LABILE PLASMA IRON (LPI) MEASUREMENT KIT

INSTRUCTIONS FOR USE v1
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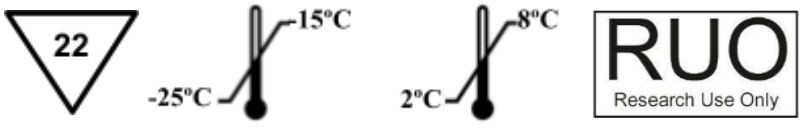

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INTENDED USE

LPI measurement is a fluorescence-based assay intended for the in vitro semi-quantitative detection of all forms of labile and chelatable iron which may be redox active. It is indicative of the total Non-Transferrin Bound Iron (NTBI) content.

INTRODUCTION

Thalassemia, hereditary Hemochromatosis and other health conditions associated with deranged metabolism or handling of iron often result in development of acute and/or chronic Iron Overload. This condition is characterized by elevated serum iron, transferrin saturation and serum ferritin.

Non-transferrin bound iron (NTBI) is commonly detected in patients with systemic iron overload whose serum total binding iron exceeds 75% transferrin saturation. Sustained presence of NTBI in plasma leads to an excessive accumulation of iron in various organs such as the liver, endocrine glands and the heart.

NTBI appears in various chemical forms depending on the origin and degree of iron overload, the history of blood transfusions, bone-marrow transplantation and treatment (chelation or phlebotomy). The forms that are potentially toxic to cells are those that are deposited on cell surfaces as redox active species. Forms that are redox active and chelatable (Labile Plasma iron (LPI)) represent the pharmacological targets of any chelation treatment aimed at preventing undesirable iron overloading of cells.

The heterogeneous composition of NTBI, that sometimes binds or complexes to small organic ligands (e.g. iron citrate and albumin), makes its determination in the clinical setting more difficult.

LPI levels can be affected by plasma components (e.g. citrate, uric acid and albumin) interacting with Iron in some different ways. As these boundaries are not strong, this iron is still toxic, but is not completely free in the plasma and this makes it undetectable. The use of a mobilizing agent in the assay overcomes the presence of those components in the plasma by causing the release of iron from the components and making all the plasma reactive (toxic) iron detectable.

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 LPI assay measures the iron-specific redox activity in serum. A reducing agent, ascorbic acid, and an oxidizing agent, O_2, cause labile iron in the tested sample to oscillate between its oxidized (Fe3+) and reduced (Fe2+) forms, generating Reactive Oxygen Species (ROS) via the Fenton reaction. The ROS are indirectly detected by an oxidation-sensitive probe (DHR), which becomes fluorescent when oxidized by ROS.

The assay employs a control reaction in which 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.

The LPI is performed in the presence of a mobilizing agent and provides a more realistic measure of the total NTBI content.

SUMMARY OF THE PROCEDURE

1. Samples are dispensed in duplicate into the wells of a microplate. Positive and Negative standards are dispensed in an identical manner as the clinical samples. **LPI Reagent** and **LPI Reference Reagent** (containing an Iron chelator; IC) are added to the microplate. 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 60 minutes kinetic fluorescence measurement is performed on a fluorometer.
3. The raw data is uploaded to the BloodGenetics LPI platform.
4. In the platform, a program first evaluates the quality metrics of the measurement and advises or warns if any sample/s or the assay does not comply with the minimum quality requirements.
5. The program then processes the data and numeric results in LPI units for each sample are obtained. Samples with levels below the pathogenic threshold (0.2 LPI units) are considered negative, samples above or equal to the pathological threshold are considered positive.

SAMPLES TO BE USED

1. Serum or heparinized plasma that was freshly collected on the day of the test, tempered Serum or heparinized plasma conserved at the fridge (5ºC) for less than 48 hours since the collection date or thawed Serum or heparinized plasma that was immediately frozen at -20 to -70°C (up to 2 hours after collection) on the day of collection. Samples can be stored at -20°C for a maximum of three months and at -70°C or below up to 6 months, provided that samples were not thawed more than twice. Do not use citrate tubes.
2. Blood samples are advised to be withdrawn at trough levels of chelator in plasma as LPI levels may be affected by the presence of iron chelators in plasma. In case of patients in treatment with chelators, it is recommended to withdraw blood just before the 1st daily intake of deferasirox or deferiprone or just before infusion of deferoxamine.

DISCLAIMER

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices. The manufacturer is not responsible for any clinical decisions that are taken.

REQUIRED EQUIPMENT

- Fluorometer (fluorescence plate reader) with excitation/emission filters for fluorescein, temperature control and kinetic measurement capability
- Vortex mixer
- Single-channel pipettes (1-10 µL, 2-20 µL, 20-200 µL and 100-1000 µL)
- Multi-channel pipette (20-200 µL)
- Pipettor (5-50 mL)
- 25 mL pipettes
- Mini centrifuge

CONTENTS OF THE KIT

The components and amounts listed above are intended for 2 assays (one per plate of 96 wells).

Component	Reference	Quantity	Vessel
96-well microplates	-	2 plates	-
Reagent Reservoirs	-	2 reservoirs	-
Falcon tubes for LPI reagents (50mL)	-	2 tubes	-
LPI Buffer*	R40010-1	1x100 mL	Plastic bottle
Iron Chelator Solution*	R40010-2	1x60 µL	Brown vial
Positive Standard*	R40010-3	1x200 µL	Vial (green cap)
Negative Standard*	R40010-4	1x200 µL	Vial (red cap)
DHR Solution*	R40010-5	2x25 µL	Brown vial
Mobilizing Agent*	R40010-6	1x60 µL	Vial (transparent cap)
Ascorbic Acid (AA) Solution Package*	R40010-7	1 bag	Aluminium Plastic Bag
AA Powder** (Vial 1)	R40010-8	1x71mg	Plastic bottle
AA dissolver** (Vial 2)	R40010-9	1x1250 µL	Vial (transparent cap)
AA working solution**(Vial 3)	R40010-10	1x1250µL	Brown vial

Table 1. Components of the kit.
* DHR is the only reagent with two separate aliquots (one per plate). All other reagents have only 1 vial/tube/bottle for 2 assays/plates. For these reagents, there is enough volume for two plates. These reagents must be stored in the fridge/freezer (see STORAGE AND STABILITY section) again just after usage for the first plate/assay (provided that the second assay is not performed on the same day).
** Components included in the Ascorbic Acid Solution Package.

STORAGE AND STABILITY

- **DHR Solution and Ascorbic Acid Powder (Vial 1) – should be stored frozen (-15 to -25°C)!**
- Keep all the other reagents at the fridge (2-8ºC) until usage, do not freeze them.
- Minimize as much as possible the time reagents spend at room temperature.
- The rest of the volume of reagents not used on the first plate/assay must be stored at the fridge (2 to 8ºC) again until usage for the second plate/test, except for AA working solution aliquot (vial 4) that should be stored in the freezer (-15 to -20ºC).
- Only thaw one aliquot of DHR solution for each plate/test performed.

ASSAY PROCEDURE

Fluorometer Setup

Previous to any step, configure your laboratory fluorometer with the conditions described below:

- Use excitation/emission filters for fluorescein (excitation peak at 450 nm and emission peak at 520 nm).

- Use the kinetic measurement mode to perform a read cycle every 2 minutes for a total of 30 cycles. The total run time is 60 minutes. Afterwards, the program will take into consideration the linear slope between 14 and 40 minutes (ΔFU/min value) for the calculations (during this time frame a linear kinetic reaction occurs).
- **Fluorometer must be pre-heated at 37ºC** before starting the assay. Otherwise, kinetic results may vary due to delay of the first time point, as measurements will not start until the fluorometer reaches the temperature set.
- Use the default settings of the plate reader, except for the following parameters:

Parameter	Setting
Read mode	Bottom
Number of flashes	25
Temperature	37°C (pre-heated)
Gain	May vary depending on the result of negative standards in the first measurement of the plate (at time 0 min).

Table 2. Fluorometer settings

General Instructions

I. Check that all components reach Room Temperature before starting the assay.

II. In each test, include the Positive and Negative Standards to normalize results and validate the test.

III. One plate is sufficient to measure up to 22 samples. Since the assay requires kinetic measurements, you can only analyze one plate at a time per fluorometer. Therefore, do not prepare LPI Reagent for more than one plate, except if more than one fluorometer is available to work in parallel.

IV. It is recommended to include a well-known LPI positive and negative sample in each plate as internal control of the test.

V. Avoid exposure to direct light during all stages of the assay, but especially when using reagents stored in brown tubes/vials.

Step by Step Protocol

1. Bring all reagents, samples and controls to room temperature (approx. 20-25ºC) before use (in exception of one aliquot of DHR, that will be kept in the freezer until the usage for the second plate).

2. Prepare the Ascorbic Acid (AA) Solution with the components included on the Ascorbic Acid Solution Package:

2.1. Add 1000 µL of the AA dissolver solution (Vial 1) into the AA Powder (Vial 2) and vortex vigorously for 10 seconds (or as much as needed to obtain a completely AA diluted solution). Do not proceed with the next steps until AA powder is completely dissolved.

2.2. Add 25µL off the dissolved AA powder (Vial 2) into the AA working solution (Vial 3) and vortex vigorously for 2-3 seconds.

2.3. Once added the AA working solution on the LPI reagent for one plate, keep the rest of the volume frozen at -20ºC until usage for the second plate or at room temperature if used immediately.

3. Samples preparation:

As a preliminary step, **pre-warm** the fluorometer to **37°C.** Also make sure that samples, controls and standards have thawed completely before proceeding with the protocol.

3.1. Vortex vigorously samples, controls and standards until the sample is homogeneous (approx. 2-3 seconds normally).

3.2. Centrifuge samples at 4,000g for 2 minutes.

3.3. Dispense 20 µL per well of each sample (avoid touching the upper lipid layer), control or standard into **four** adjacent wells of the same row as indicated in the Sample Placement Table below.

It is very **important** that the **Positive standard** is dispensed into wells **A1 to A4** and the **Negative standard** into wells **B1 to B4**. Otherwise, calculation of LPI values performed by the Blood Genetics LPI platform will be incorrect.

Sample Placement Table

	(-) IC		(+) IC		(-) IC		(+) IC		(-) IC		(+) IC	
	1	2	3	4	5	6	7	8	9	10	11	12
A	+ STD	+ STD	+ STD	+ STD+	Sample 7	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 15
B	- STD	- STD	- STD	- STD	Sample 8	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 16
C	Sample 1	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17	Sample 17
D	Sample 2	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18	Sample 18
E	Sample 3	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19	Sample 19
F	Sample 4	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20	Sample 20
G	Sample 5	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21	Sample 21
H	Sample 6	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22	Sample 22

Figure 1. Sample placement table. IC = Iron Chelator, + STD = Positive Standard, - STD = Negative Standard

3.4. It is recommended to cover the plate with a plastic cover until addition of the LPI Reagent (no later than 30 minutes to avoid drying out).

4. Prepare the LPI Reagent as follows (prepare the LPI Reagent immediately before its use):

4.1. Avoid, as much as possible, direct light exposure of reagents while preparing the mix. Covering the transparent tubes with aluminum foil or similar is recommended.

4.2. Add **22 µL of DHR** solution to the bottom of a 50 ml tube (this tube will contain the **LPI Reagent**; - IC). Add, on the same tube, **22 mL of LPI Buffer**, **22µL of Mobilizing agent** and **110 µL of AA** and vortex for 2-3 seconds.

4.3. **Transfer 11 mL** from the LPI Reagent to the **empty 50mL tube** supplied with the kit (this tube will contain the LPI Reference Reagent).

4.4. **Transfer** the **remaining LPI Reagent** (reagent without iron chelator solution: - IC) to a **reservoir** (supplied with the kit).

4.5. After removing the plastic cover, add **200 µL** of the **LPI Reagent** into each well of the **columns** indicated as **-IC** in the table above (columns 1, 2, 5, 6, 9, 10) using a multichannel pipette. Use a new set of tips for each column.

4.6. Prepare the **LPI Reference Reagent** by adding **22 µL** of the **iron chelator solution** to the **LPI Reference reagent tube** and vortex for 2-3 seconds.

4.7. Transfer the **LPI Reference Reagent** (reagent with iron chelator solution) to the **empty reservoir** (supplied with the kit).

4.8. Add **200 µL** of the **LPI Reference Reagent** into each well of the **columns** indicated as **+IC** in the table above (columns 3, 4, 7, 8, 11, 12) using a multichannel pipette. Use a new set of tips for each column.

5. Adjust the Gain of the plate to optimum values (preliminary measurement):

5.1. Introduce the plate without the plastic cover correctly oriented in the fluorometer.

5.2. Perform a **preliminary read** only of the **wells** corresponding to the **negative standard** sample to decide the appropriate gain value that will be used for the kinetics.
Choose a Gain value (usually not lower than 60 nor higher than 120) to perform one single cycle of fluorescence measurements on the four wells containing the negative standard (B1-B4). Results in FU (Fluorescence Units) must be as close as possible to the **range of observed values** indicated in the **Certificate of Lot Performance** provided with every kit. Those values change for every different lot.
The Certificate of Lot Performance can be found in the Blood Genetics LPI platform under the Gain Adjustment tab. There, you will also find guidance on how to choose the Gain. Go to the troubleshooting section on this document if the test is performed with a Varioskan Lux spectrophotometer.

5.3. Repeat the same measurement as many times as needed (changing the gain value on every new measurement) until obtaining FU values as close as possible or within the range of observed values. If results (FU units) are higher than the observed values, decrease the gain. If results are lower, increase the gain.

6. Measure the fluorescence kinetics of the microplate:

6.1. **Once the Gain is properly adjusted**, **measure** the fluorescence **kinetics for all the plate** samples with the optimum Gain and the test conditions described before (in the Fluorometer Setup section): one reading cycle every 2 minutes for a total of 30 cycles. It is recommended to start the kinetic as soon as the optimum gain is obtained to avoid displacing the linearity timeframe of the reaction.

6.2. Wait until the read is completed. Name (as desired) and save the Excel results file/s (.xlsx). Save both the kinetics measurement and each of the unique measurements of STD- samples made to adjust the gain in case the gain adjustment and the kinetics results are saved on different excel documents. The **Excel sheet with the kinetics must be named 'Sheet1'** to be able to obtain results when uploading the raw data into the Blood Genetics LPI platform.

7. Refrigerate/freeze again all the reagents that have an excess of volume that will be used on the second plate/assay. See Storage and Stability and Content of the kit sections.

Results Analysis

Once the raw data is generated by the plate reader in Excel format (.xlsx), the Excel document has to be uploaded in the BloodGenetics LPI platform. You will need to login to access the program with a username and a password provided with the kit. Once logged in, submit your lot number and the Excel file with the raw data (NOT the file with the measurements to adjust the gain) in the LPI results tab. Remember to name the sheet with kinetic results as 'Sheet1'.

Finally, the difference between slopes in the absence and presence of IC or chelator (ΔFU/min) is converted into LPI units by the program. A previous normalization of results for each sample is done by the program with the Standards slopes obtained in the same plate.

The LPI program will provide a table with results in LPI units (arbitrary units) and plots (one per sample) with the fluorescence values versus the minutes of the kinetics for each sample and standard tested in the plate/assay.

Validation of Results

Following the criteria provided by the official European Guidelines on bioanalytical method validation and study sample analysis[10], we recommend to consider LPI measurement results as valid (in terms of reliability) only when complying with the quality criteria described below.

An **assay** is only considered **valid** when its results meet all the assay Quality Criteria.
A **sample** is only considered **valid** when its results and results of the assay comply with both sample and assay Quality Criteria.
If a sample result is not valid (on a valid assay), this sample must be reanalyzed on a new assay.
If a sample result complies with sample Quality Criteria but it has been obtained on an assay that does not comply with Quality Criteria, results for this sample, as well as all the samples of the assay, are not valid. All the samples must be reanalyzed on a new assay.

Quality Criteria for validity:

1. Quality criteria for the assay:
   - Results at t=0s for Negative Standard in between **6,500-20,000 FU**
   - Positive Standard slope in between **50-300 ΔFU/min**
   - Negative Standard slope in between **0-35 ΔFU/min**
   - **CV < 25%** between standard **duplicates**
   - **Non-overflow** fluorescence values*

   The **assay** is considered **valid only when results meet the four stated quality criteria** above. If one of the results does not comply with the quality criteria, all the assay results are considered invalid for all the samples and standards and the assay must be repeated.

2. Quality criteria for samples:
   - **CV (Coefficient of Variation) < 20%** between sample **duplicate** (standards not included) results
   - **Non-overflow** fluorescence values*

*Overflow occur when an excess of light reaches the detector producing a saturation of the system.

The LPI platform shows, above the LPI units results, a table mimicking the well plate. The samples in this table are highlighted in either green or red to inform about the validity of the sample and the assay. The program then outputs a message indicating the validity of the samples and the assay and explaining the reason in case a sample/assay does not meet the quality criteria.

For better understanding of further aspects or solving questions regarding quality criteria or validation of results, consult the Troubleshooting section of this document or the ICH Guideline M10[10]. In case a problem is not solved or a question is not answered with these sources, you can ultimately contact us on info@bloodgenetics.com.

Results interpretation

The corresponding values on LPI units to 75% transferrin saturation (and therefore, serum iron pathogenic overload) are 0.2 LPI units. Equal or higher results to this threshold are considered positive.

Summary of results:

- **Negative** sample: LPI units **< 0.2**
- **Positive** sample: LPI units **≥ 0.2**

A test result of 0.2 LPI units or higher indicates potential for iron-mediated production of reactive oxygen species in the sample, and therefore, pathogenic levels of NTBI serum/plasma overload.

To help with interpretation of the Assay Results Table given by the Blood Genetics LPI platform the positive samples are highlighted in yellow. Negative samples are not highlighted.

For discussion of further aspects or solving questions regarding results interpretation, consult the Troubleshooting section of this document or contact us on info@bloodgenetics.com.

TROUBLESHOOTING

Some experimental or data-managing frequently committed errors are summarized in this section. Solutions or changes in handling of materials/data of the experiments are exposed to help avoiding or solving those errors.

Common errors/issues:

1. High **CV** between sample duplicates:
   - **I.** Avoid the creation of bubbles when dispensing volumes with a multichannel pipette. They may interfere with the measurements performed by the fluorometer.
   - **II.** Change multi-channel pipette tips for every column when dispensing LPI reagent to avoid contamination between samples/duplicates.

2. Incorrect **Gain** adjustment:
   - **I.** Make several analyses of -STD results at time point 0s on the preliminary measurement of fluorescence when adjusting the Gain for the kinetics. Try to use the Gain that outputs FU results as closer as possible to the observed values given on the Certificate of Lot Performance for the lot used. If necessary, make several measurements increasing/decreasing Gain values one unit by one until hitting the more suitable Gain value.

     Example for a lot with Observed values of 9568-10684 FU:

Measurement	Gain	Results for -STD (time 0s)				Adjustment
		Well B1	Well B2	Well B3	Well B4	
1st	70	15447	15251	14950	15725	Too high, try lower Gain: 65
2nd	65	9007	8888	8692	9109	Too low, try higher Gain: 68
3rd	68	12461	12282	12026	12602	Too high, try lower Gain: 66
4th	**66**	9959	9801	9606	10026	In the range: Optimal Gain ✔

Table 3. Gain adjustment

A gain of 66 would be used for the kinetics.

Try not to delay any longer than necessary adjusting the Gain (if possible, less than 5 minutes). Starting the kinetics measurement late makes the linearity timeframe of the reaction to be displaced. Results of reaction kinetics, in particular those performed on last measurement cycles, may be outside the linear phase.

II. If your fluorometer is a Varioskan Lux, the relative fluorescence units obtained in the assay will probably be low. A factor of 560 is recommended to be used to multiply results in order to obtain standard values that fit with the range of assay values.

3. **Invalid Standard** values:
   If slopes of ΔFU/min for Standards are outside the range of accepted values, the assay has to be considered invalid. As all the LPI samples results are normalized with Standards, having a bad established kinetic result for Standards may lead to false negative or positive results, and therefore, misdiagnosis of pathogenicity.
   To avoid obtaining invalid results for Standards, we recommend to review/double-check the following possible sources of error:

   I. Incorrect Gain adjustment Read error 2 in the Troubleshooting section.

   II. Be extremely careful when dispensing Standards into wells to avoid contamination (specially of contaminating the Negative standard with the Positive).

   III. Be extremely careful in dispensing the LPI reagent with chelator only to its correspondent columns (3, 4, 7, 8, 11 and 12).

   IV. Be meticulous when preparing the LPI reagent and avoid light exposure of DHR and Ascorbic Acid.

4. **Overflow** values
   If overflow occurs in the kinetics, the fluorometer is not able to measure the fluorescence and so it outputs *OVER* instead of numeric values. Therefore, the LPI program cannot perform its calculations. Overflow values are mostly due to an incorrect Gain adjustment. Read error 2 in the Troubleshooting section to avoid this.

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