Revised 15 August 2006 **RUO**

1 INTRODUCTION

The DRG® Hepcidin Prohormone Enzyme Immunoassay Kit provides materials for the quantitative determination of Hepcidin Prohormone in human serum.
This assay is intended for research use only.

Hepcidin is a small cysteine-rich peptide produced in the liver [1]. This molecule regulates the absorption of iron in the body similarly to the correlation of insulin and sugar.
Hepcidin was initially isolated as a 25 amino acid (aa) peptide in human plasma and urine exhibiting antimicrobial activity [2,3].
Human Hepcidin is produced from an 84 aa precursor including a putative (24 aa) signal peptide.
Application of the present ELISA allows the detection and determination of Hepcidin Prohormone (Pro-Hepcidin) in serum of patients suffering from several disorders of iron metabolism.

2 PRINCIPLE OF THE TEST

The DRG® Hepcidin Prohormone ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding.
The microtiter wells are coated with a polyclonal antibody directed towards an antigenic site on the Hepcidin Prohormone molecule (28-47 aa). Endogenous Hepcidin Prohormone of a patient sample competes with a Hepcidin Prohormone-biotin conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off.
The amount of bound biotin conjugate is reverse proportional to the concentration of Hepcidin Prohormone in the sample.
After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of Hepcidin Prohormone in the patient sample.

3 PRECAUTIONS

- This kit is intended for Research Use Only.
- For information on hazardous substances included in the kit please refer to Material Safety Data Sheets.
- All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
- Avoid contact with *Stop Solution* containing 0.5 M H_2SO_4. It may cause skin irritation and burns.
- Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
- Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
- Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
- Handling should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
- Do not use reagents beyond expiry date as shown on the kit labels.
- All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
- Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
- Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guideline or regulation.
- Safety Data Sheets for this product are available upon request directly from DRG International, Inc..
  The Safety Data Sheets fit the demands of: EU-Guideline 91/155 EC.

4 KIT COMPONENTS

4.1 Contents of the Kit

1. ***Microtiterwells***, 12x8 (break apart) strips, 96 wells;
   Wells coated with anti Pro-Hepcidin antibody (polyclonal).
2. ***Standard (Standard 0-6)***, 7 vials (lyophilized), 1 mL;
   Concentrations: 10, 50, 100, 250, 500, 1000 ng/mL of synthetic peptide Hepcidin (28-47).
   Contains < 0.3% Proclin 300 as a preservative.
   See „Preparation of Reagents“;
3. ***Control***, 1 vial (lyophilized), 1 mL,
   Control value and range please refer to vial label or QC-Datasheet.
   see „Reagent Preparation“
   Contains < 0.3% Proclin 300 as a preservative.
4. ***Assay Buffer***, 1 vial, 14 mL, ready to use,
   contains < 0.3% Proclin 300 as a preservative.
5. ***Biotin Conjugate***, 1 vial, 14 mL, ready to use,
   Pro-Hepcidin fragment conjugated to biotin;
   contains < 0.3% Proclin 300 as a preservative.
6. ***Enzyme Complex***, 1 vial, 14mL, ready to use
   contains horseradish Peroxidase
   contains 0.01% methylisothiazolone, 0.01% bromonitrodioxane as a preservative
7. ***Substrate Solution***, 1 vial, 14 mL, ready to use;
   Tetramethylbenzidine (TMB).
8. ***Stop Solution***, 1 vial, 14 mL, ready to use;
   contains 0.5M H_2SO_4.
   Avoid contact with the stop solution. It may cause skin irritations and burns.
9. ***Wash Solution***, 1 vial, 30 mL (40X concentrated);
   see „Preparation of Reagents“.

4.1.1 Equipment and material required but not provided

- A microtiter plate calibrated reader (450±10 nm), (e.g. the DRG International Microtiter Plate Reader).
- Calibrated variable precision micropipettes.
- Absorbent paper.
- Aqua dest.

4.2 Storage and stability of the Kit

When stored at 2-8°C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date. Opened reagents must be stored at 2-8°C. Microtiter wells must be stored at 2-8°C. Once the foil bag has been opened, care should be taken to close it tightly again.
Opened kits retain activity for two months if stored as described above.

4.3 Preparation of Reagents

Allow all reagents and required number of strips to reach room temperature prior to use.

Standards

Reconstitute the lyophilized contents of the standard vial with 1.0 mL Aqua dest.
Note: *The reconstituted standards are stable for 6 days at 2-8°C. For longer storage freeze at -20°C.*

Control

Reconstitute the lyophilized content with 1.0 mL Aqua dest. and let stand for 10 minutes in minimum. Mix the control several times before use.
Note: *The reconstituted control is stable for 6 days at 2-8°C. For longer storage freeze at -20°C.*

Wash Solution

Dilute 30 mL of concentrated Wash Solution with 1170 mL deionized water to a final volume of 1200 mL.
The diluted Wash Solution is stable for 2 weeks at room temperature.

4.4 Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheets (see chapter 13).

4.5 Damaged Test Kits

In case of any severe damage of the test kit or components, DRG® have to be informed written, latest one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5 SPECIMEN

Serum can be used in this assay.
Do not use haemolytic, icteric or lipaemic specimens.
Please note: Samples containing sodium azide should not be used in the assay.

5.1 Specimen Collection

Serum:
Collect blood by venipuncture (e.g. Sarstedt Monovette # 02.1388.001), allow to clot, and separate serum by centrifugation at 2500 x g for 10 min at 4°C. Aliquots should be stored at –20°C.
For other sample material a special extraction protocol may be necessary.

5.2 Specimen Storage

Specimens should be capped and may be stored for up to 24 hours at 2-8°C prior to assaying.
Specimens held for a longer time should be frozen only once at -20°C prior to assay. Thawed samples should be inverted several times prior to testing.

Note: *Samples from patients which were frozen for a longer period or which were repeatedly thawed show higher Pro-Hepcidin concentrations than freshly collected samples which were frozen only once.*

5.3 Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with *Assay Buffer* and reassayed as described in Assay Procedure.
For the calculation of the concentrations this dilution factor has to be taken into account.

Example:
a) Dilution 1:10: 10 µL Serum + 90 µL *Assay Buffer* (mix thoroughly)
b) Dilution 1:100: 10 µL dilution a) 1:10 + 90 µL *Assay Buffer* (mix thoroughly).

6 TEST PROCEDURE

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

6.2 Assay Procedure

Each run must include a standard curve.

1. Secure the desired number of Microtiter wells in the holder.
2. Dispense **100 µl** of *Assay Buffer* into appropriate wells.
3. Dispense **50 µl** of each *Standard, Control* and samples with new disposable tips into appropriate wells.
4. Dispense **100 µl** *Biotin Conjugate* into each well.
5. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
6. Incubate for **120 minutes** at room temperature (without covering the plate).
7. Briskly shake out the contents of the wells.
   Rinse the wells 5 times with diluted *Wash Solution* (400 µl per well). Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
8. Add **100 µl** of *Enzyme Complex* to each well.
9. Incubate for **60 minutes** at room temperature.
10. Briskly shake out the contents of the wells.
    Rinse the wells 5 times with diluted *Wash Solution* (400 µl per well). Strike the wells sharply on absorbent paper to remove residual droplets.
11. Add **100 µl** of *Substrate Solution* to each well.
12. Incubate for **30 minutes** at room temperature.
13. Stop the enzymatic reaction by adding **100 µl** of *Stop Solution* to each well.
14. Read the OD at **450±10 nm** with a microtiter plate reader **within 10 minutes** after adding the *Stop Solution*.

6.3 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted. For the calculation of the concentrations this dilution factor has to be taken into account.

Below is listed a typical example of a standard curve with the DRG® Hepcidin Prohormone ELISA.

Standard	Optical Units (450 nm)
Standard 0 (0 ng/mL)	1.85
Standard 1 (10 ng/mLl)	1.72
Standard 2 (50 ng/mL)	1.38
Standard 3 (100 ng/mL)	1.16
Standard 4 (250 ng/mL)	0.82
Standard 5 (500 ng/mL)	0.63
Standard 6 (1000 ng/mL)	0.48

7 EXPECTED VALUES

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

8 ASSAY CHARACTERISTICS

8.1 Assay Dynamic Range

The range of the assay is between 0 – 1000 ng/mL.

8.2 Analytical Sensitivity

The analytical sensitivity was calculated from the mean minus two standard deviations of twenty one (21) replicate analyses of *Standard 0* and was found to be < 3.95 ng/mL.

8.3 Precision

8.3.1 Intra Assay Variation

The within assay variability is shown below:

Sample	n	<X> ± SD (ng/mL)	CV (%)
1	12	426.7 ± 20.2	4.69
2	12	210.7 ± 8.58	4.07
3	12	110.7 ± 4.74	4.28

8.3.2 Inter Assay Variation

The between assay variability is shown below:

Sample	n	<X> ± SD (ng/mL)	CV (%)
1	23	431.96 ± 20.8	4.82
2	23	216.17 ± 14.44	6.68
3	23	109.8 ± 10.72	9.76

8.4 Accuracy

8.4.1 Quality Control

It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.
The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.

It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.
Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid.

In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.
After checking the above mentioned items without finding any error contact your distributor or DRG® directly.

8.4.2 Recovery

The analytical recovery of Pro-Hepcidin was estimated at 3 different concentrations in serum samples. Increasing amounts of unlabeled Pro-Hepcidin (50 ng/ml, 250 ng/ml, 500 ng/ml) were added to the samples with various initial Pro-Hepcidin concentrations. Each sample (non spiked and spiked) was assayed. The Pro-Hepcidin concentrations were measured and the percentage recovery rates were calculated.

Serum	Endogenous Pro-Hepcidin (ng/mL)	Average Recovery (%)	Range of Recovery (%)
1	273.8	93.1	91.8-94.3
2	116.8	94.7	89.2-98.7
3	82.3	97.1	94.5-105.7

8.4.3 Linearity

The linearity of the assay was evaluated by diluting of the samples (serum) having different Pro-Hepcidin levels with *Assay Buffer*. The Pro-Hepcidin content in the diluted samples was assayed by the ELISA. Three dilutions were performed for each sample and the percentage recovery rates were calculated.

Serum	Endogenous Pro-Hepcidin (ng/mL)	Average Recovery (%)	Range of Recovery (%)
1	591.6	99.1	90.6-108.2
2	157.5	107.9	106.3-107.2
3	179.4	104.6	92.3-111.6

9 LIMITATIONS OF USE

9.1 Interfering Substances

Any improper handling of samples or modification of this test might influence the results.

9.2 Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of XXXX in a sample.

9.3 High-Dose-Hook Effect

No hook effect was observed in this test.

10 LEGAL ASPECTS

10.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.
The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact DRG®.

10.2 Therapeutical Consequences

Therapeutical consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 10.1. Any laboratory result is only a part of the total clinical picture of a patient.

Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutical consequences be derived.
The test result itself should never be the sole determinant for deriving any therapeutical consequences.

10.3 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.
Claims submitted due to customer misinterpretation of laboratory results subject to point 10.2. are also invalid.
Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

11 REFERENCES

1. Kulaksiz H, et al. Pro-hepcidin: expression and cell-specific localization in the liver and its regulation in hereditary hemochromatosis, chronic renal insufficiency, and renal anemia. GUT 2004; 53: 735-743
2. Krause A, Neitz S, Magert HJ, et al. LEAP-1, a novel highly-disulfide bonded human peptide, exhibits anti-microbial activity. FEBS Lett 2000; 480; 147-150
3. Park CH, Valore EV, Waring AJ, et al. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001; 276; 7806-7810

Symbol	Português	Dansk	Svenska	Ελληνικά
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση
	Instruções de uso	Brugermanual	Användar manual	Εγχειρίδιο χρήστη
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος
		Indeholder tilsttrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις
	Temperatura de conservação	Opbevaringstemperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης
	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης
	Fabricante	Producent	Tillverkare	Κατασκευαστής
Distributed by				
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ..
Microtiterwells	Alvéolos de microtitulação	Mikrotiterbrønde	Brunnar i Mikrotiterplatta	Πηγαδάκια Μικροτιτλοδοτήσεως
Antiserum	Anti-soro	Antiserum	Antiserum	Αντιορός
Enzyme Conjugate	Conjugado enzimático	Enzymkonjugat	Enzymkonjugat	Συζευγμένο ενζυμο
Enzyme Complex	Complexo enzimático	Enzymkompleks	Enzymkomplex	Σύμπλοκο ενζύμου
Substrate Solution	Solução de substrato	Substratopløsning	Substratlösning	Διάλυμα υποστρώματος
Stop Solution	Solução de paragem	Stopopløsning	Stopp lösning	Διάλυμα τερματισμού
Zero Standard	Padrão zero	Standard 0	Standard 0	Πρότυπο Μηδέν
Standard	Calibrador	Standard	Standard	Πρότυπα
Control	Controlo	Kontrol	Kontroll	Έλεγχος
Assay Buffer	Tampão de teste	Assay buffer	Assay Buffer	Ρυθμιστικό Διάλυμα Εξέτασης
Wash Solution	Solução de lavagem	Vaskebuffer	Tvätt lösning	Διάλυμα πλύσεως
1N NaOH	1N NaOH	1N NaOH	1N NaOH	1N NaOH
1 N HCl	1 N HCl	1 N HCl	1 N HCl	1 N HCl
Sample Diluent				
Conjugate iluen				