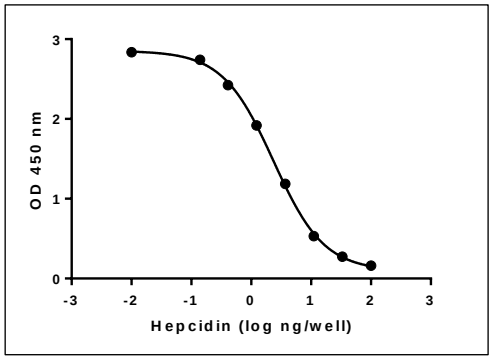
Standard Curve:
Eight standard points, including a zero, are used for each run. A typical standard curve is shown below:



Hepcidin (ng/ml)	OD 450nm
1000.00	0.162
333.30	0.275
111.10	0.531
37.00	1.185
12.30	1.918
4.10	2.424
1.40	2.741
0.01	2.836

Quality Control:
It is recommended that each laboratory select rat serum as an internal control and establish the range of this control serum over time to validate the performance of the reagents each time the assay in run.

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 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. Example for a 5% sample: 12 µl sample / 240 µl assay volume = 1/20, thus dilution factor = 20.

Converting Results:
Results are expressed in ng/ml. To convert to nmol/L, multiply results by 0.369.
Example: 100ng/ml = 36.9nmol/L

Citations:

1. Park C.H., Valore E.V., et al (2001). Journal Biological Chem 276:7806-7810.
2. Pigeon C., Ilyin G., et al (2001). Journal Biological Chem 276:7811-7819.
3. Nicolas G., Bennoun M., et al (2001). Proc Nat Acad Sciences 98: 8780-8785.
4. Ganz T. (2005). Best Practice & Research Clinical Haematology 18: 171-182.
5. Nemeth E., Rivera S., et al (2004). Journal Clinical Investigation 113:1271-1276.
6. Rivera S., Nemeth E., et al (2005). Blood 106: 2196-2199.
7. Modlinska K, Pisula W. 2020. DOI: https://doi.org/10.7554/eLife.50651

SKU# RHC-007
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INTRINSIC LIFESCIENCES
∞The BioIron Company™

Rat Hepcidin Compete™ ELISA

SKU# RHC-007

Intended Use:
The Intrinsic LifeSciences Rat Hepcidin Compete™ ELISA is designed for quantification of hepcidin in rat serum. This product does not recognize human or mouse hepcidin and these peptides do not interfere with rat hepcidin measurements using this ELISA. **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 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 increased 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.

Next to mice, rats are the second most common animal used in research (Modlinska & Pisula 2020). Data on the normal range of circulating levels of hepcidin in strains of laboratory rats (e.g. Wistar, Sprague-Dawley, Lewis) has not been determined.

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

Materials and Storage:
Store unopened kit at 2-8°C. Use kit before expiration date.

Materials Provided	1 Kit	SKU#
1. Microwell plate coated anti-rat hepcidin antibody	12x8x1	-
2. Rat Hepcidin Standard (1 glass vial, clear)	500ng	XRS-500
3. Rat Hepcidin Biotin Conjugate (1 glass vial, amber)	1 vial	XRS-140
4. SA-HRP Conjugate, 1 tube (conc., 100X)	150µl	CEC-007
5. Sample Diluent, 1 bottle (conc., 10X)	4ml	CSD-007
6. Wash Solution, 1 bottle (conc., 25X)	25ml	CWB-008
7. TMB Substrate, 1 bottle (ready to use)	12ml	CTM-007
8. Stop Solution, 1 bottle (ready to use)	12ml	CST-001
9. Microplate sealing film	2	-
10. Polypropylene 96-well Sample Set-up Plate	1	-

Materials Not Provided:
1. Precision pipettes and tips
2. Squirt bottle, manifold dispenser, or automated microplate washer
3. Reagent grade water (deionized or distilled)
4. Microplate reader (450nm), horizontal orbital microplate shaker
5. Tubes to dilute sample diluent, wash solution, rHep conjugate and HRP conjugate

Warnings and Precautions:
Kit does not contain azide- or mercury-based preservatives. For research use only.
1. It is strongly recommended to draw blood after fasting for 12 hours. Hyperlipemic, hemolyzed, heat-treated or contaminated samples may give erroneous results.
2. Do not dilute samples directly in the antibody-coated microwell plate.
3. Use separate pipette tips for each sample, standard, and reagent to avoid cross-contamination.
4. Use separate reservoirs for each reagent, especially the TMB Substrate.
5. The stop solution contains 0.5M sulfuric acid. Use appropriate protection.
6. Do not touch or scrape bottom or sides of antibody-coated wells of the microplate.
7. Incubation times and temperatures other than those specified may give erroneous results.
8. Do not allow the wells to dry once the assay has begun.
9. Do not reuse microwell plate or pour reagents back into their bottles once dispensed.
10. The components in this kit are an integral unit. All reagents should be used within 12 hours of preparation. Reagents from different lots should not be mixed. Do not refrigerate or freeze aliquots of any reagent for future use.

Specimen Collection and Handling:
Serum and urine specimens can be used for the assay.
Serum samples are optimal; use of plasma requires further investigation. It is strongly recommended to fast the rat for at least 12 hours before blood collection. Collect serum samples according to standard techniques. Centrifuge samples 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. Thaw samples and allow them to equilibrate to room temperature for 30 minutes before use; samples must be mixed before analysis.

Sample Dilution:
It is recommended to use a 5% sample dilution. Therefore 12µl of sample is required to begin quantification of hepcidin in duplicate. Samples can be diluted from 2.5-10% of the recommended 100µl assay volume. Avoid sample volumes >10% due to interference.

Preparation of Reagents:
This kit has sufficient reagents and is designed to run an 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 reagent grade water.
2. 1X Sample Diluent: Transfer contents of the concentrated **Sample Diluent** bottle (4ml) to 36ml of reagent grade water.
3. 1X SA-HRP Conjugate: Pipette 120µl of the concentrated **SA-HRP Conjugate** into 12ml of the 1X Sample Diluent.
4. Biotin Conjugate: Add 0.5 ml of 1X Sample diluent into **Rat Hepcidin Biotin Conjugate** vial, mix by vortex then transfer into 15ml tube containing 13.5ml of 1X Sample Diluent. Mix gently by inverting the tube a few times.
5. Biotin Conjugate for Standard: We recommend using a 5% sample dilution. Prepare 4 ml by mixing 0.2 ml of 1X Sample Diluent with 3.8 ml of Biotin Conjugate.

Preparation of Standard and Samples:
Standard:
1. Add 0.5 ml Biotin Conjugate for Standard to the **Rat Hepcidin Standard** vial. Mix by pipette.
2. Into the sample set-up plate, transfer 400µl of 1X Rat Hepcidin Standard (well A1) and 250µl Biotin Conjugate for Standard (wells B1-H1). Perform a 125µl serial dilution from well A1 to well G1; leave well H1 undiluted.

Sample:
3. Transfer 228µl of Biotin Conjugate to columns 2 through 6 of the sample set-up plate. Pipette 12µl of sample to achieve a final volume of 240µl/well (5% sample dilution).

Assay Procedure:
1. After warming to room temperature, remove microwell plate from the foil pouch.
2. Transfer 100µl/well of standard curve and samples from the sample set-up plate to the microwell plate in duplicate.
3. Apply sealing film and incubate on an orbital shaker (350 rpm) at room temperature for **2 hours**.
4. Wash microwell plate three times with 1X Wash Solution (300µl/well).
5. 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.
6. Wash microwell plate three times with 1X Wash Solution (300µl/well).
7. Develop the microwell plate by adding 100µl/well TMB Substrate. Incubate for **15 minutes** at room temperature. Protect from ambient light during incubation.
8. Stop the reaction by adding 100µl/well Stop Solution exactly 15 minutes after the addition of the TMB Substrate.
9. Measure absorbance at 450nm of the microwell plate using a plate reader.