FERRITIN ELISA

Direct immunoenzymatic determination of Ferritin in human serum or plasma

INTENDED USE
Immunoenzymatic colorimetric method for quantitative determination of Ferritin concentration in human serum or plasma. Ferritin ELISA kit is intended for research use only.

1. CLINICAL SIGNIFICANCE
Ferritin is a globular protein found mainly in the liver, which can store about 2250 iron (Fe²⁺) ions. The ferritin molecule consists of a protein shell (apoferritin) composed of heavy and light subunits, which surrounds a crystalline core containing iron oxide and phosphate.

Ferritin is synthesized in the liver, spleen and numerous other body tissues, with major concentrations found in the liver, spleen, bone marrow, and intestinal mucosa.

The ferritin levels measured have a direct correlation with the total amount of iron stored in the body. If ferritin is high there is iron in excess, which would be excreted in the stool. If ferritin is low there is a risk for lack in iron, which sooner or later could lead to anaemia.

In the setting of anaemia, serum ferritin is the most sensitive lab test for iron deficiency anaemia. In contrast, serum ferritin levels are normal or increased in anaemia associated with chronic disease. Elevated serum ferritin levels have been observed in acute and chronic liver disease and lymphoid malignancy (leukemia and Hodgkin lymphoma). High serum ferritin levels have also been associated with an elevated risk for myocardial infarction in men. Ferritin is also used as a marker for iron overload disorders, such as haemochromatosis in which the ferritin level may be abnormally raised.

Ferritin is an acute-phase reactant, it is often elevated in the course of disease.

Free iron is toxic to cells, and the body has an elaborate set of protective mechanisms to bind iron in various tissue compartments. Within cells, iron is stored complexed to protein as ferritin or hemosiderin. Apoferritin binds to free ferrous iron and stores it in the ferric state. Under steady state conditions, the serum ferritin level correlates with total body iron stores; thus, the serum ferritin level is the most convenient laboratory test to estimate iron stores.

2. PRINCIPLE
Diametra Ferritin ELISA test is based on simultaneous binding of human Ferritin to two monoclonal antibodies, one immobilized on microwell plates and the other conjugated with horseradish peroxidase (HRP).

After incubation the bound/free separation is performed by a simple solid-phase washing. Then the enzyme HRP in the bound-fraction reacts with the Substrate (H₂O₂) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H₂SO₄) is added. The colour intensity is proportional to the Ferritin concentration in the sample. The Ferritin concentration in the sample is calculated based on a standard curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION
3.1. Reagents and materials supplied in the kit
1. Calibrators (6 vials)
   - CAL0 (3 mL)
   - CAL1 (1 mL)
   - CAL2 (1 mL)
   - CAL3 (1 mL)
   - CAL4 (1 mL)
   - CAL5 (1 mL)
   - REF DCE002/3906-0
   - REF DCE002/3907-0
   - REF DCE002/3908-0
   - REF DCE002/3909-0
   - REF DCE002/3910-0
   - REF DCE002/3911-0
2. Control (1 vial, 1 mL)
   - Control concentration is indicated on the Certificate of Analysis
     - REF DCE045/3903-0
3. Conjugate (1 vial, 12 mL)
   - Anti Ferritin antibody conjugated with Horseradish peroxidase (HRP)
     - REF DCE002/3902-0
4. Coated Microplate (1 breakable microplate)
   - Anti Ferritin antibody adsorbed on microplate
     - REF DCE002/3903-0
5. TMB Substrate (1 vial, 15 mL)
   - H₂O₂-TMB 0.26 g/L (avoid any skin contact)
     - REF DCE004-0
6. Stop Solution (1 vial, 15 mL)
   - Sulphuric acid 0.15 mol/L (avoid any skin contact)
     - REF DCE005-0
7. 10X Conc. Wash Solution (1 vial, 50 mL)
   - Phosphate buffer 0.2M, pH 7.4
     - REF DCE054-0
3.2 Reagents necessary not supplied

Distilled water.

3.3 Auxiliary materials and instrumentation

Automatic dispenser.

Microplates reader (450 nm, 620-630 nm)

Note

Store all reagents between 2-8°C in the dark. Open the bag of reagent 4 (Coated Microplate) only when it is at room temperature and close it immediately after use; once opened, it is stable until the expiry date of the kit.

Do not remove the adhesive sheets on the strips unutilised.

4. WARNINGS

- This kit is intended for in vitro use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- All human source material used in the preparation of the reagents has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, Calibrators and Controls should be handled in the same manner as potentially infectious material.
- Some reagents contain small amounts of Proclin 300® as preservative. Avoid the contact with skin or mucosa.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H2O2 to directed sunlight, metals or oxidants. Do not freeze the solution.
- This method allows the determination of Ferritin from 5 to 1000 ng/mL.

5. PRECAUTIONS

- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background. To improve the performance of the kit on automatic systems is recommended to increase the number of washes.
- 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 minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of reagents.
- Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1 Preparation of the Calibrators (C0…C5)

The Calibrators are ready to use, are calibrated against the WHO 1st IS Ferritin 80/602 and have the following concentrations:

<table>
<thead>
<tr>
<th>ng/mL</th>
<th>C0</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
<td>20</td>
<td>100</td>
<td>400</td>
<td>1000</td>
</tr>
</tbody>
</table>

For sample with concentration over 1000 ng/mL dilute the sample with C0.

Once opened, the Calibrators are stable 6 months at 2-8°C.

6.2 Preparation of Wash Solution

Dilute the content of each vial of the “10X Conc. Wash Solution” with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.

In concentrated wash solution it is possible to observe the presence of crystals; in this case mix at room temperature until the complete dissolution of crystals; for greater accuracy, dilute the whole bottle of concentrated wash solution to 500 mL, taking care to transfer completely the crystals, then mix until crystals are completely dissolved.
6.3. Preparation of the Sample
Ferritin determination should be done in human serum or plasma. Specimen can be stored at 2-8°C for at short time (max five days). For longer storage the specimen should be frozen at -20°C. Avoid repeated freezing and thawing. The Control is ready to use.

6.4. Procedure
- **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.** At the end of the assay, store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C₀–C₅), two for each Control, two for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Sample/Control</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample/Control</td>
<td></td>
<td>20 µL</td>
<td></td>
</tr>
<tr>
<td>Calibrator C₀–C₅</td>
<td>20 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjugate</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

Incubate for 1 hour at room temperature (22-28°C). Remove the content from each well. Wash the wells 3 times with 300 µL of diluted wash solution.

**Important note:** during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel.

**Automatic washer:** if you use automated equipment, wash the wells at least 5 times.

**TMB Substrate** 100 µL 100 µL 100 µL

Incubate for 10 minutes at room temperature (22-28°C) in the dark.

**Stop Solution** 100 µL 100 µL 100 µL

Shake the microplate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.

7. QUALITY CONTROL
Each laboratory should assay controls at normal, high and low levels range of Ferritin 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. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. 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. RESULTS

8.1. Mean Absorbance
Calculate the mean of the absorbencies (Em) corresponding to the single points to the calibration curve (C₀–C₅) and of each sample.

8.2. Calibration curve
Plot the values of absorbance (Em) of the Calibrators (C₀–C₅) against concentration. Draw the best-fit curve through the plotted points. (es: Cubic Spline or Four Parameter Logistic).

8.3. Calculation of Results
Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in ng/mL.

9. REFERENCE VALUES
The serum values are comprised in the following intervals:

<table>
<thead>
<tr>
<th></th>
<th>Mean (ng/mL)</th>
<th>Range (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>53</td>
<td>6 – 180</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>105</td>
<td>8 – 350</td>
</tr>
<tr>
<td>Men</td>
<td>175</td>
<td>20 – 400</td>
</tr>
</tbody>
</table>

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.
10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision

10.1.1. Intra Assay
Within run variation was determined by replicate (15x) the measurements of three different control sera in one assay. The within assay variability is ≤ 7.5%.

10.1.2. Inter Assay
Between run variations was determined by replicate (16x) the measurements of three different control sera in different lots. The between assay variability is ≤ 6.1%.

10.2. Specificity
The cross reaction of the antibody calculated on a weight/weight basis are shown in the table:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Human Iso-Ferritin</td>
<td>100%</td>
</tr>
<tr>
<td>Spleen Human Iso-Ferritin</td>
<td>80%</td>
</tr>
<tr>
<td>Hearth Human Iso-Ferritin</td>
<td>12%</td>
</tr>
</tbody>
</table>

10.3. Accuracy
The recovery of 12.5 – 25 – 50 – 100 – 200 ng/mL of Ferritin added to sample gave an average value (±SD) of 98.66% ± 2.90%.
The dilution test performed on 3 samples diluted up to 8 times gave an average value (±SD) of 102.11% ± 5.32%.

10.4. Sensitivity
The lowest detectable concentration of Ferritin that can be distinguished from the Calibrator 0 is 0.04 ng/mL at the 95% confidence limit.

10.5. Correlation with RIA
Diametra Ferritin ELISA kit was compared to another commercially available Ferritin assay. Serum samples of 22 females and 32 males were analysed according in both test systems.
The linear regression curve was calculated
\[(\text{Ferritin Diametra}) = 1.11*(\text{Ferritin Diasorin}) - 10.46\]
\[r^2 = 0.972\]

10.6. Hook Effect
Ferritin ELISA kit, a competitive enzyme immunoassay, shows no Hook Effect up to 50000 ng/mL

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation