

Human Hepcidin, (Hepc) ELISA

Cat No: K12-1020

Principle:

This is sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Hepcidin in samples. Standards or Samples are added to the microtiter well which is pre-coated with Human Hepcidin monoclonal Antibody. Biotinylated Human Hepcidin antibodies are added to the microplate to form a complex. Subsequently Streptavidin-HRP conjugate is pipetted. After incubation and a washing step TMB Substrate A and B, are added. Blue color develops on incubation and the reaction is stopped with a Stop Solution to form a yellow color. The concentration of the Human Hepcidin in the samples is directly proportional to the yellow color developed in the wells.

Intended Use:

This kit is used to assay the level of Human Hepcidin in Human serum and plasma samples.

Materials provided in the kit:

1. Anti-Human Hepcidin Coated Microtitre Plate (96 wells) – 1 no
2. Biotinylated Human Hepcidin Antibody – 1 ml
3. Human Hepcidin Standard (1600 ng/ml) – 0.5 ml
4. Streptavidin-HRP Conjugate – 6 ml
5. (30X) Wash Buffer– 20 ml
6. Standard Diluent – 3 ml
7. TMB Substrate A– 6 ml
8. TMB Substrate B – 6 ml
9. Stop Solution – 6 ml
10. Instruction Manual

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Semi-log graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

Storage Information:

1. All reagents should be stored at 2°C to 8°C.
2. All the reagents and wash solutions are stable until the expiration date of the kit.
3. 30 minutes prior before use, bring all components to room temperature (18-25°C). Store all the components of the kit at its appropriate storage condition after use.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

1. The kit cannot test samples which contain NaN_3 , because NaN_3 inhibits HRP activity.
2. Extract as soon as possible after specimen collection as per relevant procedure. The samples should be tested as soon as possible after the extraction. Alternately the extracted samples can be kept in -20°C. Avoid repeated freeze-thaw cycles.
3. **Serum-** Coagulate at room temperature for 10-20 minutes; centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.

- Plasma-** Use EDTA or citrate plasma as an anticoagulant, mix for 10-20 minutes; centrifuge for 20-min at the 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.

Note: Grossly hemolyzed samples are not suitable for use in this assay.

Reagent Preparation (all reagents should be diluted immediately prior to use):

- Bring all reagents to Room Temperature prior to use.
- To make 1X Wash Solution, add 10 ml of 30X Wash Buffer in 290 ml of DI water.

Procedural Notes:

- In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
- High Dose Hook Effect may be observed in samples with very high concentrations of Human Hepcidin. High Dose Hook Effect is due to excess of antibody for very high concentrations of Human Hepcidin present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Human Hepcidin of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
- Avoid assay of Samples containing Sodium Azide (NaN₃), as it could destroy the HRP activity resulting in under-estimation of the amount Human Hepcidin.
- It is recommended that all Controls and Samples be assayed in duplicates.
- Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
- If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
- The plates should be read within 30 minutes after adding the Stop Solution.
- Make a work list in order to identify the location of Controls and Samples.

Assay Procedure:

- Bring all reagents to room temperature prior to use. It is strongly recommended that all Standards and Samples should be run in duplicates or triplicates. A standard curve is required for each assay.
- Standards Dilution: Prepare the standards as per the table given below using the provided standard Concentration and standard diluent.

Standard Concentration	Standard No	Dilution Particulars
1600 ng/ml	Standard, concentrated	Original Standard provided in the Kit
800 ng/ml	Standard No.5	120 ul Original Standard + 120 ul Standard diluent
400 ng/ml	Standard No.4	120 ul Standard No.5 + 120 ul Standard diluent
200 ng/ml	Standard No.3	120 ul Standard No.4 + 120 ul Standard diluent
100 ng/ml	Standard No.2	120 ul Standard No.3 + 120 ul Standard diluent
50 ng/ml	Standard No.1	120 ul Standard No.2 + 120 ul Standard diluent

** refer accompanying sheet with the Standard, concentrated in the kit*

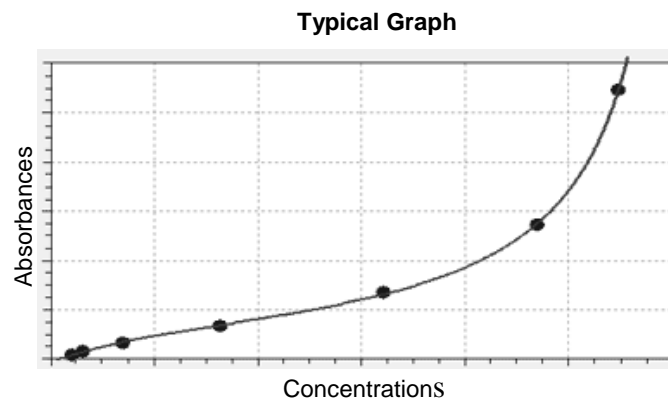
- The quantity of the plates depends on the quantities of samples and standards to be tested. It is suggested to remove the number of strips required for the assay.
- Pipette **50 ul of Standards** and **40 ul Samples** into the respective wells as mentioned in the work list.
Note do not add the sample, Biotin Conjugate and Streptavidin-HRP to the blank well.
- Pipette **10 ul of Biotinylated Human Hepcidin Antibody** into each sample well.
Do not pipette into the blank and standards wells.
- Pipette **50 ul of HRP Conjugate** into each sample and standards well.
Do not pipette into the Blank well.
- Cover the plate and incubate for 1 hour at 37°C in the incubator.
- Aspirate and wash plate 4 times with **1X Wash Buffer** and blot residual buffer by firmly tapping the plate on an absorbent paper. Wipe off any liquid from the bottom of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
- Then add **TMB Substrate A 50 ul**, then **TMB Substrate B 50 ul** to each well including Blank well. Gently mixed,
- Incubate for 10 min at 37°C in dark.
- Pipette **50 ul of Stop Solution**. Wells should turn from blue to yellow in color.

12) Read the absorbance at 450 nm within 15 minutes after adding the Stop Solution. Blanking the zero standard for net absorbance.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit or a polynomial regression to the 2nd order is best recommended for automated results.

**Precautions:**

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

Performance Characteristics:

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Sensitivity:

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to be **5.123 ng/ml**.

Specificity:

The antibodies used in the kit for capture and detection are specific for human Hepsidin.

Assay Range: 50 ng/ml – 800 ng/ml**Precision:**

Intra-Assay: CV<10%

Inter-Assay: CV<12%

Linearity:

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of human Hepcidin and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1:2	1:4	1:8
serum (n=5)	84-120%	87-108%	82-112%
EDTA plasma (n=5)	83-102%	83-115%	83-118%
heparin plasma (n=5)	83-99%	80-95%	82-93%

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Human Hepcidin, (Hepc) ELISA ASSAY PROCEDURE

1	Bring all reagents to room temperature before use.		
2	Pipette Standards 1 - 6 Samples	50 ul	40 ul
3	Pipette Hepcidin Biotin Detection Antibody		10 ul
4	Pipette Streptavidin :HRP Conjugate	50 ul	50 ul
5	Incubate 60 minutes (37°C)		
6	Wash 1X Wash Buffer Decant, 4 x 300 ul		
7	Pipette Substrate (A)	50 ul	50 ul
8	Pipette Substrate (B)	50 ul	50 ul
9	Incubate in the dark 10 minutes (37°C)		
10	Pipette Stop Solution	50 ul	50 ul
11	Measure 450 within 15 mins		

Troubleshooting:

Problem	Possible cause	Investigation/Actions
High Absorbances	<ol style="list-style-type: none"> 1. Cross-contamination from other specimens 2. Insufficient or inefficient washing or reading 3. Wavelength of filter not correct. 4. High assay background. 5. Contaminated TMB 6. Incubation time too long or incubation temperature too high. 7. Incorrect dilution of serum 	<ul style="list-style-type: none"> > Repeat assay taking care when washing and pipetting. > Check washer efficiency > Check that the wavelength is 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. > Repeat assay and include a well that contains only sample diluent or sample absorbent (i.e. a blank well). > Check that TMB is colorless or faint blue. > Check incubation time and temperature. > Check incubator is at the correct temperature. > Repeat assay, ensuring correct serum dilution is used.
Low Absorbances	<ol style="list-style-type: none"> 1. Incubation time too short or incubation temperature too low. 2. Incorrect dilution or pipetting of sera 3. Incorrect filter wavelength. 4. Contaminated Conjugate solution. 5. Kit has expired. 6. Air blank reading high. 7. Incorrect storage of kit. 8. Kit reagents not equilibrated at room temperature 9. Incorrect reagents used. 10. Over washing of plate (e.g. inclusion of a long soak step). 	<ul style="list-style-type: none"> > Ensure time and temperature of assay incubation are correct. > Check incubator is set at the correct temperature. > Repeat assay ensuring correct dilutions and volumes are used. > Ensure controls are sufficiently mixed. > Check the wavelength is set at 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650nm. > Dispense conjugate directly from the bottle using clean pipette tip; avoid transferring Conjugate to another container if possible. > Do not return unused Conjugate to bottle. > Ensure all pipettes and probes used to dispense the Conjugates are clean and free from serum, detergent and bleach. > Check expiration date of kit and do not use if expired. Investigate causes of high background absorbance. > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue/purple. > Allow sufficient time for reagents to equilibrate to room temperature prior to assay. > Check the reagents used match those listed on the specification sheet. > Repeat assay using recommended wash procedure.
Poor Duplicates	<ol style="list-style-type: none"> 1. Poor mixing of samples. 2. Poor pipette precision 3. Addition of reagents at inconstant timing intervals; reagent addition takes too long, air bubbles when adding reagents. 4. Inefficient washing - Wash buffer left in wells, inconsistent washing, inadequate washing. 5. Reader not calibrated or warmed up prior to plate reading. 6. Optical pathway not clean 7. Spillage of liquid from wells 8. Serum samples exhibit microbial growth, haemolysis or lipaemia. 9. Uneven well volumes due to evaporation. 	<ul style="list-style-type: none"> > Mix reagents gently and equilibrate to room temperature. > Calibration may need to be checked. > Check pipetting technique-change pipette tip for each sample and ensure excess liquid is wiped from the outside of the tip. > Use consistent timing when adding reagents. > Ensure all dilutions are made before commencing addition to plate. > Improve pipetting technique and skill. > Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled and aspirated when washing. > Check reader precision > Check reader manual to ascertain warm up time of instrument. > Gently wipe bottom of plate. > Check reader light source and detector are clean. > Repeat assay, taking care not to knock the plate or splash liquid > It is not recommended to use serum samples exhibiting microbial growth, haemolysis or lipaemia. > Cover plate with a lid or plate sealer (not provided).
All wells yellow	<ol style="list-style-type: none"> 1. Contaminated TMB. 2. Contaminated reagents (e.g. Conjugate, Wash buffer). 3. Incorrect dilution of serum. 4. Incorrect storage of kit. 5. Inefficient washing- Wash buffer left in wells, inconsistent washing, inadequate washing. 6. If Conjugate reconstitute is required – Conjugate reconstituted incorrectly. 	<ul style="list-style-type: none"> > Check TMB is colorless or faint blue. > Check reagents for turbidity. > Repeat assay, ensuring correct serum dilution is used. > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue / purple. > Tap out wash buffer after washing. > Check wells are sufficiently and uniformly filled and aspirated when washing. > Repeat assay ensuring Conjugate is reconstituted according to assay method.

All wells negative

1. Test not performed correctly – correct reagents not added or not added in the correct sequence.
 - > Check procedure and check for unused reagents.
 - > Ensure that Stop Solution was not added before Conjugate or TMB.
 - > Ensure that serum was diluted in correct Sample diluent; e.g. do not use Sample Absorbent for an IgG ELISA.
2. Contaminated Conjugate solution.
 - > Dispense Conjugate directly from the bottle using a clean pipette tip; avoid transferring Conjugate to another container if possible.
 - > Do not return unused Conjugate to bottle.
 - > Ensure all pipettes and probes used to dispense the Conjugate are clean and free from serum, detergent and bleach.
 - > Repeat assay using recommended wash procedure.
3. Over- washing of plate (e.g. inclusion of a long soak step).
4. Incorrect storage of kit.
 - > Ensure kit is stored at 2-8°C, plate is sealed in foil pouch and desiccant sachet is blue / purple.
5. Wash Buffer made up with Stop Solution instead of Wash Buffer Concentrate
 - > Ensure Wash Buffer is made up correctly.