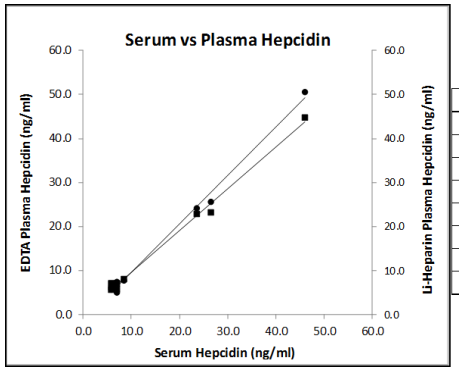
Example of Hepcidin Standard Curve Data

Hepcidin-25 Standard	Concentration (ng/mL)	Absorbance (450nm)
1	0	2.36
2	2.5	2.14
3	10	1.61
4	25	0.90
5	50	0.47
6	100	0.24
7	250	0.10
8	1000	0.03

Correlation of Serum Hepcidin with Plasma Hepcidin

The graph below represents regression analysis of serum versus plasma hepcidin from 8 human blood samples (low, medium, high hepcidin-25) obtained and prepared as serum, lithium heparin plasma, and EDTA plasma using standard procedures. Hepcidin-25 concentrations were measured using the Intrinsic Hepcidin IDx™ ELISA kit. A strong correlation was observed between serum and EDTA plasma (●, y = 1.096x - 1.25, R^2 = 0.994) and serum and lithium heparin plasma (■, y = 0.946x + 0.19, R^2 = 0.994).



Sample ID	Serum	Plasma EDTA	Plasma-Li-Heparin
Patient 1	8.4	7.7	8.1
Patient 2	7.0	7.4	7.1
Patient 3	46.0	50.6	44.7
Patient 4	6.9	5.0	5.9
Patient 5	23.6	24.2	22.9
Patient 6	26.5	25.7	23.1
Patient 7	5.8	6.3	7.1
Patient 8	5.8	5.8	5.7

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U.S. Pat. No. 9,657,098; NZ Pat. No. 631098; EP Pat. No. 2968503; Australia Patent No. 2014236677; Russia Pat. No. 2668391
Pat. Pending in the US and abroad

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INTRINSIC LIFESCIENCES
∞The BioIron Company™

Intrinsic Hepcidin IDx™ ELISA

Catalog No. ICE-007 (96 Tests)

The Intrinsic Hepcidin IDx™ ELISA kit is an ELISA test for the quantitative measurement of hepcidin in human serum and plasma. For **IVD Use Only**.

SUMMARY AND EXPLANATION

Hepcidin is a 25 amino acid hormone and the master regulator for iron homeostasis (metabolism) in humans.[1] Hepcidin regulates dietary iron absorption from the duodenum [2], controls the recycling of senescent erythrocyte iron by macrophages, and manages iron transport from hepatocytes into plasma for production of blood. Hepcidin is positively regulated by plasma iron and IL-6 (inflammation, infection) and is suppressed by erythropoiesis via erythroferrone. [3] Abnormally low serum hepcidin is associated with iron deficiency anemia (IDA) and hereditary hemochromatosis, and high serum hepcidin can lead to iron sequestration and to anemia of inflammation (anemia of chronic disease) observed in chronic kidney disease (CKD), rheumatoid arthritis (RA), cancers, and iron refractory iron deficiency anemia (IRIDA).[4] Intrinsic LifeSciences developed the first ELISA test for hepcidin and measured hepcidin in key clinical iron disorders. [5] Recently hepcidin was shown to be useful in predicting response to oral iron therapy based on the hepcidin level and, for this indication, it is superior to both ferritin and %transferrin saturation (%Tsat). [6]

PRINCIPLE OF THE TEST

The Intrinsic Hepcidin IDx™ ELISA kit is a patented competitive binding assay based on a monoclonal antibody (mAb) that binds with high affinity to the N-terminus of hepcidin-25, which is required for bioactivity and binding to ferroportin. [7] This antibody also binds low abundance, N-terminus isomers of hepcidin-25 with lower affinities. The Intrinsic Hepcidin IDx™ ELISA kit is a competitive binding assay between Hepcidin-25 in the test specimen and a biologically active biotinylated human hepcidin-25 tracer for a constant number of high affinity anti-hepcidin-25 N-terminal-specific mAb binding sites. To begin, purified hepcidin-25 standard, hepcidin-25 controls (Control 1 and Control 2), and patient samples (all in duplicate) are added to the mAb coated microwell strip plate and incubated with biotinylated hepcidin-25 tracer for 60 minutes. The biotinylated hepcidin-25 tracer competes with native or reference hepcidin for a fixed number of N-terminal specific antibody binding sites. Thus, the amount of biotinylated hepcidin-25 tracer bound progressively decreases with increasing concentration of native serum hepcidin bound from the patient sample. Unbound biotinylated hepcidin-25 tracer is washed away and streptavidin-horseradish peroxidase (HRP) conjugate is added to the wells. The wells are incubated for 30 minutes and washed to remove unbound streptavidin-HRP. TMB substrate is added for 15 minutes and the reaction is stopped with the addition of stop solution. Absorbance at 450nm is measured using a microwell plate reader and the data recorded.

	MATERIALS PROVIDED	96 Tests
1.	Hepcidin-25 mAb coated wells, 96 Microwell Solid Plate	12x8x1
2.	Hepcidin-25 Standard, 8 vials ready to use	8 x 0.5mL
3.	Hepcidin-25 Control 1, 1 vial ready to use	0.5mL
4.	Hepcidin-25 Control 2, 1 vial ready to use	0.5mL
5.	Biotinylated Hepcidin-25 Tracer, 1 bottle ready to use	13mL
6.	Streptavidin-HRP Conjugate, 1 bottle ready to use	12mL
7.	TMB Substrate, 1 bottle ready to use	12mL
8.	Stop Solution, 1 bottle ready to use	12mL
9.	Wash Buffer, 1 bottle of 20X solution, dilute before use	25mL
10.	Sample Diluent, 1 bottle ready to use	3mL
11.	Polypropylene (PP) 96 Microwell Plate, 2 adhesive covers	1 package

MATERIALS NOT PROVIDED

1. Distilled or deionized water
2. Precision pipettes (single and multichannel), disposable pipette tips (100 and 1000µl)
3. Microwell plate reader capable of reading absorbance at 450nm, benchtop centrifuge
4. Flat-head vortex mixer, microplate shaker, absorbent paper
5. Curve fitting software, graph paper

STORAGE & STABILITY

1. Store the kit at 2-8°C.
2. Keep all microwell strips sealed in a dry bag with desiccant.
3. The reagents are stable until expiration date of the kit.
4. Do not expose test reagents to heat, sun, or strong light.

WARNINGS AND PRECAUTIONS

1. Potential biohazardous materials: The standards and controls contain human source components which have been tested and found non-reactive for hepatitis B surface antigen as well as HIV antibody with FDA licensed reagents. However, no test method can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent. These reagents should be handled as Biosafety Level 2 material. [8]
2. This kit is designed for **IVD Use Only.**
3. Do not pipette by mouth. Do not smoke, eat, or drink in the areas in which specimens or kit reagents are handled.
4. The components in this kit are an integral unit. Reagents from different lots should not be mixed. It is recommended that standards, control and patient samples be run in duplicate. Optimal results are obtained by strict adherence to this protocol.
5. Accurate and precise pipetting, careful washing and droplet removal, and exact timing at specified temperatures are essential. Deviation from recommendations may yield invalid data.
6. Check the Hepcidin-25 standard concentration value on each vial. This value might vary from lot-to-lot.
7. Patient samples with an Absorbance (A) 450nm equal to or less than the A450nm for the 250 ng/ml Hepcidin-25 standard (standard 7) should be diluted and re-run (see Instructions For Diluting Samples).

SPECIMEN COLLECTION AND HANDLING

1. Blood specimens may be collected in serum, serum separator, lithium or sodium heparin plasma or EDTA plasma tubes. Serum and plasma specimens may be stored refrigerated at 2-8°C for up to 24 hours from the time of draw.
2. If storage is required for more than 24 hours, the plasma or serum may be stored frozen at -20° to -80°C for up to one year. Avoid multiple freeze-thaw cycles.
3. Prior to assay, frozen sera or plasma should reach room temperature. If visible precipitates are present, gently mix and centrifuge the sample prior to use. Do not use hemolyzed, contaminated or lipemic samples.

REAGENT PREPARATION

All reagents and specimens must be allowed to reach room temperature before use. All reagents must be GENTLY mixed without foaming. Once the procedure has started, all steps should be completed without interruption. Prepare 1X Wash Buffer by adding the contents of the bottle (25 mL, 20X) to 475 mL of distilled or deionized water. Store Wash Buffer at room temperature (18-24°C).

STANDARD ASSAY PROCEDURE

For best results premix the samples, standards, controls and tracer for the ELISA in the 96-well polypropylene (PP) plate provided in the kit.

1. Using a multichannel pipette, transfer 110µl of biotinylated hepcidin-25 tracer to required wells of PP plate.
2. Transfer 22µl of each hepcidin standard, sample(s) and each hepcidin control to the PP plate. If a patient sample requires further dilution see the instructions for diluting samples described below.
3. Using a multichannel pipette, carefully mix the solution. Transfer 120µl of this solution from the PP plate to the corresponding wells in the 96 microwell strip plate.
4. Incubate the microwell strip plate for 60 minutes at room temperature, with agitation (350 rpm). Briskly shake out the contents of the wells into a waste reservoir.
5. Dispense 300µl of 1X Wash Buffer into each well, and then briskly shake out the Wash Buffer into a waste reservoir. Strike the wells sharply on absorbent paper to remove residual droplets. Repeat 2 additional times for a total of 3 washes.
6. Dispense 100µl of Streptavidin-HRP Conjugate into each well.
7. Incubate 30 minutes at room temperature with agitation (350 rpm). Briskly shake out the contents of the wells into a waste reservoir.
8. Dispense 300µl of 1X Wash Buffer into each well, and then briskly shake out the Wash Buffer into a waste reservoir. Strike the wells sharply on absorbent paper to remove residual droplets. Repeat 2 additional times for a total of 3 washes.
9. Using a multichannel pipette, dispense 100µl of TMB Substrate into each well. Incubate for 15 minutes at room temperature, preferably in the dark.
10. Dispense 50µl of Stop Solution into each well to stop the enzymatic reaction. Carefully mix plate contents for 20 - 30 seconds.
11. Read absorbance at 450nm within 15 minutes of addition of the stop solution.

Instructions for Diluting Samples

If the hepcidin concentration in the sample approaches or exceeds the upper limit of quantitation (~ 250 ng/ml), dilute the sample and re-run the ELISA (see WARNINGS AND PRECAUTIONS). Perform the dilutions in the Sample Diluent (supplied) in a low protein binding 96 microwell plate (not included) or in individual low protein binding microcentrifuge tubes (not included). See sample dilution example depicted in table below. Transfer 22µl of the diluted sample(s) to the 96-well PP plate as described in STANDARD ASSAY PROCEDURE (above).

Sample Dilution Factor	**2**	**5**	**10**
Volume Sample (µl)	15.0	6.0	3.0
Volume Sample Diluent (µl)	15.0	24.0	27.0
Total Sample Volume (µl)	30.0	30.0	30.0

NOTE: When performing the data calculations, always multiply the sample results by the sample dilution factor.

OPTIONAL ASSAY PROCEDURE

Prior to performing assay, bring all reagents to room temperature. Gently mix all reagents before use.

1. Dispense 20µl of Hepcidin standards, controls and samples into each well.
2. Dispense 100µl of Biotinylated Hepcidin-25 Tracer, into each well.
3. **INCUBATION #1 -** Incubate microwell strip plate for 60 minutes at room temperature, with agitation (350rpm). Briskly shake out the contents of the wells into a waste reservoir.
4. **WASH #1 -** Dispense 300µl of 1X Wash Buffer into each well, and then briskly shake out the Wash Buffer into a waste reservoir. Strike the wells sharply on absorbent paper to remove residual droplets. Repeat 2 additional times for a total of 3 washes.
5. Dispense 100µl of Streptavidin-HRP Conjugate into each well.
6. **INCUBATION #2 -** Incubate 30 minutes at room temperature with agitation (350rpm). Briskly shake out the contents of the wells into a waste reservoir.
7. **WASH #2 -** Dispense 300µl of 1X Wash Buffer into each well, and then briskly shake out the Wash Buffer into a waste reservoir. Strike the wells sharply on absorbent paper to remove residual droplets. Repeat 2 additional times for a total of 3 washes.
8. Using a multichannel pipette, dispense 100µl of TMB Substrate into each well.
9. **INCUBATION #3 -** Incubate for 15 minutes at room temperature, preferably in the dark.
10. **STOP -** Dispense 50µl of Stop Solution into each well to stop the enzymatic reaction. Carefully mix plate contents for 20 - 30 seconds.
11. Read absorbance at 450nm within 15 minutes of addition of the stop solution.

CALCULATION OF RESULTS

Software Calculation: A standard curve is generated and sample hepcidin concentration determined using either Four Parameter logistic (4PL) or Point-to-Point curve fitting algorithms with GraphPad Prism (Version 6.01) software (www.graphad.com). This test was developed and validated using 4PL curve fitting. Both methods yield very similar results. We recommend one method be used consistently once chosen.

Manual Calculation: To construct the standard curve, plot the absorbance (OD 450nm) for the Hepcidin-25 standards (vertical axis) versus Hepcidin-25 standard concentrations (horizontal axis) on a linear graph paper. Draw the best curve through the points. Read the absorbance for controls and each unknown sample from the curve and record.