



Ferritin Test System
Product Code: 2825-300

1.0 INTRODUCTION

Intended Use: 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 these conditions. However, an assay of serum Ferritin is simply more sensitive and reliable means of demonstration these disorders.

Ferritin is present in blood in very low concentrations. Normally, approximately 1% of plasma iron is contained in Ferritin. The plasma ferritin, is in equilibrium with body stores, and variations of iron storage. The plasma concentrations of ferritin decline very early in anemic conditions like development of iron deficiency, long before the changes are observed in the blood hemoglobin concentration, size of the erythrocytes and TIBC. Thus measurements of serum ferritin can serve as an early indicator of iron deficiency that is uncomplicated by other concurrent conditions. At the same time a large number of chronic conditions can result in elevated levels of serum ferritin. These include chronic infections, chronic inflammatory diseases such as rheumatoid arthritis, heart disease and some other malignancies, especially lymphomas, leukemia, breast cancer and neuroblastoma. In patients who have these chronic disorders together with iron deficiency, serum ferritin levels are often normal. An increase in circulating 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.

Circulating ferritin levels have been used by clinicians, as an aid, in the diagnosis of several other disorders. It has proved as a valuable tool in differential diagnosis of anemia due to iron deficiency and anemias due to other disorders and, in exposing the depletion of iron reserves long 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 dialysis. 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 blood transfusions or iron replacement therapy.

In this method, ferritin calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal antibody (specific for ferritin) is added and the reactants mixed. Reaction results between the biotinylated ferritin antibody and

native ferritin to form an immune complex that is deposited on the streptavidin coated well. The excess serum proteins are washed away via a wash step. Another ferritin specific antibody, labeled with an enzyme, 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 generated by the addition of a substrate. The intensity of the color generation is directly proportional to the concentration of the ferritin in the sample.

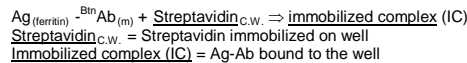
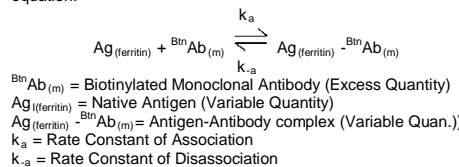
The employment of several serum references of known ferritin levels permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with ferritin concentration.

3.0 PRINCIPLE

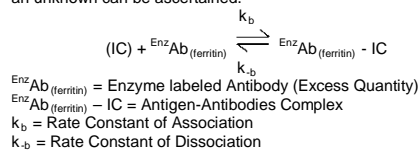
Immunoenzymometric sequential assay (TYPE 4):

The essential reagents required for an immunoenzymometric assay include high affinity 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 assay at the surface of a microplate well 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, reaction results between 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:



After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells. Excess enzyme is washed off via a wash step. A suitable substrate is added to produce color measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly 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:

- A. Ferritin Calibrators – 1 ml / vial - Icons A-F**
 Six (6) vials of Ferritin calibrators at levels of 0(A), 10(B), 50(C), 150(D), 400(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 a reference preparation, which was assayed against the WHO 3rd IS 94/572
- B. Ferritin Biotin Reagent – 13ml/vial - Icon ▽**
 One (1) vial containing biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°C.

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

- One (1) vial containing Horseradish Peroxidase (HRP) labeled anti-ferritin IgG in buffer, dye and preservatives. Store at 2-8°C.
- D. Streptavidin Coated Plate – 96 wells – Icon ↓**
 One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.
- E. Wash Solution Concentrate – 20 ml/vial - Icon ⬇**
 One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.
- F. Substrate A – 7ml/vial - Icon S^A**
 One (1) vial containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- G. Substrate B – 7ml/vial - Icon S^B**
 One (1) vial containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.
- H. Stop Solution – 8ml/vial - Icon ⊞^{STOP}**
 One (1) vial containing a strong acid (1N HCl). Store at 2-8°C.

I. Product Instructions.

- 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:

1. Pipette capable of delivering 0.025 and 0.050ml (25 & 50µl) volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100 and 0.350ml (100 and 350µl) volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. 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 licensed reagents. 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 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, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot for 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, 0.050ml (50µl) of the specimen is required.

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.

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.

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' wells for each serum reference, 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 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 coated 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 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or 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, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100µl) of the Ferritin Enzyme Conjugate to each well.
DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION
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 350µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes.
12. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section).
DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
13. Incubate at room temperature for fifteen (15) minutes.
14. Add 0.050ml (50µl) 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 620-630nm to minimize 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.

- Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
- Plot the absorbance for each duplicate serum reference versus the corresponding ferritin concentration in ng/ml on linear graph paper.
- Draw the best-fit curve through the plotted points.
- 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 duplicates 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).

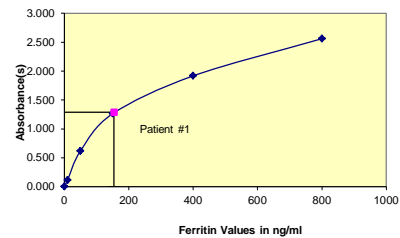
Note: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such software is utilized, the validation of the software should be ascertained.

EXAMPLE 1

| Sample ID | Well | Abs | Mean Abs (B) | Conc |
|-----------|------|-------|--------------|-------|
| Cal A | A1 | 0.002 | 0.003 | 0 |
| | B1 | 0.003 | | |
| Cal B | C1 | 0.110 | 0.112 | 10 |
| | D1 | 0.113 | | |
| Cal C | E1 | 0.586 | 0.617 | 50 |
| | F1 | 0.647 | | |
| Cal D | G1 | 1.204 | 1.262 | 150 |
| | H1 | 1.320 | | |
| Cal E | A2 | 1.947 | 1.917 | 400 |
| | B2 | 1.887 | | |
| Cal F | C2 | 2.586 | 2.561 | 800 |
| | D2 | 2.536 | | |
| Ctrl 1 | E2 | 0.707 | 0.721 | 66.1 |
| | F2 | 0.734 | | |
| Patient 1 | G2 | 1.289 | 1.287 | 154.0 |
| | H2 | 1.285 | | |
| Patient 2 | A3 | 1.647 | 1.659 | 301.6 |
| | B3 | 1.671 | | |

*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.

Figure 1



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 F should be ≥ 1.3
- The absorbance of the A calibrator should be ≤ 0.05
- 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 is 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 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.
- 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 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 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.
- Patient specimens with ferritin concentrations above 800 ng/ml may be diluted (for example 1/10) with normal serum stripped of ferritin and re-assayed. The sample's concentration is obtained by multiplying the result by the dilution factor (10).
- Each component in one assay should be of the same lot number and stored under identical conditions.

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

| Males | 16-220 ng/ml |
|---------|--------------|
| Females | 10-124 ng/ml |

In addition to the above the following ranges were assigned based on the available literature. However, these ranges were confirmed using AccuBind® Ferritin Microplate Elisa Procedure with limited number of samples.

| Newborn | 22-220 ng/ml |
|---------------|----------------|
| 1-2 Months | 190-610 ng/ml |
| 2-5 Months | 50-220 ng/ml |
| 6Mos - 16 Yrs | 10 - 160 ng/ml |

It is important to keep in mind that any normal range establishment is dependent upon a multiplicity of factors like the specificity of the method, the locale, the population tested and the precision of the method in the hands of technicians. 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 technicians 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 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.

| TABLE 2 Within Assay Precision (Values in ng/ml) | | | | |
|---|----|-------|----------|------|
| Sample | N | X | σ | C.V. |
| Level 1 | 20 | 43.5 | 1.36 | 3.1% |
| Level 2 | 20 | 110.5 | 6.10 | 5.5% |
| Level 3 | 20 | 349.6 | 7.54 | 2.2% |

| TABLE 3 Between Assay Precision* (Values in ng/ml) | | | | |
|---|----|-------|----------|------|
| Sample | N | X | σ | C.V. |
| Level 1 | 10 | 41.2 | 2.33 | 5.5% |
| Level 2 | 10 | 113.2 | 8.11 | 7.2% |
| Level 3 | 10 | 372.4 | 11.80 | 3.2% |

*As measured in ten experiments in duplicate.

14.2 Sensitivity

The minimum detectable dose (*Sensitivity*) is defined as the apparent concentration 2σ above the absorbance for zero calibrator. 2σ of the mean absorbance for twenty replicates for zero calibrator for the ferritin AccuBind™ ELISA test system gave a sensitivity of 0.17 ng/ml.

14.3 Specificity

The cross-reactivity of the ferritin AccuBind® ELISA test system 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 Ferritin needed to produce the same absorbance.

| Substance | Cross Reactivity |
|----------------------|------------------|
| Liver Ferritin | 100% |
| Spleen Ferritin | 100% |
| Human Heart Ferritin | <1.0% |
| Hemoglobin | <0.1% |

14.4 High Dose Effect

Since the assay is sequential in design, high concentrations of ferritin do not show the hook effect. Samples with concentrations over 50,000 ng/ml demonstrated extremely high levels of absorbance

15.0 REFERENCES

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| Reagent (fill) | Size | 96(A) | 192(B) |
|----------------|------|----------|----------|
| | A) | 1ml set | 1ml set |
| | B) | 1 (13ml) | 2 (13ml) |
| | C) | 1 (13ml) | 2 (13ml) |
| | D) | 1 plate | 2 plates |
| | E) | 1 (20ml) | 1 (20ml) |
| | F) | 1 (7ml) | 2 (7ml) |
| | G) | 1 (7ml) | 2 (7ml) |
| | H) | 1 (8ml) | 2 (8ml) |

For Orders and Inquiries, 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)

