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 of iron storage. Normal ranges for body's total iron binding capacity (TIBC) have been widely used as aids in the determination of these conditions. However, an assay of serum ferritin is simply more sensitive and reliable means of diagnosis these disorders. Ferritin is present in blood in very low concentrations. Normally, approximately 1% of plasma iron is contained in Ferritin. The plasma concentrations of ferritin decline very early in anemic conditions like development of iron deficiency, long before the iron stores become depleted in the blood in parallel to the decrease in body's total iron binding capacity. This is due to the increased rate of release of intracellular ferritin into the plasma. Ferritin can be released as an acute phase reactant, for example, in patients with viral hepatitis or after a toxic liver injury when iron is released from the injured liver cells. Elevated serum ferritin levels are found with viral hepatitis or after a toxic liver injury as a release of ferritin from the injured liver cells. Elevated serum ferritin levels are also found in patients with hemochromatosis and hemosiderosis. In patients who have these chronic disorders together with iron deficiency, serum ferritin levels are often normal. An increase in serum ferritin is observed in patients with viral hepatitis or after a toxic liver injury as a release of ferritin from the injured liver cells. Elevated serum ferritin levels are found in patients with hemochromatosis and hemosiderosis.

2.1 IMMUNOGENETIC SEQUENTIAL ASSAY (TYPE 4)

The essential reagents required for an immunoenzymometric assay like this assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in excess and antibody-antigen complex. An immobilization step is placed between the antibody bound to the well and the excess antibody bound to the antibody-antigen complex. The interaction is illustrated by the following equation:

\[ \text{Ag(ferritin)} + 
\text{BtnAb(m)} 
\rightarrow \text{Ag(ferritin)} - \text{BtnAb(m)} \]

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

Six (6) vials of Ferritin calibrators at levels of 0(A), 10(B), 50(C), 100(D), 500(E) and 800(F) ng/ml. Store at 2-8°C. A preservative has been added.

Note: The calibrators, human serum based, were calibrated using the native antigen, which was assayed against the WHO 3rd 64 94572

B. Ferritin Biotin Reagent – 13 ml/vial – Icon V

One (1) vial containing biotinylated mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

C. Ferritin Enzyme Reagent – 13 ml/vial–Icon E

One (1) vial containing Horseradish Peroxidase (HRP) labeled anti-ferritin (goat in buffer, dye and preservatives. Store at 2-8°C.

D. Streptavidin Coated Plate – 96 wells – Icon C

One (1) pack describing streptavidin coated plastic, with 8 vials per pack. Store at 2-8°C.

E. Wash Solution Concentrate – 20 ml/vial–Icon O

One (1) vial containing tertamethylbenzidine (TMB) in buffer. Store at 2-8°C.

F. Substrate A – 7 ml/vial – Icon S

One (1) vial containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.

G. Substrate B – 7 ml/vial – Icon S

One (1) vial containing hydrochloric acid (HCl) in buffer. Store at 2-8°C.

H. Stop Solution – 8 ml/vial –Icon C

One (1) vial containing stop solution. Store at 2-8°C.

I. Kit Expiration Date: 1 year after receipt, if stored at temperatures of -20°C for up to 60 days.

2.2 SPECIMEN COLLECTION AND PREPARATION

1. Dilute contents of wash solution to 1000 ml 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: Pipette 0.05 ml of Substrate A into the clear vial labeled Substrate B. Place the yellow cap on the clear vial for easy identification. Mix and label accordingly.

Note 1: Do not use the working substrate if it looks blue.

Note 2: Do not use reagents that are contaminated or have bacteria growth.

3. QUALITY CONTROL

7.1 SPECIMEN COLLECTION AND PREPARATION

1. Centrifuge the specimen to separate the serum from the cells.

2. Dispense 0.025 ml (25 µl) of the appropriate serum reference, control or specimen into the assigned well.

3. Add 0.050 ml of Ferritin Biotin Reagent to each well.

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

5. Incubate 30 minutes at room temperature.

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

7. Add 350 µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) and 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, ensure that the bottle is inverted to remove all air bubbles (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.

8. Add 0.050 ml (50 µl) of Ferritin Enzyme Reagent to conjugate to each well.

9. Incubate 30 minutes at room temperature.

10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.

11. Add 350 µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) and aspirate. Repeat two (2) additional times for a total of three (3) washes.

12. Add 0.100 ml (100 µl) of working sample solution to all wells, mix and cover for 15 minutes.

13. Add 350 µl of Stop Solution (see Reagent Preparation Section), decant (tap and blot) and aspirate. Repeat two (2) additional times for a total of three (3) washes.

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

15. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630 nm) to minimize any well imperfections in a microplate reader. The results should be read within thirty (30) minutes of adding the stop solution.

Note: Always add reagents in the same order to minimize reaction time differences between wells.
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 average 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 duplicate of the unknown may be averaged as indicated. In the following example, the average absorbance (1.287) intersects the dose response curve at 154 ng/ml ferritin concentration (See Figure 1).

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

EXAMPLE 1

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Well</th>
<th>Abs</th>
<th>Mean Abs</th>
<th>Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal A</td>
<td>A1</td>
<td>0.022</td>
<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td>Cal B</td>
<td>C1</td>
<td>0.110</td>
<td>0.112</td>
<td>10</td>
</tr>
<tr>
<td>Cal C</td>
<td>E1</td>
<td>0.586</td>
<td>0.617</td>
<td>50</td>
</tr>
<tr>
<td>Cal D</td>
<td>G1</td>
<td>2.024</td>
<td>3.928</td>
<td>150</td>
</tr>
<tr>
<td>Cal E</td>
<td>A2</td>
<td>1.947</td>
<td>1.917</td>
<td>400</td>
</tr>
<tr>
<td>Cal F</td>
<td>C2</td>
<td>2.588</td>
<td>2.561</td>
<td>800</td>
</tr>
</tbody>
</table>

The within and between assay precisions of the ferritin AccuBind ELISA test system were determined by analyses on three different levels 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.

11.0 Q.C. PARAMETERS

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

1. The absorbance (OD) of the ‘OD’ blank should be > 1.8
2. The absorbance of the A calibrator should be < 0.05
3. Three of four out quality control pools should be within the established ranges.

12.0 RISK ANALYSIS

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