1.0 INTRODUCTION

Intended Use: The Quantitative Determination of Folate Concentration in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric.

2.0 SUMMARY AND EXPLANATION OF THE TEST

Folate supplementation has escalated over recent years with the knowledge of its many benefits. As one of the B vitamins, folate, or Vitamin B9, is involved in many bodily functions and deficiency can cause disease in not only the elderly, but infants too. Folate deficiency is associated with megaloblastic anemia, neural tube defects, and cardiovascular diseases.1,2

Folate plays an important role in brain development and therefore is vital during growth. The most common defects resulting from folate deficiency include neural tube defects. With a vital role in nucleic acid synthesis, folate has been found to be beneficial as supplementation during pregnancy and other times of rapid tissue growth. Folate plays a vital role in maintaining proper balance of homocysteine, a contributing factor in occurrences of occlusive vascular diseases and stroke. Individuals with susceptibility to heart disease and several forms of cancer may also benefit from supplementation.3,4

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-methylfolate 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 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 several different forms of folate including folic acid and N-methyltetrahydrofolate. The interaction between folic acid and folate binding protein is greater than methyltetrahydrofolate. Current assays on the market require an extraction step to release the folate derivatives from the folate binding protein.3,5

In the past, folate has been quantified in samples using such methods as microbiological assays, bio-specific procedures and HPLC-MS techniques; however, this rapid rise in knowledge of folate, its importance, and subsequently folate supplementation has caused a higher demand for improved testing methods.3

3.0 PRINCIPLE

Competitive Binding Protein Assay (TYPE 8): The essential reagents required for a competitive binding assay include specific binding protein, enzyme-antigen conjugate and native antigen. Upon mixing enzyme-antigen conjugate, biotinylated binding protein and a serum containing the native antigen, a competition reaction results between the native antigen and the enzyme-antigen conjugate for a limited number of binding sites. The interaction is illustrated by the followed equation:

\[
\text{EnzAg} + \text{Ag} + \text{BPBtn} \rightarrow \text{AgBPBtn} + \text{EnzAgBPBtn}
\]

Note 1: Do not use reagents beyond the kit expiration date. Note 2: Do not use reagents that are contaminated or have exceeded the expiration date. Note 3: Reopened reagents are stable for 60 days (60) when stored at 2-8°C. Kit and component stability are identified on the package insert. Above reagents are for a single 96-well microplate.

4.0 REAGENTS

Materials Provided:

A. Folate Calibrators – 1.0 ml/vial - Icon A-F
B. Folate Enzyme Reagent – 1.0 ml/vial – Icon E
E. Wash Solution Concentrate – 20.0 ml/vial - Icon W
C. Folate Biotin Reagent – 7.0 ml/vial - Icon B
D. Strepavidin CW = Streptavidin immobilized on well
F. Substrate Reagent – 12.0 ml/vial - Icon SN
G. Glass test tubes for calibrator, control, and patient sample preparation.

G. Glass test tubes for calibrator, control, and patient sample preparation.

H. Glass test tubes for calibrator, control, and patient sample preparation.

I. Stabilizing Agent – 0.7 ml/vial - Icon S
J. Neutralizing Buffer – 0.25 ml/vial - Icon N
K. Serum Evacuation Tube
L. Microplate washer or a squeeze bottle (optional).

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, are not FDCA approved tests. To date, immunometric tests have offered complete assurance that infectious agents are absent, 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, 1989, HHS Publication No. (CDC) 88-8385.

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 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 red top (with or without additives) tube for plasma use evacuated tube(s) containing heparin. Allow the blood to clot for neutralization reaction to go to completion (see above). Samples may be refrigerated at 2-8°C for a maximum period of two (2) 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 7 days. Avoid use of contaminated devices and repetitive freezing and thawing. When assayed in duplicate, 0.100 (100 µl) 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 demonstrate 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 trend. 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

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°C) for up to 60 days.

2. EXTRACTION AGENT
Add an aliquot of the stabilizing agent in order to prepare a 1:400 (stabilizing agent / releasing agent) dilute solution. For example, to make 4ml (400µl), add 0.100ml (100µl) stabilizing agent to 3.9ml (390µl) releasing agent.

3. SAMPLE EXTRACTION (See Note 3)
Obtain at least 1ml of whole blood for preparation of all patient samples, controls, and calibrators. Dispense 0.10ml (100µl) of the microplate 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 neutralization buffer and mix. After the neutralization buffer is added and mixed, let the reaction go to completion by warming an additional 5 min before dispensing into the microplates.

Note 1: Do not use the working substrate if it looks blue.
Note 2: Do not use reagents that are contaminated or have exceeded the expiration date.
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 add it near the bottom of the glass tubes at an angle while touching the side of the tubes.

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**

1. Prepare all samples according to the “Sample Extraction” procedure in section “8.0 Reagent Preparation”, it is important to wait 5 min before proceeding to allow the contaminated reaction to go to completion (see above).

2. Format the microttes wells for each calibrator, control and patient specimen to be assayed in duplicate. Replace any unused microttes strips 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 Enzyme Reagent to all wells

5. Mix the microttes gently for 20-30 seconds.

6. Add 0.050 ml (50 µl) of the Folate Biotin Reagent to all wells

7. Mix the microttes gently for 20-30 seconds.

8. Wait for 20 minutes. The cells will float to the surface.

9. Discard the contents of the microttes by decantation or aspiration. If decanting, blot the plate dry with absorbent paper without disturbing the cells.

10. Add 0.350 ml (350 µl) of wash buffer (see Reagent Preparation Section) to the calibrator, control and all patient sample wells. 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.

11. Add 0.100 ml (100 µl) of substrate reagent to all wells. Always add the substrate solution to each well in a single addition in 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 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 optical density at 305nm using a reference wavelength of 620-630nm. The results should be read within thirty (30) minutes of adding the stop solution.

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

Note: 1: Do not use reagents beyond the kit expiration date. 2: Do not use reagents that are contaminated or have exceeded the expiration date. 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 add it near the bottom of the glass tubes at an angle while touching the side of the tubes.**
10.0 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Folate 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 calibrator versus the corresponding Folate concentration in ng/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 Folate for an unknown, locate the average absorbance of the duplicates for each position on the X-axis and find the intersecting point on the curve, and read the concentration (in ng/ml) from the vertical axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (1.021) intersects the dose response curve at 11.9 ng/ml Folate concentration (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.

*The data, figure and table below are for example only. Do not use it for calculating your results.

11.0 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 ng/ml should be ≥ 1.3.
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 extend beyond ten (10) minutes to avoid assay drift.
3. Highly lipemic, hemolysed 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 recommended 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 steps 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 invalid 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.

12. Risk Analysis - as required by CE Mark IVDD Directive 98/79/EC - for this and all other devices, made by Monobind, can be requested via email at info@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 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 Folate AccuBind® 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>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>X</th>
<th>a</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>12</td>
<td>3.32</td>
<td>0.32</td>
<td>9.6</td>
</tr>
<tr>
<td>Level 2</td>
<td>12</td>
<td>8.85</td>
<td>0.68</td>
<td>7.7</td>
</tr>
<tr>
<td>Level 3</td>
<td>12</td>
<td>12.86</td>
<td>1.15</td>
<td>8.9</td>
</tr>
</tbody>
</table>

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

14.2 Sensitivity

The Folate AccuBind® ELISA Test System has a sensitivity of 0.52ng/ml. The sensitivity was ascertained by determining the variability of the 0 ng/ml serum calibrator and using the 2σ (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The Folate AccuBind® ELISA 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 samples was 30. The least square regression equation and the correlation coefficient were computed for this ELISA ELISA in comparison with the reference method. The data obtained is displayed in Table 4.

13.0 EXPECTED RANGES OF VALUES

In agreement with established reference intervals for a “normal” population the expected ranges for the Folate AccuBind® ELISA Test System are detailed in Table 1.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Interference</th>
<th>Bilirubin</th>
<th>LDH*</th>
<th>Lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Lypase</td>
<td>ND*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ND=Not Detectable

15.0 REFERENCES