Quantikine® ELISA

Human Hepcidin Immunoassay

Catalog Number DHP250

For the quantitative determination of human Hepcidin concentrations in cell culture supernates, serum, plasma, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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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 (1, 2). It is synthesized as a preprohormone that is cleaved intracellularly and secreted as a mature 25 amino acid peptide (1, 3, 4). Hepcidin contains eight cysteine residues that form four disulfide bonds which appear to be important for stability in biological fluids (5). It is predominantly expressed, processed, and secreted by hepatocytes (2, 6). Hepcidin expression is positively regulated by inflammation via IL-6/JAK2/STAT3 signaling, endoplasmic reticulum stress, and BMP-6 (7-11). BMP-6-dependent Hepcidin induction involves RGM-C/Hemojuvelin, which acts as a co-receptor for BMP-6 (11-13). Conversely, Hepcidin expression is negatively regulated by MMP-15/MT2-MMP and multiple erythropoietic stimuli, including anemia, hypoxia, and Erythropoietin (14-18). MMP-15 downregulates Hepcidin expression by interacting with and cleaving RGM-C (19).

Hepcidin was originally identified in human blood and urine as an antimicrobial peptide (1, 3). 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 (20-22). Degradation of Ferroportin results in reduced iron release from macrophages, hepatocytes, and duodenal enterocytes, suggesting that Hepcidin may be an effector of inflammatory hypoferremia (20). Pathologies have been associated with both deficiency and overexpression of Hepcidin. Overexpression of Hepcidin results in lower iron levels and may contribute to the development of anemia in many human disorders (23). Higher Hepcidin levels have also been linked to poor survival in patients with non-Hodgkin lymphoma (24). Hepcidin deficiency results in increased iron levels and is the pathogenic cause of iron overload in most forms of hereditary hemochromatosis (25, 26).

The Quantikine® Human Hepcidin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Hepcidin in cell culture supernates, serum, plasma, and urine. It contains synthetic Hepcidin and antibodies raised against synthetic Hepcidin. This immunoassay has been shown to accurately quantitate synthetic and naturally occurring human Hepcidin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Hepcidin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Hepcidin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Hepcidin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Hepcidin bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Hepcidin Microplate	894894	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Hepcidin.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Hepcidin Standard	894896	2 vials of synthetic Hepcidin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a fresh standard for each assay. Discard after use.
Human Hepcidin Conjugate	894895	21 mL of a monoclonal antibody specific for human Hepcidin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-21	895215	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:4 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human Hepcidin Controls (optional; R&D Systems®, Catalog # QC220).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly icteric samples are not suitable for use in this assay.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell culture supernate samples require a 2-fold dilution due to a matrix effect. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

Serum and plasma samples require at least a 5-fold dilution due to matrix effect. A 20-fold dilution is recommended due to endogenous levels. Due to the wide range of sample values, multiple dilutions may be necessary. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

Urine samples require at least a 10-fold dilution due to endogenous levels. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

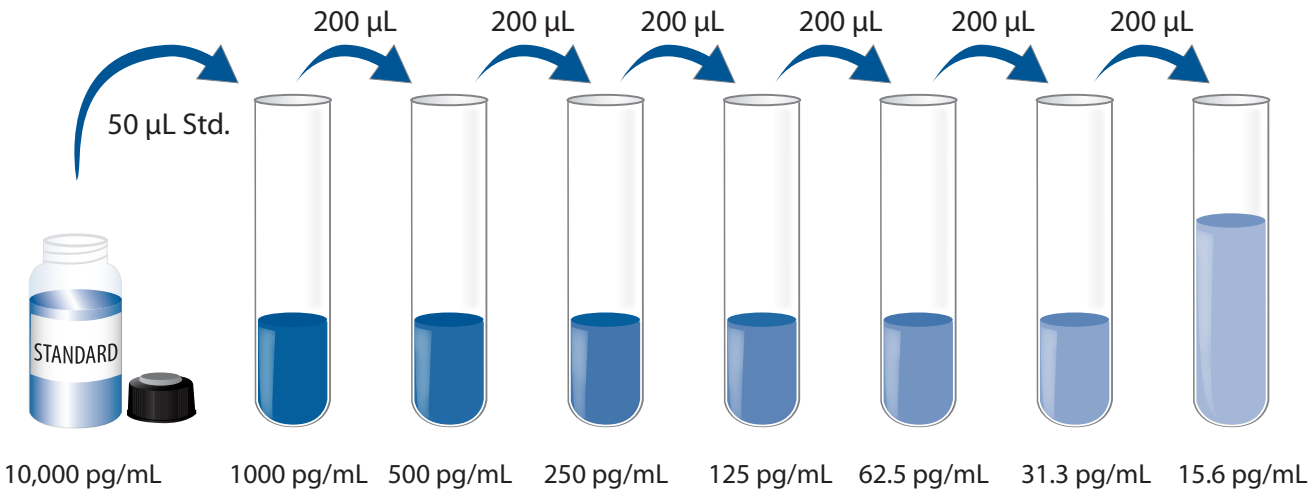
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:4) - Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Human Hepcidin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Hepcidin Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-26 (diluted 1:4) into the 1000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-21 to each well.
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human Hepcidin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

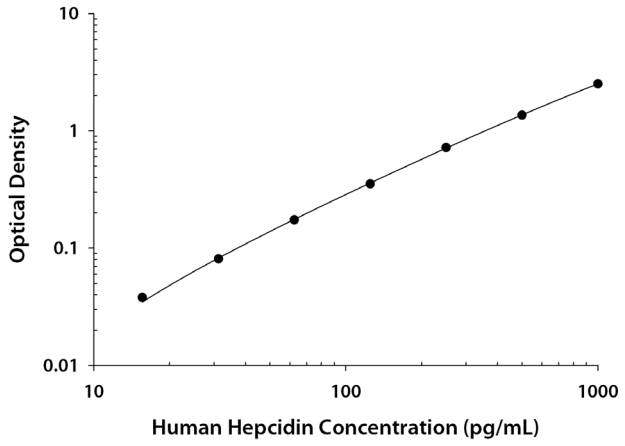
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Hepcidin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.008 0.010	0.009	—
15.6	0.047 0.047	0.047	0.038
31.3	0.089 0.090	0.090	0.081
62.5	0.182 0.184	0.183	0.174
125	0.358 0.366	0.362	0.353
250	0.721 0.736	0.729	0.720
500	1.351 1.390	1.371	1.362
1000	2.506 2.535	2.521	2.512

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	175	350	726	164	328	647
Standard deviation	7.51	10.8	23.5	18.0	26.1	39.8
CV (%)	4.3	3.1	3.2	11.0	8.0	6.2

RECOVERY

The recovery of human Hepcidin spiked to levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	111	99-118%
Serum (n=4)	106	96-118%
EDTA plasma (n=4)	103	95-115%
Heparin plasma (n=4)	104	93-117%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human Hepcidin were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	94	100	98	98	95
	Range (%)	92-98	100-102	97-101	96-100	95-97
1:4	Average % of Expected	92	101	95	97	95
	Range (%)	87-98	99-103	92-99	95-102	92-99
1:8	Average % of Expected	92	101	96	97	96
	Range (%)	86-98	97-104	90-103	93-103	95-97
1:16	Average % of Expected	92	98	94	97	95
	Range (%)	90-94	90-106	84-104	87-106	92-100

SENSITIVITY

Nineteen assays were evaluated and the minimum detectable dose (MDD) of human Hepcidin ranged from 0.446-3.81 pg/mL. The mean MDD was 1.70 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is standardized against synthetic Hepcidin.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Hepcidin in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=40)	18,365	79.0-49,400	14,677
EDTA plasma (n=40)	19,399	82.4-56,700	15,506
Heparin plasma (n=40)	17,467	83.3-49,201	14,191

Sample Type	Mean (µg/g Creatinine)	Range (µg/g Creatinine)	Standard Deviation (µg/g Creatinine)
Urine (n=12)	69,054	1768-195,947	64,158

Cell Culture Supernates:

HepG2 human hepatocellular carcinoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were unstimulated or stimulated with 10 ng/mL of recombinant human IL-6, 10 ng/mL of recombinant human IL-1β, and 10 ng/mL of recombinant human TNF-α for 3 days. Aliquots of the cell culture supernates were removed, assayed for human Hepcidin, and measured 4455 pg/mL and 20,000 pg/mL, respectively.

HepG2 human hepatocellular carcinoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate under normoxic conditions or in a hypoxia workstation at 1% O_2 for 3 days. Aliquots of the cell culture supernates were removed, assayed for human Hepcidin, and measured 1804 pg/mL and 3540 pg/mL, respectively.

SPECIFICITY

This assay recognizes natural human Hepcidin.

The factors listed below were prepared in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors in a mid-range recombinant human Hepcidin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinants:
human Ferroportin
human Pro-Hepcidin (aa 25-85)
mouse Hepcidin-2

Natural proteins:
human α_2-Macroglobulin
human Serum Albumin

This kit also recognizes human Hepcidin-20, -22, -24, and -25.

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