

**REF**

GD7000 00

# FERRITIN

Enzyme-immunoassay for the quantitative determination of Ferritin in serum or plasma

**IVD**

## INDICATION

Iron is present in human organism in essential compounds (haemoglobin, myoglobin, and enzymes), reserve (ferritin) or transport (transferring). Ferritin molecule consists of a proteic shell (apoferritin) constituted by 24 polypeptidic subunits, with a molecular weight of 450.000 kDa, and by a core of ferric hydroxyphosphate that can be composed by a variable number of atoms (up to 4000 per apoferritin molecule).

Two types of polypeptidic subunits can be distinguished: H subunits, more acid (pI 4.8-5.2) and heavy (PM 21.000), and L subunits more basic (pI 5.3-5.8) and light (PM 19.000). Different combinations of the two types give origin to different forms of ferritin, called *isoferritins*. H types are found in heart and kidney (acid isoferritin), while L types are found in liver and spleen (basic isoferritin). In serum mainly basic isoferritin is found, with a very low iron content.

Plasma ferritin concentration in men is threefold fertile women one and is related to the total reserves of the organism.

In case of iron deficit or overload, ferritin concentration in plasma is correlated to its concentration in tissues, while in case of tissue damages (acute or chronic liver diseases) or tumors (leukaemia) plasma concentration is elevated.

Measurement of plasma ferritin is indicated in case of: differential diagnosis of anaemia (sideropenic a., pernicious a., or caused by chronic disease), acute or chronic liver diseases, chronic renal failure (patients in haemodialysis), iron overload monitoring (patients with  $\beta$  thalassemia major, politransfused subjects, idiomatic hemochromatosis) substitutive iron therapy monitoring, iron reserve monitoring in pregnant women and in blood donors, tumor marker, particularly in case of acute myeloblastic leukaemia, Hodgkin's disease, and several solid tumors.

## PRINCIPLE OF THE ASSAY

The present method uses two high affinity and specificity monoclonal antibodies, directed against different epitopes of ferritin molecule. One is coated on the solid phase and the other one is labelled with the enzyme horseradish peroxidase (HRP).

The two monoclonals bind to ferritin present in the sample forming on the solid phase a stable soluble "sandwich" complex. Unbound components are removed by aspiration and washing step. The enzyme activity bound to the solid phase, proportional to the analyte concentration in the sample, is detected by the specific chromogen-substrate solution (TMB/H<sub>2</sub>O<sub>2</sub>). Colorimetric reaction is blocked by an acid solution and the colour intensity is photometrically detected at 450 nm. Unknown ferritin concentration in the sample is calculated by interpolation on a standard curve.

## KIT CONTENT

### 1. Reagent A – Microplate

12x8 strip.

8 wells breakable strips, coated with monoclonal antibody anti-human ferritin. The strips are assembled on a plastic frame and contained in a sealed bag with desiccant. Bring the strips to room temperature before use, to prevent any moisture formation inside the bag.

### 2. Reagent B – Enzymatic Tracer

1 vial of 13 ml.

Ready to use proteic buffer solution containing anti-human liver ferritin monoclonal antibody, conjugated with Horseradish peroxidase (HRP). Contains stabilizers and Gentamicin sulphate 0.01% as preservative.

### 3. Reagent C – Washing Solution 20x

1 vial of 60 ml.

Concentrated solution to be diluted 1:20 with distilled water. It contains PBS buffer pH 7.4 with detergents and preservatives (Kathon GC 0.1%).

### 4. Reagent D – Chromogen

1 vial of 12 ml.

Ready to use solution containing Tetramethylbenzidine (TMB) with activators and stabilizers.

**Avoid light exposure.**

### 5. Reagent E – Substrate

1 vial of 12 ml.

Ready to use solution containing Hydrogen peroxide and activators.

### 6. Reagent F – Stop Solution

1 vial of 16 ml.

It contains a mixture of 1 M Hydrochloric and Phosphoric acid.

The reagent is irritant: **Xi R36/37/38; S(1/2)26-45**  
Handle with care.

### 7. Standard O – Diluent

1 vial of 3.5 ml.

"Ferrin-free" proteic buffer solution with Gentamicin 0.01% as preservative. Use this solution as Standard 0 ng/ml (S<sub>0</sub>) and to dilute samples with high ferritin concentration.

### 8. Ferritin Standards

5 vials of 1 ml each.

Human liver ferritin diluted in proteic buffer with preservative (Gentamicin sulphate 0.01%) at the following concentrations:

**S<sub>1</sub>**: 5 ng/ml, **S<sub>2</sub>**: 20 ng/ml, **S<sub>3</sub>**: 100 ng/ml,

**S<sub>4</sub>**: 300 ng/ml, **S<sub>5</sub>**: 600 ng/ml.

(Calibrated against WHO 1<sup>st</sup> IS Ferritin, human liver, 80/602)

### 9. Cardboard sealers

2 cardboard sealers to be used to cover the plate during the incubations.

### 10. Package insert: instruction for use GD7000 00 it/ing.

## MICROBIOLOGICAL STATE AND CLEANING GRADE

1. All the materials of human origin resulted negative to HbsAg, HIV 1&2 and HCV FDA approved tests. Anyhow, as no test can guarantee the absolute absence of infective agents, handle reagents as potentially infected, especially standards, controls and samples. All objects come in direct contact with samples and all residuals of the assay should be treated or eliminated as potentially infected. Best procedures for inactivation are treatments with autoclave at 121°C for 30 minutes or with sodium hypochlorite at a final concentration of 2.5 % for 24 hours.
2. Avoid any contact with skin and mucous membrane, in particular for Stop Solution.
3. Use protective disposable talk-free gloves.
4. Avoid contaminating reagents when taking them from the vials. We recommend to use automatic pipettes with disposable tips. When dispensing reagents, do not touch with tips the wall of wells in order to avoid cross-contaminations.
5. For the washing step, use only the Washing Solution provided in the kit and follow carefully the indications reported in "WASHING INSTRUCTION".
6. Avoid the substrate/chromogen to come in contact with oxidizing agents or metallic surfaces; avoid intense light exposure during incubation or reagent preparation.

## STORAGE AND STABILITY

1. The kit has to be stored at 2-8 °C and used before the expiry date stated on the label.
2. Unused strips have to be placed in the bag containing the desiccant and firmly sealed before re-store at 2-8 °C. After opening the strips are stable up to the expiry date stated on the label.
3. The diluted washing solution can be stored for one week at room temperature or 3 weeks at +2-8 °C.
4. When preparing chromogen/substrate we recommend the use of plastic disposable containers. The chromogen/substrate solution is stable for 4 hours at room temperature, protected from light.
5. All other reagents can be repeatedly used up to exhaustion if stored at 2-8 °C, provided that they are handled carefully to avoid any environment contamination. Under these conditions the reagents are stable up to the expiry date stated on the labels.

## AUXILIARY MATERIALS

- Semi automatic pipettes of 10, 200 and 1000 µl
- Vortex mixer and absorbent paper
- Chronometer
- Ultrapure Elisa grade water
- Photometric reader of microplates or microstrips, linear up to at least 2 OD and supplied with filters of 450 nm and 620- 630 nm.
- Microplate incubator set at a 37 (±1) °C.
- Automatic microplates washing device or manual apparatus capable of aspirating and dispensing volumes of 300 µl.

## SAMPLES

Either serum or plasma can be used (ACD, heparin). If the assay is not immediately performed, the samples should be

kept at 2-8 °C for 48 hours; otherwise they should be stored at - 20 °C. Avoid repeated freeze-thaw cycles. Samples must not be turbid, lipemic, haemolyzed and microbiologically contaminated.

## REAGENTS PREPARATION

- WASHING SOLUTION: dilute 1:20 with distilled or ELISA grade water (ex. 60 ml of Reagent C + 1200 ml of distilled water) and mix carefully before use. It is recommended to store diluted washing solution at room temperature for immediate use.
- CHROMOGEN/SUBSTRATE: prepare in disposable plastic container, according to needs, the substrate/chromogen solution by mixing Reagent D with Reagent E in equal volumes.

## WASHING INSTRUCTION

A good washing procedure is essential to obtain correct and precise analytical results.

We therefore recommend to use a good quality ELISA microplate washer, maintained at a good level of washing mechanical performances.

Generally, 3-5 automatic washing cycles of 0.3 ml/well are sufficient to avoid false positive reactions and remove high background. Anyhow we recommend to calibrate the washing system on the kit itself so to match the declared analytical performances.

In case of manual washing, we suggest to perform 5 washing cycles, dispensing and aspirating 0.3 ml/well per cycle.

In any case the liquid washed out from the plates must be inactivated with a sodium hypochlorite solution at a final concentration of 2.5 %, before being thrown away or autoclaved, as it must be considered as potentially infected.

## ASSAY PROCEDURE

1. At least one hour before use, bring all reagents, standards and samples to room temperature (18-30°C), mixing them carefully on vortex.
2. Do not mix reagents from different lots.
3. We recommend to distribute standards and samples in duplicate.
4. Distribution and incubation times must be the same for all wells in the same analysis.
5. Avoid long interruptions between each step of the assay procedure.
6. It is suggested to eliminate the excess of washing solution from the microplate after washing by blotting it gently on an absorbent paper pad.
7. The colour developed in the last incubation is stable for a maximum of one hour. Otherwise, in case of reading after 10-15 min after dispensing stop solution, immediately place the strips **in the dark**.
8. We recommend to read the plate with an ELISA automatic reader able to subtract the background at 620-630 nm and to read the absorbance of samples and standards at 450 nm. The "blanking" of the instrument is to be carried out in the blank reagent well where only substrate-chromogen and stop solutions are added.

### ASSAY SCHEME

If expected sample concentration exceeds Standard 600 ng/ml ( $S_5$ ), dilute with Standard 0 ng/ml ( $S_0$ ).  
Follow the scheme:

|                        | Antibody anti-Ferritin coated wells  |             |             |             |
|------------------------|--|-------------|-------------|-------------|
|                        | REAGENTS   | Blank       | Standard    | Sample      |
| Immunological reaction | Standard   | -           | 20 $\mu$ l  | -           |
|                        | Sample   | -           | -           | 20 $\mu$ l  |
|                        | Reagent B (Enzymatic Tracer)   | -           | 100 $\mu$ l | 100 $\mu$ l |
|                        | - Cover the strips with cardboard sealer<br>- Incubate <b>60 minutes at room temperature</b> (20-25 °C) or <b>30 minutes at 37 (<math>\pm</math> 1) °C</b>                                       |             |             |             |
| Washing                | - Peel out the cardboard sealer and aspirate the reaction solution from all wells<br>- Rinse 5 times with 300 $\mu$ l of diluted washing solution, carefully aspirating off the remaining liquid |             |             |             |
| Colorimetric reaction  | Reagent D+E (Chromogen-Substrate) mixed 1:1  | 200 $\mu$ l | 200 $\mu$ l | 200 $\mu$ l |
|                        | - Cover the strips with cardboard sealer<br>- Incubate <b>10 minutes at room temperature (20-25 °C)</b> , avoiding light exposure  |             |             |             |
|                        | Reagent F (Stop Solution)  | 50 $\mu$ l  | 50 $\mu$ l  | 50 $\mu$ l  |
|                        | Read the absorbance of each well against Blank at 450 and 620-630 nm   |             |             |             |

### CALCULATION OF RESULTS

- Calculate the mean value of the OD 450 nm obtained for each duplicate for standards and samples.
- Draw a standard curve by plotting the absorbance of the standards (y axis) with the corresponding concentrations in ng/ml (x axis) on a log-log paper.
- Ferritin concentrations of the samples are obtained by interpolating the mean absorbance value of each sample on the calibration curve.

#### Note:

- It is recommended to read all wells at three wavelengths: 405-450-630 nm and perform the following calculation on the assay data:  
Compute the ratio  $K = OD\ 450\ nm / OD\ 405\ nm$  on standard or sample with an OD 450 value between 1.3 and 1.9.  
For the samples which gave an OD 450 nm value greater than the linear-reading capability of the available instrument, we suggest to use the following method in order to compute the equivalent:  
Equivalent OD 450 nm =  $K \times OD\ 405\ nm$   
Express all the values in OD 450 nm.  
For the most spread readers the K value is between 3.3 and 3.7.
- In case of using automated systems for the assay and results calculation, the use of "four parameter logistic" or "cubic spline" is recommended.
- Samples exceeding measure range have to be diluted with Standard 0 and retested. Multiply the obtained results by the used dilution factor.

#### Example of calculation

Example of calibration curve, do not utilize for the calculation of results.

| Standard (ng/ml) | OD 450 nm | OD 405 nm |
|------------------|-----------|-----------|
| S0 (0 ng/ml)     | 0.023     | 0.003     |
| S1 (5 ng/ml)     | 0.061     | 0.013     |
| S2 (20 ng/ml)    | 0.187     | 0.056     |
| S3 (100 ng/ml)   | 0.791     | 0.242     |
| S4 (300 ng/ml)   | 1.824     | 0.585     |
| S5 (600 ng/ml)   | 2.340     | 0.987     |

### VALIDITY OF THE ASSAY

To validate the obtained results it is necessary that the following acceptability criterions are respected:

- The OD 450 nm of the blanking well is lower than 0.100.  
Higher values indicate a chromogen/substrate contamination. In such a case, repeat the assay carefully checking the reagent.
- After subtracting the blank value, the mean OD 450 nm value for the Standard 0 ng/ml has to be lower than 0.200. Higher values indicate an erroneous washing procedure. In such a case, check the efficiency of the washing device.
- The OD 450 nm of the Standard 5 ng/ml has to be higher than the Standard 0 ng/ml one.
- The OD 450 nm value of the Standard 600 ng/ml has to be higher than 1.000. Lower values indicate kit or calibrator decay. Before repeating the assay, check the expiry date of the kit.

### EXPECTED VALUES

Based on data obtained by Minias Globe Diagnostics, the following expected values are suggested.

However, it is recommended each laboratory establish its own reference range according to its population.

| Subjects               | n. | Mean (ng/ml) | Range (ng/ml) |
|------------------------|----|--------------|---------------|
| Males                  | 60 | 183          | 21 - 385      |
| Females                | 55 | 49           | 7 - 187       |
| Post menopause females | 24 | 105          | 10 - 346      |

## ANALYTICAL PERFORMANCES

### Precision

#### a. Intra-assay precision (n = 28)

| Sample   | Mean (ng/ml) | SD (ng/ml) | %CV |
|----------|--------------|------------|-----|
| Serum #1 | 14.8         | 0.75       | 5.1 |
| Serum #2 | 60.3         | 1.20       | 2.0 |
| Serum #3 | 204.0        | 7.95       | 3.9 |

#### b. Inter-assay precision (n = 12)

| Sample   | Media (ng/ml) | DS (ng/ml) | %CV |
|----------|---------------|------------|-----|
| Sample   | 14.9          | 1.05       | 7.1 |
| Serum #1 | 59.6          | 3.21       | 5.4 |
| Serum #2 | 201.0         | 1.16       | 5.8 |

### Sensitivity

The sensitivity of the method, in terms of limit of detection (LOD) is 1 ng/ml.

### Specificity

The test shows cross-reactions with spleen and heart iso-ferritin of 90% and 15%, respectively.

### "Hook" effect

Samples with concentration of ferritin between 1.000 and 50.000 ng/ml gave always absorbance values higher than the absorbance of the last standard included in the kit.

### Accuracy

#### a. Parallelism test

| Sample   | Dilution | ng/ml measured | ng/ml expected | Recovery (%) |
|----------|----------|----------------|----------------|--------------|
| Serum #1 | -        | 1243           | -              | -            |
|          | 1:2      | 590            | 621.5          | 94.9         |
|          | 1:5      | 258            | 248.6          | 103.8        |
|          | 1:10     | 123            | 124.3          | 99.0         |
|          | 1:50     | 24             | 24.9           | 96.4         |
| Serum #2 | -        | 1010           | -              | -            |
|          | 1:2      | 474            | 505            | 93.9         |
|          | 1:5      | 226            | 202            | 111.9        |
|          | 1:10     | 114            | 101            | 112.9        |
|          | 1:50     | 19             | 20             | 95.0         |

#### b. Recovery

| Sample   | Dilution | ng/ml measured | ng/ml expected* | Recovery (%) |
|----------|----------|----------------|-----------------|--------------|
| Serum #1 | -        | 72             | -               | -            |
|          | 20       | 49             | 46              | 106.5        |
|          | 50       | 66             | 61              | 108.2        |
|          | 150      | 115            | 111             | 103.6        |
|          | 450      | 262            | 261             | 100.4        |
| Serum #2 | -        | 143            | -               | -            |
|          | 40       | 96             | 91.2            | 105          |
|          | 100      | 118            | 121.5           | 97.1         |
|          | 200      | 166            | 171.5           | 96.8         |

\* The expected value was calculated taking into consideration the dilution factor (1:2) due to the addition of known ferritin solution to the initial sample.

### c. Correlation with other methods

Results (y) using the present kit were compared to values obtained by a commercial available immunoradiometric kit (x) on a population of 50 samples with values of ferritin included between 2 – 495 ng/ml.

Linear regression analysis of data gave the following result:

$$y = 1.05x + 3.13 \text{ ng/ml} \quad r = 0.990$$

### Interferences

Rheumatoid factors (up to 1000 IU/ml), Bilirubin (up to 40 mg/dl), Haemoglobin (up to 1000 mg/dl), Triglycerides (up to 1900 mg/dl) and Cholesterol (up to 800 mg/dl) do not interfere with the present test.

## PRECAUTIONS IN USE

**Reagent F is irritant (Xn).** Refer to Safety Data Sheet.

The other reagents contain inactive components such as preservatives (Sodium azide or others), surfactants etc. The total concentration of these components is lower than the limits reported by 67/548/EEC and 88/379/EEC directives about classification, packaging and labelling of dangerous substances. However, the reagents should be handled with caution, avoiding swallowing and contact with skin, eyes and mucous membranes.

The use of laboratory reagents according to good laboratory practice is recommended.

### Waste Management

Please refer to local legal requirements.

## REFERENCES

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