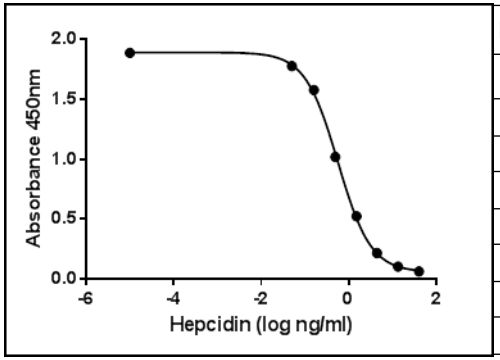
Standard Curve:
Eight point standard curve, including a zero, are used for each run. An example of a typical standard curve is shown below:



Hepcidin (ng/ml)	OD 450nm
400	0.0648
133.3	0.1043
44.4	0.2168
14.8	0.5247
4.9	1.0188
1.65	1.5767
0.55	1.7773
0.0001	1.8880

Quality Control:
It is recommended that each laboratory establish human EDTA plasma controls and utilize the hepcidin-25 concentration of the controls to validate performance of the kit.

Calculating Results:
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit (Recommended: Graphpad Prism Software, www.graphpad.com). There is no need to correct the OD values for background. Calculate the concentration of hepcidin-25 corresponding to the mean absorbance from the standard curve. Since samples were diluted, the concentration extrapolated from the standard curve must be multiplied by the dilution factor. For a 10% sample, the dilution factor is 10 (240µl assay volume / 24µl sample). If the quantity of hepcidin-25 in the sample exceeds 400 ng/ml, it is recommended to dilute the sample (dilute 15µl of sample in 15µl of sample diluent) and use 24µl of this diluted sample to rerun the assay in duplicate. The dilution factor for this example would be 20.

Converting Results:
Results are expressed in ng/ml. To convert to nmol/L, multiply results by 0.355
Example: 100ng/ml = 35.5nmol/L

Citations:

1. Park CH, Valore EV et al. (2001). J Biological Chemistry 276:7806-7810.
2. Pigeon C, Ilyin G et al. (2001). J Biological Chemistry 276:7811-7819.
3. Nicolas G, Bennoun M et al. (2001). PNAS 98:8780-8785.
4. Ganz T (2005). Clinical Haematology 18:171-182.
5. Nemeth E, Rivera S, et al. (2004). J Clinical Investigation 113:1271-1276.
6. Rivera S, Nemeth E, et al. (2005). Blood 106:2196-2199.

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SKU# ICE-004
For orders and inquires, please contact: Intrinsic LifeSciences
505 Coast Blvd South, Ste. 408, La Jolla, CA, 92037 USA
Tel (858) 459-1758, Fax (858) 459-3794
www.intrinsiclifesciences.com; custserv@intrinsiclifesciences.com

INTRINSIC LIFESCIENCES
∞The BioIron Company™

Intrinsic Hepcidin IDx™ EDTA ELISA

SKU# ICE-004

Intended Use:
The Intrinsic Hepcidin IDx™ EDTA competitive ELISA is designed for quantification of hepcidin-25 in human EDTA plasma samples. Quantification of hepcidin-25 from other body fluids or sample matrices has not been evaluated.

For Research Use Only.

This kit does not contain azide- or mercury-based preservatives.

Summary and Explanation:
Ganz and colleagues discovered hepcidin as an antimicrobial peptide produced in the liver (Park et al., 2001) and, together with other investigators in the field (Pigeon et al., 2001; Nicolas et al., 2001), identified hepcidin-25 as a peptide hormone that regulates extracellular iron in response to changes in dietary and systemic iron load, anemia, hypoxia, erythropoiesis, and inflammation (Ganz 2005). Hepcidin is an acute phase protein that is elevated in anemia of inflammation. IL-6 is a principal regulator of hepcidin during inflammation (Nemeth et al., 2004). Hepcidin inhibits cellular iron efflux by binding to and inducing degradation of the sole known iron channel, ferroportin. Synthetic hepcidin injected into mice binds to ferroportin-rich tissues and rapidly lowers serum iron levels (Rivera et al., 2005). It is now well established that hepcidin is the master regulator of iron homeostasis in vertebrates.

Principle of the Test:
This kit is a solid-phase enzyme-linked immunosorbent assay (ELISA), based on the principles of competitive binding. Human plasma EDTA samples, standards, or controls are mixed with buffer containing hepcidin-25 biotin conjugate. This mixture is then incubated in an anti-human hepcidin monoclonal antibody coated ELISA microwell plate. The more human hepcidin-25 present in the sample, the less hepcidin-25 biotin conjugate will bind to the antibody coated well due to “competition” for antibody binding sites between native hepcidin-25 and hepcidin-25 biotin conjugate. The plate is then washed to allow the removal of unbound hepcidin-25 biotin conjugate, streptavidin conjugated horseradish peroxidase (HRP) is added and the amount of hepcidin-25 biotin conjugate bound by the antibody is quantified by the addition of TMB. The reaction produces a blue color and is halted with the addition of the stop solution and the absorbance is read at 450 nm. A standard curve is produced by plotting the concentration of the standard curve versus the absorbance. The intensity of the color is inversely proportional to the concentration of human hepcidin-25 in the sample. The total assay run time is less than 3 hours.

Materials and Storage:
Store unopened kit at 2-8°C. Do not use after the kit expiration date and do not mix components from different kit lots.

Materials Provided	1 Kit	SKU#
1. Microwell plate coated anti-human hepcidin antibody	96-well x1	-
2. Human Hepcidin-25 Standard (1 glass vial, clear)	200 ng	XHS-200
3. Hepcidin-25 Biotin Conjugate (1 glass vial, amber)	1 bead	XHT-010
4. HRP Conjugate, 1 tube (conc., 100X)	150 µl	CEC-001
5. Sample Diluent, 1 bottle (conc., 10X)	4 ml	CSD-004
6. Wash Solution, 1 bottle (conc., 25X)	25 ml	CWB-008
7. TMB Substrate, 1 bottle (ready to use)	12 ml	CTM-001
8. Stop Solution, 1 bottle (ready to use)	12 ml	CST-001
9. Microplate sealing film	2	-
10. Polypropylene (PP) 96-well Sample Setup Plate	1	-

Materials Not Provided:
1. Precision single channel and multi-channel pipettes and tips
2. Squirt bottle (8 channel), manifold dispenser, or automated microplate washer
3. Deionized or distilled water
4. Horizontal orbital microplate shaker
5. Microplate reader (450nm)
6. Statistical analysis software

Warnings and Precautions:
Kit does not contain azide- or mercury-based preservatives. For research use only.

1. Use separate pipette tips for each sample, standard, and reagent to avoid cross-contamination.
2. Use separate reservoirs for each reagent, especially the TMB Substrate.
3. The Stop Solution contains 0.5M sulfuric acid. Use appropriate protection and safety precautions.
4. Hemolyzed, hyperlipemic, heat-treated, or contaminated samples may give erroneous results.
5. Do not dilute samples directly in the antibody coated microwell plate.
6. Do not touch or scrape the bottom or sides of the antibody coated microwell plate wells.
7. Incubation times and temperatures other than those specified may give erroneous results.
8. Do not allow the wells in the microplate to dry once the assay has begun.
9. Do not reuse microwell plate or pour reagents back into their bottles once dispensed.

Specimen Collection and Handling:
Only use EDTA plasma samples for this assay.
EDTA plasma samples are optimal; use of other types of body fluids require further investigation. Samples should be diluted to 10% of the recommended 100µl assay volume. It is recommended to begin at 10% and adjust if necessary. Therefore, to quantify the hepcidin-25 in the sample (in duplicate) will require 24µl of EDTA plasma sample. Collect EDTA plasma samples according to standard techniques. Samples should be centrifuged to remove lipids and cellular debris.

For long term sample storage, aliquot in small volumes and freeze at -80°C. Avoid repeated freeze-thaw cycles. Samples should be thawed and allowed to equilibrate to room temperature 30 minutes before use; samples must be mixed before analysis.

Preparation of Reagents:
This kit has sufficient reagents and is designed to run a duplicate 8-point standard curve and 40 samples in **duplicate only**.
Bring all samples and reagents to room temperature (20-25°C) before use.
1. 1X Wash Solution: Transfer contents of concentrated **Wash Solution** bottle (25ml) to 600ml of deionized or distilled water.
2. 1X Sample Diluent: Transfer contents of the concentrated **Sample Diluent** bottle (4ml) to 36ml of deionized or distilled water.
3. 1X HRP Conjugate: Pipette 120µl of the concentrated **HRP Conjugate** into 12ml of the 1X Sample Diluent.
4. Biotin Conjugate: Carefully transfer lyophilized **Hepcidin-25 Biotin Conjugate** bead to 20ml of the 1X Sample Diluent. Mix gently by inversion or briefly vortex.

Preparation of Standard
1. Biotin Conjugate for Standard: Prepare 4ml by combining 3.6ml of Biotin Conjugate with 0.4ml of 1X Sample Diluent.
2. Add 0.5ml of Biotin Conjugate for Standard to the **Human Hepcidin-25 Standard** vial. Mix by repeated pipetting.
3. Into the PP 96-well sample setup plate, transfer 400µl **Human Hepcidin-25 Standard** to well A1 and 250µl Biotin Conjugate for Standard to wells B1-H1. Perform a 125µl serial dilution from well A1 to well G1; leave well H1 undiluted.

Preparation of EDTA Plasma Sample
Transfer 216µl of Biotin Conjugate to the sample set-up plate and pipette 24µl of sample to achieve a final volume of 240µl/well (10% sample in assay solution).

Assay Procedure:
1. Transfer 100µl/well of standard curve and samples from the PP 96-well sample set-up plate to the microwell assay plate in duplicate.
2. Apply microplate sealing film and incubate on an orbital shaker (350 rpm) at room temperature for **1 hour**.
3. Wash microwell plate three times with 1X Wash Solution (300µl/well).
4. Transfer 100µl/well of the 1X HRP Conjugate solution to the microwell plate and incubate for **30 minutes** at room temperature on the orbital shaker.
5. Wash microwell plate three times with 1X Wash Solution (300µl/well).
6. Develop the microwell plate by adding 100µl/well TMB Substrate. Incubate for **10 minutes** at room temperature. Protect from ambient light.
7. Stop the reaction by adding 100µl/well Stop Solution precisely 10 minutes after the addition of the TMB Substrate.
8. Measure absorbance at 450nm of the microwell plate using a plate reader.