

Human Hepcidin ELISA Kit

Cat #: orb1473462 (manual)

Sandwich Enzyme-Linked Immunosorbent Assay for Quantitative Detection of Human Hepcidin Concentrations in Cell culture supernates, Serum, Plasma, Urine.

For research use only. Not for diagnostic or therapeutic procedures.

INTRODUCTION

Hepcidin, also known as Liver Expressed Antimicrobial Protein 1 (LEAP-1), is a peptide hormone that is involved in the regulation of iron metabolism. It is synthesized as a prohormone that is cleaved intracellularly and secreted as a mature 25 amino acid peptide. Hepcidin contains eight cysteine residues that form four disulfide bonds which appear to be important for stability in biological fluids. It is predominantly expressed, processed, and secreted by hepatocytes. Hepcidin expression is positively regulated by inflammation via IL-6/JAK2/ STAT3 signaling, endoplasmic reticulum stress, and BMP-6. BMP-6-dependent Hepcidin induction involves RGM-C/Hemojuvelin, which acts as a co-receptor for BMP-6. Conversely, Hepcidin expression is negatively regulated by MMP-15/MT2-MMP and multiple erythropoietic stimuli, including anemia, hypoxia, and Erythropoietin. MMP-15 downregulates Hepcidin expression by interacting with and cleaving RGM-C. Hepcidin was originally identified in human blood and urine as an antimicrobial peptide. It has since been shown to regulate iron metabolism. Hepcidin binds the cellular iron exporter Ferroportin, and this interaction results in Ubiquitin-mediated degradation of both Hepcidin and Ferroportin. Degradation of Ferroportin results in reduced iron release from macrophages, hepatocytes, and duodenal enterocytes, suggesting that Hepcidin may be an effector of inflammatory hypoferremia.

ASSAY PRINCIPLES

The Biorbyt Human Hepcidin ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Human Hepcidin in Cell culture supernates, Serum, Plasma, Urine. This assay employs an antibody specific for Human Hepcidin coated on a 96-well plate. Standards and samples are pipetted into the wells and Hepcidin present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human Hepcidin antibody is added. After washing away unbound biotinylated antibodies, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of Hepcidin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

KIT COMPONENTS

| Component | Volume |
|--|---------------------|
| 96-well Plate Coated With Anti-Human Hepcidin Antibody | 8 wells x 12 Strips |
| Human Hepcidin Standard | 0.5 ng x 2 |
| Biotin-Labeled Detection Antibody (100X) | 120 μ l |
| Streptavidin-HRP (100X) | 120 μ l |
| Standard/Sample Diluent | 30 ml |
| Detection Antibody Diluent | 12 ml |
| Streptavidin-HRP Diluent | 12 ml |
| Wash Buffer (20X) | 30 ml |
| TMB Substrate Solution | 12 ml |
| Stop Solution | 12 ml |
| Plate Adhesive Strips | 3 Strips |
| Technical Manual | 1 Manual |

STORAGE AND STABILITY

All kit components are stable at 2 to 8 °C. Standard (recombinant protein) should be stored at -20 °C or -80 °C (recommended at -80 °C) after reconstitution. Opened Microplate Wells or reagents may be stored for up to 1 month at 2 to 8 °C. Return unused wells to the pouch containing the desiccant pack, and reseal along the entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20 °C. Avoid repeated freeze-thaw cycles.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and pipette tips to deliver 2 µl to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1-liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. Computer and software for ELISA data analysis.
8. Tubes to prepare standard or sample dilutions.

HEALTH AND SAFETY PRECAUTIONS

1. Reagents provided in this kit may be harmful if ingested, inhaled, or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.
2. Stop Solution contains 2 N Sulfuric Acid (H₂SO₄) and is an extremely corrosive agent. Please wear proper eye, hand, and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution before disposing of the plate.

REAGENT PREPARATION

1. Sample Preparation

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

Cell culture supernates: Remove particulates by centrifugation, assay immediately or aliquot, and store samples at -20°C.

Serum: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 minutes. Analyze the serum immediately or aliquot and store samples at -20°C.

Plasma: Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1500 X g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C.

Cell Lysates: Collect cells and rinse cells with PBS. Homogenize and lysate cells thoroughly in lysate solution. Centrifuge cell lysates at approximately 10000 X g for 5 minutes to remove debris. Aliquots of the cell lysates were removed and assayed.

Bone Tissue: Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors. Dissolve the final sample in 2 M Guanidine-HCl.

Tissue Homogenates: The preparation of tissue homogenates will vary depending upon tissue type. Rinse tissue with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS, and stored overnight at ≤ -20 °C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. The supernate was removed immediately and assayed. Alternatively, aliquot and store samples at ≤ -20 °C.

Note: Some lysis buffers, such as RIPA can not be used. Some components will affect the binding.

Urine: Urinary samples should be cleared by centrifugation and then can be used directly without dilution. Storage at -20°C.

2. Human Hecpidin Standard Preparation

Reconstitute the lyophilized Human Hecpidin Standard by adding 1 ml of Standard/Sample Diluent to make the 500 pg/ml standard stock solution. Allow the solution to sit at room temperature for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting. Two tubes of the standard (0.5 ng per tube) are included in each kit. Use one tube for each experiment.

Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (7.8 pg/ml - 500 pg/ml) as below. Standard/Sample Dilution Buffer serves as the zero standard (0 pg/ml).

| Standard | Add | Into |
|------------|-------------------------------------|---------------------------------------|
| 500 pg/ml | | |
| 250 pg/ml | 500 µl of the Standard (500 pg/ml) | 500 µl of the Standard/Sample Diluent |
| 125 pg/ml | 500 µl of the Standard (250 pg/ml) | 500 µl of the Standard/Sample Diluent |
| 62.5 pg/ml | 500 µl of the Standard (125 pg/ml) | 500 µl of the Standard/Sample Diluent |
| 31.2 pg/ml | 500 µl of the Standard (62.5 pg/ml) | 500 µl of the Standard/Sample Diluent |
| 15.6 pg/ml | 500 µl of the Standard (31.2 pg/ml) | 500 µl of the Standard/Sample Diluent |
| 7.8 pg/ml | 500 µl of the Standard (15.6 pg/ml) | 500 µl of the Standard/Sample Diluent |
| 0 pg/ml | 1 ml of the Standard/Sample Diluent | |

Note: The standard solutions are best used within 2 hours. The 500 pg/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

3. Biotin-Labeled Detection Antibody Working Solution Preparation

The Biotin-Labeled Detection Antibody should be diluted in 1: 100 with the Detection Antibody Diluent and mixed thoroughly. The solution should be prepared no more than 2 hours prior to the experiment.

4. Streptavidin-HRP Working Solution Preparation

The Streptavidin-HRP should be diluted in 1: 100 with the Streptavidin-HRP Diluent and mixed thoroughly. The solution should be prepared no more than 1 hour prior to the experiment.

5. Wash Buffer Working Solution Preparation

Pour entire contents (30 ml) of the Wash Buffer Concentrate into a clean 1, 000 ml graduated cylinder. Bring the final volume to 600 ml with glass-distilled or deionized water (1: 20).

ASSAY PROCEDURE

The Streptavidin-HRP Working Solution and TMB Substrate Solution must be kept warm at 37°C for 30 minutes before use. When diluting samples and reagents, they must be mixed completely and evenly. A standard detection curve should be prepared for each experiment. The user will decide on sample dilution fold by crude estimation of protein amount in samples.

1. Add 100 µl of each standard and sample into appropriate wells.
2. Cover well and incubate for 90 minutes at room temperature or overnight at 4°C with gentle shaking.
3. Remove the cover, discard the solution, and wash the plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 100 µl of Biotin-Labeled Detection Antibody Working Solution into each well and incubate the plate at 37°C for 60 minutes.
5. Wash the plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Discard the Wash Buffer Working Solution and blot the plate onto paper towels or other absorbent material.
6. Add 100 µl of Streptavidin-HRP Working Solution into each well and incubate the plate at 37°C for 45 minutes.
7. Wash the plate 5 times with Wash Buffer Working Solution, and each time let the wash buffer stay in the wells for 1 - 2 minutes. Discard the wash buffer and blot the plate onto paper towels or other absorbent material.
8. Add 100 µl of TMB Substrate Solution into each well and incubate the plate at 37°C in the dark for 10-20 minutes.
9. Add 100 µl of Stop Solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 minutes after adding the Stop Solution.

For calculation, $(\text{the relative O.D.450}) = (\text{the O.D.450 of each well}) - (\text{the O.D.450 of Zero well})$. The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The concentration of the samples can be interpolated from the standard curve.

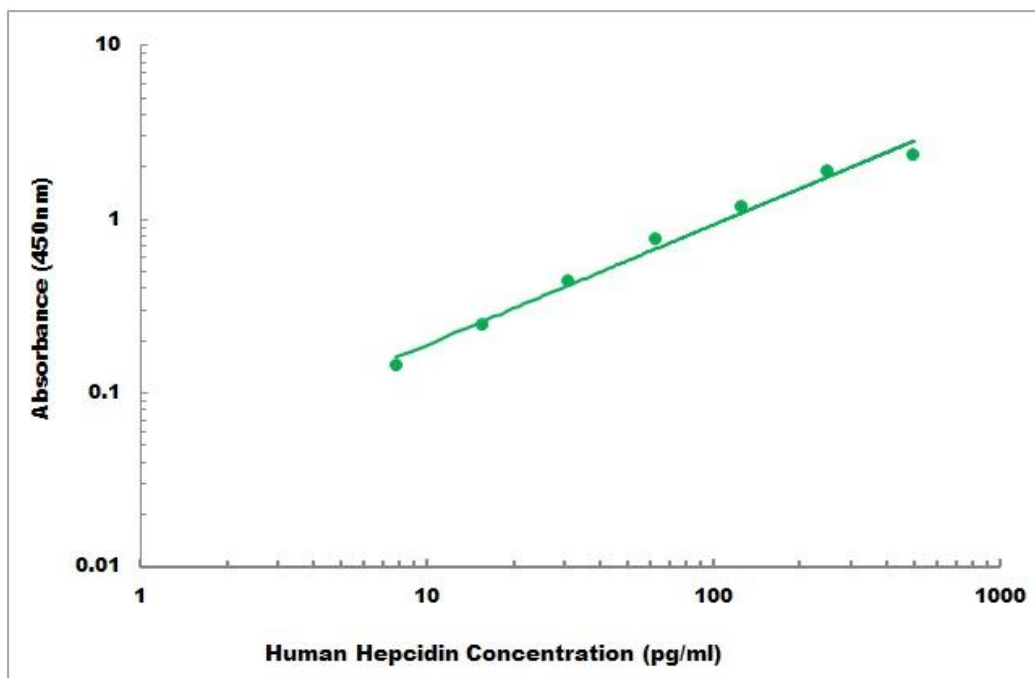
Note: If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution.

ASSAY PROCEDURE SUMMARY

- Prepare all reagents, samples and standards
- Add 100 μ l Standard or Sample
- Wash plate 3 times with Wash Buffer Working Solution
- Add 100 μ l Biotin-Labeled Detection Antibody Working Solution
- Wash plate 3 times with Wash Buffer Working Solution
- Add 100 μ l Streptavidin-HRP Working Solution
- Wash plate 5 times with Wash Buffer Working Solution
- Add 100 μ l TMB Substrate Solution
- Add 100 μ l Stop Solution
- Read the plate at 450nm

TYPICAL DATA

The standard curve is for demonstration only. A standard curve must be run with each assay.



SENSITIVITY

The minimum detectable dose of Human Hepcidin is typically less than 4 pg/ml.

SPECIFICITY

The Human Hepcidin ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human Hepcidin proteins within the range of 7.8 pg/ml - 500 pg/ml.

CROSS REACTIVITY

No detectable cross-reactivity with other relevant proteins.

TROUBLESHOOTING GUIDE

| Problem | Possible Cause | Solution |
|--|--|--|
| High signal and background in all wells | • Insufficient washing | • Increase number of washes • Increase the time of soaking between in-wash |
| | • Too much Streptavidin-HRP | • Check dilution, titration |
| | • Incubation time too long | • Reduce incubation time |
| | • Development time too long | • Decrease the incubation time before the stop solution is added |
| No signal | • Reagent added in incorrect order or incorrectly prepared | • Review protocol |
| | • Standard has gone bad (If there is a signal in the sample wells) | • Check the condition of stored standard |
| | • Assay was conducted from an incorrect starting point | • Reagents are allowed to come to 20 - 30 °C before performing the assay |
| Too much signal-whole plate turned uniformly blue | • Insufficient washing-unbound Streptavidin-HRP remaining | • Increase the number of washes carefully |
| | • Too much Streptavidin-HRP | • Check dilution |
| | • Plate sealer or reservoir reused, resulting in the presence of residual Streptavidin-HRP | • Use fresh plate sealer and reagent reservoir for each step |
| Standard curve achieved but poor discrimination between point | • Plate not developed long enough | • Increase substrate solution incubation time |
| | • Improper calculation of standard curve dilution | • Check dilution, make a new standard curve |
| No signal when a signal is expected, but the standard curve looks fine | • Sample matrix is masking detection | • More diluted sample Recommended |
| Samples are reading too high, but the standard curve is fine | • Samples contain protein levels above the assay range | • Dilute samples and run Again |
| Edge effect | • Uneven temperature around the work surface | • Avoid incubating plates in areas where environmental conditions vary • Use plate sealer |