FeROS™ LPI kit

For the Detection of Redox-Active Labile Plasma Iron (LPI)

INSTRUCTIONS FOR USE

REF: 8801 V.6

For Research Use Only

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INTENDED USE

The FeROS™ LPI kit is a fluorescence-based assay intended for the *in vitro* semi-quantitative detection of the redox active LPI (Labile Plasma Iron) fraction of NTBI (Non-Transferrin Bound Iron) and is **for research use only**.

INTRODUCTION

Thalassemia, hereditary Hemochromatosis and other health conditions associated with deranged metabolism or handling of iron, often result in the development of acute and/or chronic Iron Overload. This condition is characterized by elevated serum iron, transferrin saturation and serum ferritin, and in many cases by the appearance of non-transferrin bound iron (NTBI). In many iron-overloaded patients, a fraction of the NTBI is redox active and may catalyze the formation of reactive oxygen species (ROS) in the circulation and in iron overloaded cells. The accumulation of oxidized proteins and metabolites could ultimately lead to damage in sensitive tissues such as liver, pancreas and especially the heart.

LPI (Labile Plasma Iron) represents a component of NTBI that is both redox active and chelatable, capable of permeating into organs and inducing tissue iron overload. LPI measurement can serve not only as indicators of impending iron overload but also as measures of the efficacy of iron chelation in eliminating a potentially toxic agent from plasma. LPI measures the iron-specific capacity of a given sample to produce reactive oxygen species (ROS).

WARNINGS AND PRECAUTIONS

- Universal Precautions should be taken when handling material of human origin, such as patient specimens.
- All samples should be considered potentially infectious. Handling of samples, their use, storage and disposal should be in accordance with procedures defined by the applicable national or local biohazard safety guidelines or regulations.

PRINCIPLE OF THE TEST

The FeROS™ LPI assay measures the iron-specific redox activity in serum. A reducing agent, such as ascorbic acid and an oxidizing agent, (atmospheric O$_2$) cause labile iron in the tested sample to oscillate between its oxidized (Fe$^{3+}$) and reduced (Fe$^{2+}$) forms, generating Reactive Oxygen Species (ROS) via the Fenton reaction. The ROS are detected by an oxidation-sensitive probe (DHR), which becomes fluorescent when oxidized by ROS.

The assay employs a control reaction where a selective iron chelator blocks redox cycling of iron to specifically identify iron-mediated ROS generation. Comparison of the kinetics of fluorescence generated in the reaction in the presence and absence of the iron chelator translates into a measurement of the quantity of LPI in the tested sample.
SUMMARY OF THE PROCEDURE

1. Samples are dispensed into wells of a microplate. Positive and Negative standards are processed in an identical manner as the clinical samples. **LPI Reagent** and **LPI Reference Reagent** containing an Iron chelator (IC) are added to the samples. The LPI Reagent identifies the total level of detectable ROS generated in the sample, while the LPI Reference Reagent identifies any ROS that are not directly generated by iron.

2. A 40-minute kinetic fluorescence measurement is performed and the slope of fluorescence increase in each well is determined (ΔFU/min).

3. The difference between the slopes obtained with the LPI Reagent and the LPI Reference Reagent is calculated (ΔFU/min values).

4. The ΔFU/min value of each sample is converted into LPI units using an equation based on the standards values obtained in the same test (an MS Excel template which performs the calculation is provided).

DISCLAIMER

Results should be used and interpreted only in the context of the overall clinical picture. The manufacturer is not responsible for any clinical decisions that are taken.

The user of this kit should emphasize these points when reporting results to the diagnosing clinician.

EQUIPMENT REQUIRED

- Fluorometer (fluorescence plate reader) with excitation/ emission filters for fluorescein, temperature control and kinetic measurement capability.

- Vortex mixer

- Multichannel pipettor (for 200 µL)

- Pipettor (5-50µL)

- 25 mL Pipettes

- Mini centrifuge
## CONTENTS OF THE KIT

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Vessel</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well microplate</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent Reservoir</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube for LPI reagent</td>
<td>2</td>
<td>Empty 50 mL tube</td>
<td></td>
</tr>
<tr>
<td>LPI Buffer</td>
<td>1</td>
<td>Bottle</td>
<td>60 mL</td>
</tr>
<tr>
<td>Iron Chelator Solution</td>
<td>1</td>
<td>Vial</td>
<td>60 µL</td>
</tr>
<tr>
<td>Positive Standard I</td>
<td>1</td>
<td>Vial</td>
<td>200 µL</td>
</tr>
<tr>
<td>Positive Standard II</td>
<td>1</td>
<td>Vial</td>
<td>200 µL</td>
</tr>
<tr>
<td>Negative Standard</td>
<td>1</td>
<td>Vial</td>
<td>200 µL</td>
</tr>
<tr>
<td>DHR Solution</td>
<td>2</td>
<td>Brown vial</td>
<td>25 µL</td>
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</tbody>
</table>

## STORAGE AND STABILITY

- **DHR Solution** - Immediately upon receiving the kit, spin down the tubes to remove drops from the cap and store frozen (-18 to -30°C)!!

- Keep the kit at 2-8°C; Do not freeze.

- Do not use the kit beyond its expiration date (marked on box label). Stability is maintained even when components are re-opened several times.

- Minimize the time reagents spend at room temperature.

- This kit has been calibrated and tested as a unit; do not mix reagents from kits with different lot numbers.
ASSAY PROCEDURE

Fluorometer Setup

- Use excitation/ emission filters for fluorescein
- Use kinetic measurement mode: one reading cycle every 2 minutes for a total of 20 cycles. Total run time is 40 minutes. Set the reader software to calculate the linear slope between 15 and 40 minutes (FU/min value). The kinetic reaction becomes linear only after approximately 15 minutes.
- Use default settings of the plate reader. In addition, set the following parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
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<tbody>
<tr>
<td>Read mode</td>
<td>Bottom</td>
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<tr>
<td>Number of flashes</td>
<td>3</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
</tbody>
</table>

Gain

When running the first plate, calibrate the Gain parameter so that negative standard wells without ‘IC’ will obtain 700 to 1500 Fluorescence Units (wells C3 and C4 in “Sample Placement table”).

General Instructions

I. **Samples to be used:** Serum or heparinized plasma that was freshly collected on the day of the test or immediately frozen at -20 to -70°C (up to 2 hours after collection). Use frozen samples up to 3 months. Avoid repeatedly freezing and unfreezing the samples. Do not use citrate or EDTA tubes.

II. In each test include the Positive Standard I, Positive Standard II and Negative Standard.

III. One plate is sufficient for 21 samples. Since the assay requires kinetic measurements you can only analyze one plate at a time. Therefore, do not prepare LPI Reagent for more than one plate.

IV. It is recommended to include a well-known LPI positive sample in each plate.

V. Prior to running the test, spin down all vials to remove drops from the cap.

VI. **Avoid exposure to direct light during all stages of the assay.**

Step by Step Protocol

1. Prepare Ascorbic Acid (AA) working solution according to the instruction provided with the AA solution package.

2. **Preparation of the samples**

   2.1 Pre-warm the fluorometer to 37°C.

   2.2 Bring samples to ambient temperature. Centrifuge at 4,000g for 2 minutes immediately before using.

   2.3 Dispense into the microplate 20 µL from each sample and standard (use the
supernatant, avoid touching the upper lipid layer) into four adjacent wells in the same row as indicated in the table below. Always dispense the positive standard I into wells A1 to A4, the positive standard II into wells B1 to B4 and the negative standard in wells C1 to C4.

Sample Placement table

<table>
<thead>
<tr>
<th></th>
<th>IC</th>
<th>- IC</th>
<th>+ IC</th>
<th>+ IC</th>
<th>+ IC</th>
<th>- IC</th>
<th>- IC</th>
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<td>B</td>
<td>Positive II Standard</td>
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<td>Sample 15</td>
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<tr>
<td>C</td>
<td>Negative Standard</td>
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<td>Negative Standard</td>
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<td>Sample 8</td>
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<td>5</td>
<td>Sample</td>
<td>5</td>
<td>Sample</td>
<td>5</td>
<td>Sample</td>
<td>19</td>
</tr>
</tbody>
</table>

2.4 Cover the plate with the plastic cover. Add LPI reagent within 30 minutes.

3. Preparation of LPI Reagents

Prepare the LPI Reagents immediately before its use as follows:

3.1. Defrost one aliquot of DHR solution and one aliquot of AA working solution just before use.

3.2 Add 22 µL DHR solution to the bottom of the "LPI Reagent" labeled tube supplied in the kit. Add to the same tube 22 mL LPI Buffer.

3.3. Add to the tube 44 µL AA Working Solution. Vortex for 2-3 seconds.

3.4. Transfer 11 mL from the LPI Reagent to the reagent reservoir supplied with the kit.

3.5 Add 200 µL of the LPI Reagent into two wells of each sample (-IC) using a multichannel pipettor as indicated in the table above (columns 1,2,5,6,9,10).

Use a new set of tips for each column.

3.6 Discard the Reagent remaining in the reservoir and wipe the droplets.

3.7 Prepare LPI Reference Reagent by adding 22 µL of iron chelator solution to the remaining LPI Reagent and vortex for 2-3 seconds.

3.8 Fill up the reagent reservoir with the LPI Reference Reagent.

3.9 Add 200 µL of the LPI Reference Reagent into two wells of each sample (+IC) using a multichannel pipettor as indicated in the table above (columns 3, 4, 7, 8, 11,12).

Use a new set of tips for each column.

3.10 Cover the plate with the plastic cover

3.11 Start the fluorescence kinetic readings immediately.
Analysis of results

- An MS Excel template is provided for calculating the LPI units from the fluorescence values. In the template the difference between the slopes in the absence and presence of IC (ΔFU/min) is converted into LPI units, using an equation based on the ΔFU/min values obtained from standards in the same test.

- Copy the slope values (FU/min) obtained and paste the values only into the green colored frame of the Raw Data table in the "LPI-template" Excel worksheet. Confirm that the standards were placed as indicated in the "Sample Placement" table. The calculated values (average FU/min, ΔFU/min, LPI units) will be displayed in the table labeled "Analyzed Results".

Validation of results

Results are valid only if the values of the standards are within the values specified as follows:

Positive Standard I: 150-250 ΔFU/min
Positive Standard II: 50-100 ΔFU/min
Negative Standard: 0-15 ΔFU/min

Interpretation of results

LPI - Negative (Normal range): ≤0.4 LPI units
LPI - Low positive: 0.4-0.6 LPI units (blue colored values)
LPI - Positive: ≥0.6 LPI units (red colored values)

*A test result of 0.6 units of LPI or more indicates a potential for iron-mediated production of reactive oxygen species in the sample.
References


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