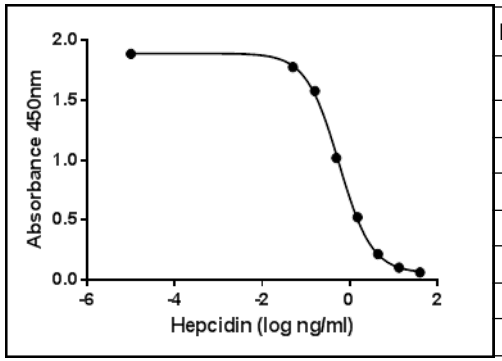
Standard Curve:
Eight point standard curve, including a zero, are used for each run. A typical standard curve is shown below:



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 baboon or cynomolgus monkey serum controls and utilize the hepcidin-25 concentration of the controls to validate the 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. Example for 10% sample: dilution factor = 24 µl sample / 240 µl assay volume = 1/10.

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 C.H., Valore E.V., et al (2001). The Journal of Biological Chemistry 276:7806-7810.
2. Pigeon C., Ilyin G., et al (2001). The Journal of Biological Chemistry 276:7811-7819.
3. Nicolas G., Bennoun M., et al (2001). PNAS 98: 8780-8785.
4. Ganz T. (2005). Best Practice & Research. Clinical Haematology 18: 171-182.
5. Nemeth E., Rivera S., et al (2004). The Journal of Clinical Investigation 113:1271-1276.
6. Rivera S., Nemeth E., et al (2005). Blood 106: 2196-2199.

SKU# PHC-001
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INTRINSIC LIFESCIENCES
∞The BioIron Company™

Primate-Hepcidin Compete™ ELISA

SKU# PHC-001

Intended Use:
The Intrinsic LifeSciences Primate-Hepcidin Compete™ competitive ELISA is designed for quantification of hepcidin-25 in baboon or cynomolgus monkey serum. Quantification of hepcidin-25 from other primate species or from plasma or other body fluids has not been evaluated.

This kit does not contain any 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 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.

Principle of the Test:
This kit is a solid-phase enzyme-linked immunosorbent assay (ELISA), based on the principles of competitive binding. Primate samples, standards, or controls are mixed with buffer containing hepcidin-25 biotin conjugate. This mixture is then incubated in a anti-primate hepcidin monoclonal antibody coated ELISA microwell plate. The more primate 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 hepcidin-25 biotin conjugate 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 primate 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 component lots.

Materials Provided	1 Kit	SKU#
1. Microwell plate coated anti-primate hepcidin antibody	96-well x1	-
2. Primate Hepcidin-25 Standard (1 glass vial, clear)	500 ng	XPS-200
3. Hepcidin-25 Biotin Conjugate (1 glass vial, amber)	1 bead	XPT-028
4. HRP Conjugate, 1 tube (conc., 100X)	150 µl	CEC-008
5. Sample Diluent, 1 bottle (conc., 10X)	4 ml	CSD-008
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 Plate	1	-

Materials Not Provided:
1. Precision pipettes and tips
2. Squirt bottle, manifold dispenser, or automated microplate washer
3. Deionized or distilled water
4. Horizontal orbital microplate shaker
5. Microplate reader (450nm)

Warnings and Precautions:
Kit does not contain any 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.
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 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:
Serum samples can be used for the assay.
Serum samples are optimal; use of plasma or other types of body fluids requires further investigation. Samples should be diluted to 5 or 10% of the recommended 100µl assay volume. It is recommended to begin at 10% and adjust if necessary. Therefore, to begin assessment the determination of hepcidin-25 in duplicate requires 24µl of serum. Collect serum 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 for Samples: Transfer lyophilized **Hepcidin-25 Biotin Conjugate** bead to 14ml of the 1X Sample Diluent. Mix gently by inversion or vortex.
5. Biotin Conjugate for Standard: Prepare 4ml; Dilute *Biotin Conjugate for Samples* based on the desired sample dilution:

Sample Dilution of 10%	Sample Dilution of 5%
3.6ml Biotin Conjugate 0.4ml 1X Sample Diluent	3.8ml Biotin Conjugate 0.2ml 1X Sample Diluent

Preparation of Standard and Samples:
Standard:
1. Add 0.5ml *Biotin Conjugate for Standard* to the **Primate Hepcidin-25 Standard** vial. Mix by pipette.
2. Into the PP 96-well sample set-up plate, transfer 400µl primate 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.

Sample:
3. Transfer the appropriate volume of Biotin Conjugate for Samples to the sample set-up plate and pipette in sample to achieve a final volume of 240µl/well:

Sample Dilution of 10%	Sample Dilution of 5%
216µl Biotin Conjugate 24µl sample	228µl Biotin Conjugate 12µl sample

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 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.