**1.0 INTRODUCTION**

The Quantitative Determination of Circulating Ferritin Concentrations in Human Serum by a Microplate Enzyme Immunoassay, Colorimetric

**2.0 SUMMARY AND EXPLANATION OF THE TEST**

Ferritin in circulation, as measured in serum levels is a satisfactory index of body’s iron storage. The iron storage is directly measured by quantitative phlebotomy, iron absorption studies, liver biopsies and microscopic examinations of bone marrow aspirates. Iron deficiency (Anemia) and iron overload (Hemochromatosis) are conditions associated with body’s iron storage or lack thereof. Measurements of total iron binding capacity (TIBC) have widely been used as aids in the determination of iron deficiency. A reverse essay of serum ferritin, however, if not carefully performed, can result in overestimation of body iron stores.

Ferritin is present in blood in very low concentrations. Normally, approximately 1% of plasma iron is contained in Ferritin. The half life of Ferritin is about two weeks. In patients with certain chronic disorders, however, the half life of Ferritin can be significantly reduced. Circulating ferritin levels have been used by clinicians, as an aid, in the diagnosis of several other disorders. It has proved to be a valuable tool to aid in the diagnosis of iron deficiency and anemia due to other disorders and, in exposing the depletion of iron stores before the onset of anemia. Serial determinations have been used to monitor, non-invasively, the erosion of iron storage during pregnancy and in patients undergoing parenteral iron therapy. Serum ferritin is routinely used as a screen for iron deficiency for a variety of populations like blood donors and people who are receiving regular transfusions or iron replacement therapy.

In this method, ferritin calibrator, patient specimen or control is directly measured by quantitative phlebotomy, iron absorption studies, liver biopsies and microscopic examinations of bone marrow aspirates. Iron deficiency (Anemia) and iron overload (Hemochromatosis) are conditions associated with body’s iron storage or lack thereof. Measurements of total iron binding capacity (TIBC) have widely been used as aids in the determination of iron deficiency. A reverse essay of serum ferritin, however, if not carefully performed, can result in overestimation of body iron stores.

**3.0 PRINCIPLE**

Immunoenzymometric sequential assay (TYPE 4):

The essential reagents required for an immunoenzymometric assay include highly specific and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the formation of antigen-antibody complexes through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-ferritin antibody. Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen and the antibody, forming an antibody-antigen complex. Simultaneously the biotin attached to the antibody binds to the streptavidin coated on the microwells resulting in immobilization of the complex. The interaction is illustrated by the following equation:

\[
A_{\text{Ab}} + A_{\text{Ab(b)} + Ag(ferritin)} \rightarrow A_{\text{AbAb}} \rightarrow A_{\text{AbAb}} + Ag(ferritin)
\]

**4.0 REAGENTS**

Materials Provided:

A. Ferritin Calibrators – 1ml / vial - Icons A-F

B. Ferritin Enzyme Reagent – 13 ml/vial-Icon C

C. Ferritin Antibody (specific for ferritin) is added to the wells. The enzyme labeled antibody binds to the ferritin already immobilized on the well. Excess enzyme is washed off via a wash step. A color is observed. For accurate comparison to established normal values, precautions in the collection of venipuncture samples should be taken. The specimens shall be blood serum in type and the usual regulatory and statutory requirement.

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. The controls and patient specimen should be assayed within two (2) days, 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, 0.050ml (50µl) of the specimen is required.

**5.0 PRECAUTIONS**

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All procedures contain human serum have been found to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. Since no known test can certify that all enzyme labeled reagents that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory practices must be followed. Find guidelines for the use of the Center for Disease Control / National Institute of Health, “Guidelines for the Use of Biological and Biomedical Laboratories,” 2nd Edition, 1988, HHS Publicaton No. (CDC) 88-9395. Safe Disposal of kit components must be according to local regulatory and statutory requirement.

**6.0 SPECIMEN COLLECTION AND PREPARATION**

The specimens shall be blood serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, precautions in the collection of venipuncture samples should be taken. The specimens shall be blood serum in type and the usual regulatory and statutory requirement.

**7.0 QUALITY CONTROL**

Each laboratory should assay controls at levels in the low, normal and elevated 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. 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 1000ml with distilled or deionized water in a suitable storage container. Store at 2-30°C for up to 60 days.

2. Working Substrate Solution – Stable for one year

Pour the contents of the amber vial labeled Solution ‘A’ into the clear vial labeled Solution ‘B’. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.

**9.0 TEST PROCEDURE**

Before proceeding with the assay, bring all reagents, serum reference calibrators and controls to room temperature (20-27°C). Test Procedure should be performed by a skilled individual or trained professional!

1. Format the microplates for each serum, 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 0.025 ml (25µl) of the appropriate serum reference, control or patient specimen into the assigned well.

3. Add 0.100 ml (100µl) of the Ferritin Biotin Reagent to each well. It is very important to dispense all reagents close to the bottom of the microplate. Store at 2-8°C.

4. Swirl the microplate gently for 20-30 seconds to mix and cover.

5. Incubate 30 minutes at room temperature.

6. Dispense contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with a paper towel.

7. Add 3.50µl of wash buffer (see Reagent Preparation Section), and 7.00µl (3.50+3.50µl) of substrate mixture will be used. For 96-well microplates, a total volume of 20 µl per well should be used.

8. Add 0.100 ml (100µl) of the Ferritin Enzyme Conjugate to each well.

9. Incubate 30 minutes at room temperature.

Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with a paper towel.

10. Read the absorbance in each well at 450nm (using a reference calibrators and controls to room temperature (20-27°C).

11. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with a paper towel.

12. Add 0.050ml of wash buffer to each well. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly. Store at 2-8°C.

13. Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store at 2-30°C for up to 60 days.

14. Add 0.050ml (50µl) of substrate solution to each well and mix gently for 15-20 seconds.

15. Read the absorbance capability.

**10.0 CALCULATION OF RESULTS**

A dose response curve is used to ascertain the concentration of ferritin 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 ferritin concentration in ng/ml on linear graph paper.
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of ferritin for an unknown, locate the absorbance 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 ng/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 of the four repeats of the assay should be used in lieu of a dose response curve.
5. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
6. If 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.
7. Plate readers measure vertically. Do not touch the bottom of the wells.
8. It is important that the time of reaction in each well is held constant to achieve reproducible results.
9. 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 maintenance.
10. The within and between assay precisions of the ferritin AccuBind® ELISA test system were determined by analyses on three different in-house ranges can be determined by the technicians using the method of control sera. The number (N), mean value (X), standard deviation (σ), and coefficient of variation (C.V.) for each of these control sera are presented in Table 2 and Table 3.
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. The cross-reactivity of the ferritin AccuBind® ELISA test system to other iron-related proteins and substances were 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 Ferritin needed to produce the apparent concentration.

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>11</td>
<td>1.3</td>
<td>11.8%</td>
</tr>
<tr>
<td>Level 2</td>
<td>13</td>
<td>2.0</td>
<td>15.4%</td>
</tr>
<tr>
<td>Level 3</td>
<td>15</td>
<td>2.5</td>
<td>16.7%</td>
</tr>
</tbody>
</table>

8.11 2.33 1.36

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>X</th>
<th>σ</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>10</td>
<td>1.0</td>
<td>10.0%</td>
</tr>
<tr>
<td>Level 2</td>
<td>11</td>
<td>1.1</td>
<td>9.1%</td>
</tr>
<tr>
<td>Level 3</td>
<td>12</td>
<td>1.2</td>
<td>9.7%</td>
</tr>
</tbody>
</table>

#### 11.0 G. C. PARAMETERS

In order for the assay results to be considered valid the following criteria should be met.
1. The absorbance (OD) of Calibrator F should be ≥ 1.3
2. The absorbance of the A calibrator should be ≤ 0.05
3. Four out of six quality control pools should be within the established ranges.

#### 12.0 RISK ANALYSIS

#### 13.0 EXPECTED RANGE OF VALUES

Approximate reference ranges for normal males and female adults were established by using 400 normal sera with the Ferritin AccuBind® ELISA test system.

### Table 1

#### Example 1

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Well</th>
<th>Abs</th>
<th>Mean Abs (B)</th>
<th>Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>0.002</td>
<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>0.003</td>
<td>0.004</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cal B</td>
<td>C1</td>
<td>0.110</td>
<td>0.112</td>
<td>10</td>
</tr>
<tr>
<td>D1</td>
<td>0.115</td>
<td>0.116</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cal C</td>
<td>E1</td>
<td>0.586</td>
<td>0.617</td>
<td>50</td>
</tr>
<tr>
<td>F1</td>
<td>0.647</td>
<td>0.677</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Cal D</td>
<td>G1</td>
<td>1.204</td>
<td>1.262</td>
<td>150</td>
</tr>
<tr>
<td>H1</td>
<td>1.350</td>
<td>1.399</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Cal E</td>
<td>A2</td>
<td>1.947</td>
<td>1.917</td>
<td>400</td>
</tr>
<tr>
<td>B2</td>
<td>1.887</td>
<td>1.877</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>Cal F</td>
<td>C2</td>
<td>2.586</td>
<td>2.561</td>
<td>800</td>
</tr>
<tr>
<td>D2</td>
<td>2.539</td>
<td>2.524</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>Cal E</td>
<td>E2</td>
<td>0.707</td>
<td>0.721</td>
<td>66.1</td>
</tr>
<tr>
<td>F2</td>
<td>0.734</td>
<td>0.749</td>
<td>66.1</td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>A1</td>
<td>1.285</td>
<td>1.287</td>
<td>154.0</td>
</tr>
<tr>
<td>B2</td>
<td>1.285</td>
<td>1.287</td>
<td>154.0</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>A1</td>
<td>1.671</td>
<td>1.647</td>
<td>301.6</td>
</tr>
</tbody>
</table>

### Figure 1

The absorbance obtained from the printout of the microplate reader as outlined in Example 1 and 2.

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipette reader as should not extend beyond ten (10) minutes to avoid assay drift.