td>Cal B</td>
<td>B1</td>
<td>89714</td>
</tr>
<tr>
<td>Cal C</td>
<td>C1</td>
<td>6319</td>
</tr>
<tr>
<td>Cal D</td>
<td>D1</td>
<td>46841</td>
</tr>
<tr>
<td>Cal E</td>
<td>E1</td>
<td>51278</td>
</tr>
<tr>
<td>Cal F</td>
<td>F1</td>
<td>6991</td>
</tr>
<tr>
<td>Cal G</td>
<td>G1</td>
<td>84531</td>
</tr>
<tr>
<td>Cal H</td>
<td>H1</td>
<td>100687</td>
</tr>
<tr>
<td>Cal I</td>
<td>I1</td>
<td>148</td>
</tr>
<tr>
<td>Cal J</td>
<td>J1</td>
<td>7243</td>
</tr>
<tr>
<td>Cal K</td>
<td>K1</td>
<td>6748</td>
</tr>
<tr>
<td>Cal L</td>
<td>L1</td>
<td>63819</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Expected Values - Vitamin B12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population pg/ml pmol/L</td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td>Adult</td>
</tr>
<tr>
<td>160 - 1300</td>
<td>118 - 959</td>
</tr>
<tr>
<td>110 - 800</td>
<td>81 - 590</td>
</tr>
</tbody>
</table>
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 assay precision of the Folate 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 3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>24</td>
<td>19.47</td>
<td>0.304</td>
<td>1.7</td>
</tr>
<tr>
<td>Level 2</td>
<td>24</td>
<td>10.213</td>
<td>0.504</td>
<td>4.9</td>
</tr>
<tr>
<td>Level 3</td>
<td>24</td>
<td>14.932</td>
<td>0.613</td>
<td>4.1</td>
</tr>
</tbody>
</table>

The within 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 4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>σ</th>
<th>C.V.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>24</td>
<td>424.9</td>
<td>36.4</td>
<td>8.6</td>
</tr>
<tr>
<td>Normal</td>
<td>24</td>
<td>592.4</td>
<td>30.2</td>
<td>5.1</td>
</tr>
<tr>
<td>High</td>
<td>24</td>
<td>724.4</td>
<td>35.2</td>
<td>4.9</td>
</tr>
</tbody>
</table>

14.2 Sensitivity
The Folate/Vit B12 VAST® AccuLite® CLIA Test System has sensitivity for different analytes as listed in the following Table 5. The specificity was ascertained by determining the variability of the 0ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate</td>
<td>0.933 ng/ml</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>4.12 pg/ml</td>
</tr>
</tbody>
</table>

14.3 Accuracy
The Folate AccuLite® CLIA Test System was compared with a reference method. Biological specimens were used with values that ranged from 3.2ng/ml – 13.7ng/ml. The total number of such specimens was 56. The least square regression equation and the correlation coefficient were computed for this Folate CLIA in comparison with the reference method. The data obtained is displayed in Table 6.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean (x)</th>
<th>Least Square Regression Analysis</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>This Method (Y)</td>
<td>7.76</td>
<td>y = 0.162 + 1.07x</td>
<td>0.984</td>
</tr>
<tr>
<td>Reference (X)</td>
<td>8.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The sensitivity for different analytes as listed in the following Table 5.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>0.0003</td>
</tr>
<tr>
<td>Rheumatoid Factor</td>
<td>0.0008</td>
</tr>
<tr>
<td>Cobinamide</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lipemia</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

15.0 REFERENCES