



Ferritin

Product Code: 2825-300

Intended Use: The Quantitative Determination of Circulating Ferritin Concentrations in Human Serum by a Microplate Immunoassay

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.

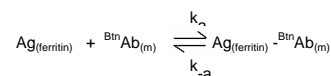
The employment of several serum references of known ferritin levels permits the construction of a dose response curve of activity and concentration. From comparison to the dose response curve, an unknown specimen's activity can be correlated with ferritin concentration.

PRINCIPLE

Immunoassay sequential assay (TYPE 4):

The essential reagents required for an immunoassay sequential 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:



$\text{B}^{\text{in}}\text{Ab}_{(\text{m})}$ = Biotinylated Monoclonal Antibody (Excess Quantity)

$\text{Ag}_{(\text{ferritin})}$ = Native Antigen (Variable Quantity)

$\text{Ag}_{(\text{ferritin})} - \text{B}^{\text{in}}\text{Ab}_{(\text{m})}$ = Antigen-Antibody complex (Variable Quan.)

k_a = Rate Constant of Association

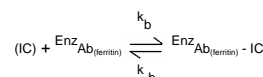
k_a = Rate Constant of Disassociation

$\text{Ag}_{(\text{ferritin})} - \text{B}^{\text{in}}\text{Ab}_{(\text{m})} + \text{Streptavidin}_{\text{C.W.}} \rightarrow \text{immobilized complex (IC)}$

$\text{Streptavidin}_{\text{C.W.}}$ = Streptavidin immobilized on well

Immobilized complex (IC) = Ag-Ab bound to the well

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.



$\text{Enz Ab}_{(\text{ferritin})}$ = Enzyme labeled Antibody (Excess Quantity)

$\text{Enz Ab}_{(\text{ferritin})} - \text{IC}$ = Antigen-Antibodies Complex

k_b = Rate Constant of Association

k_d = Rate Constant of Dissociation

REAGENTS

Provided:

- Ferritin Calibrators --1ml / 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
- Ferritin Biotin Reagent —13ml/vial - Icon**
One (1) vial containing biotinylated monoclonal mouse IgG in buffer, dye, and preservative. Store at 2-8°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.
- 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.
- Wash Solution-- 20 ml - Icon**
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.
- Substrate A --7ml/vial - Icon S^A**
One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.
- Substrate B -- 7ml/vial - Icon S^B**
One (1) bottle containing hydrogen peroxide (H₂O₂) in buffer. Store at 2-8°C.
- Stop Solution -- 8ml/vial - Icon**
One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.
- Product Insert.**

Note 1: Do not use reagents beyond the kit expiration date.

Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.

Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

- Pipette capable of delivering 25, & 50µl volumes with a precision of better than 1.5%.
- Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
- Microplate washers or a squeeze bottle (optional).
- Microplate Reader with 450nm and 620nm wavelength absorbance capability.
- Absorbent Paper for blotting the microplate wells.
- Plastic wrap or microplate cover for incubation steps.
- Vacuum aspirator (optional) for wash steps.
- Timer.
- Quality control materials.

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.

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.

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 repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the specimen is required.

REAGENT PREPARATION:

- Wash Buffer**
Dilute contents of wash solution to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature (20-27°C) for up to 60 days.
- Working Substrate Solution**
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: Do not use the working substrate if it looks blue.

TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27° C).

- 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.**
- Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.
- 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.**
- Swirl the microplate gently for 20-30 seconds to mix and cover.
- 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 absorbent paper.
- Add 300µl 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.**
- Add 0.100 ml (100µl) of the Ferritin Enzyme Conjugate to each well.
DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION
- Incubate 30 minutes at room temperature.
- Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
- Add 300µl 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.
- Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section).
DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
- Incubate at room temperature for fifteen (15) minutes.
- Add 0.050ml (50µl) of stop solution to each well and mix gently for 15-20 seconds.
- 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.

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.

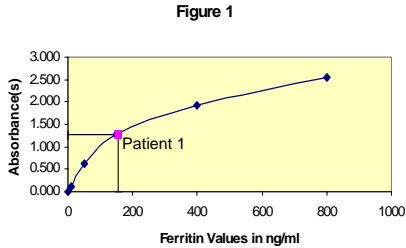
CALCULATION OF RESULTS

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 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 IEMA Elisa assays may also be used for the data reduction.

*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.



EXAMPLE 1

I.D.	Well Position	Absorbance	Mean Absorbance (B)	Concentration
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		

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 F should be ≥ 1.3 .
- 2. The absorbance of the A calibrator should be ≤ 0.1 .
- 3. Four out of six quality control pools should be within the established ranges.

LIMITATIONS OF PROCEDURE

A. Assay Performance

- 1. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten (10) minutes to avoid assay drift. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
- 2. Addition of the substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the addition of the substrate and the stopping solution should be added in the same sequence to eliminate any time deviation during reaction.
- 3. Plate readers measure vertically. Do not touch the bottom of the wells.
- 4. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
- 5. Sample(s), which are contaminated microbiologically, should not be used in the assay. Highly lipemic or hemolysed specimen(s) should similarly not be used.
- 6. 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).
- 7. Each component in one assay should be of the same lot number and stored under identical conditions.

B. Interpretation

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.

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.

PERFORMANCE CHARACTERISTICS

A. 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.

B. 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 1.0 ng/ml.

C. 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%

D. 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

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Instruments & Applications

Monobind's immunoassay products are designed to work in both manual and automated lab environments. AccuBind™ and AccuLite™ are compatible with any open-ended instrumentation, including chemistry analyzers, microplate readers and microplate washers. There may or may not be an application developed for your particular instrument, please visit the instrument section of our website, or contact techsupport@monobind.com

Monobind offers several instruments, including the Impulse 2 Luminometer CLIA Plate Reader designed hand-in-hand with our products and capable of 2-point calibration. Visit our website for more information.