Hepcidin 25 LC-MS/MS Kit

*For the determination of hepcidin in serum*

Valid from 13.05.2014

**Manual**

Distribuito in ITALIA da

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**REF** KM4000

Σ

96

+2°C

-20°C

**CAL 1**

**CAL 2**

**CTRL 1**

**CTRL 2**

**INT STD**

**IVD**

**CE**
# Table of Contents

1. **INTENDED USE** ........................................................................................................ 15  
2. **INTRODUCTION** ...................................................................................................... 15  
3. **PRINCIPLE OF THE TEST** ...................................................................................... 15  
4. **MATERIAL SUPPLIED** ........................................................................................... 16  
5. **MATERIAL REQUIRED BUT NOT SUPPLIED** .......................................................... 16  
6. **PREPARATION AND STORAGE OF REAGENTS** ...................................................... 17  
   - Mobile phases ........................................................................................................... 17  
   - Calibrators, controls and internal standard ............................................................... 17  
7. **SAMPLE PREPARATION** .......................................................................................... 17  
8. **TEST PROCEDURE** .................................................................................................. 18  
9. **CHROMATOGRAPHIC CONDITIONS** ....................................................................... 18  
10. **MS/MS-METHOD (LISTED AS AN EXAMPLE FOR A WATERS QUATTRO PREMIER XE TANDEM MASS SPECTROMETER)** ................................................. 19  
    - MRM transitions (m/z) .............................................................................................. 19  
11. **CALCULATION** ..................................................................................................... 19  
12. **EXAMPLES OF CHROMATOGRAMS** ..................................................................... 20  
13. **QUALITY CONTROL** .............................................................................................. 21  
14. **PERFORMANCE CHARACTERISTICS** ................................................................... 21  
    - Precision and reproducibility .................................................................................. 21  
    - Detection limit ......................................................................................................... 21  
15. **DISPOSAL** ............................................................................................................. 21  
16. **PRECAUTIONS** ..................................................................................................... 22  
17. **TECHNICAL HINTS** .............................................................................................. 22  
18. **GENERAL NOTES ON THE TEST AND TEST PROCEDURE** .............................. 22  
19. **REFERENCES** ....................................................................................................... 22
1. INTENDED USE
The described LC-MS/MS application is intended for the quantitative determination of hepcidin in serum. For \textit{in vitro} diagnostic use only.

2. INTRODUCTION
Hepcidin is a small cystein-rich peptide produced in the liver. It regulates the absorption of iron in the body. Hepcidin was initially isolated as a circulating, antimicrobial 25 amino acid peptide in human urine and blood. Human hepcidin is synthesized as prohepcidin, a 84 amino acid precursor, including a putative signal peptide of 24 amino acids. From human urine, two predominant forms, hepcidin-20 and hepcidin-25, differing by amino-terminal truncation, were characterized as 20 and 25 amino acid residues with 8 cysteines connected by intramolecular disulfide bonds. An over-expression of hepcidin was shown to result in severe iron deficiency anemia in transgenic mice, indicating that hepcidin plays a pivotal role in iron metabolism. Recent studies have found abnormal hepcidin expression and disrupted hepcidin regulation in hemochromatosis gene. A disturbed hepcidin regulation mechanism has a direct effect on the iron metabolism in the organism. Decreased hepcidin expression, e.g. due to a genetic defect, results in a direct iron overload, known as the iron disease hemochromatosis. Based on these observations, it has been suggested that hepcidin is a key component of iron homeostasis that acts as a negative regulator of iron absorption in the small intestine and of iron release from macrophages. Hepcidin can be used independently of accompanying diseases as an early predictive marker for functional iron deficiency and/or anomalous iron utilization.

3. PRINCIPLE OF THE TEST
For the determination of Hepcidin 25 samples, we developed two sample extraction methods depending on the LC-MS/MS system used.

1. Suitable for all LC-MS/MS systems is the procedure using a SPE \textmu Elution 96 well plate.
2. Suitable for high sensitive LC-MS/MS systems is also a SPE procedure using 1 ml cartridges.
4. MATERIAL SUPPLIED

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Label</th>
<th>Kit components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KM4000LA</td>
<td>MOPHA A</td>
<td>Mobile phase A</td>
<td>1000 ml</td>
</tr>
<tr>
<td>KM4000LB</td>
<td>MOPHA B</td>
<td>Mobile phase B</td>
<td>1000 ml</td>
</tr>
<tr>
<td>KM4000KA</td>
<td>CAL 1</td>
<td>Calibrators 1 and 2 (-20°C; lyophilized; concentration, see product specification)</td>
<td>3 vials each</td>
</tr>
<tr>
<td></td>
<td>CAL 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KM4000KO</td>
<td>CTRL 1</td>
<td>Controls 1 and 2 (-20°C; lyophilized; concentration, see product specification)</td>
<td>3 vials each</td>
</tr>
<tr>
<td></td>
<td>CTRL 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KM4000IS</td>
<td>INT STD</td>
<td>Internal Standard (lyophilized)</td>
<td>5 x 2 ml</td>
</tr>
<tr>
<td>KM4000AC</td>
<td>ACTSOL</td>
<td>Activation solution</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>KM4000W1</td>
<td>WASH 1</td>
<td>Wash solution 1</td>
<td>50 ml</td>
</tr>
<tr>
<td>KM4000W2</td>
<td>WASH 2</td>
<td>Wash solution 2</td>
<td>20 ml</td>
</tr>
<tr>
<td>KM4000EL</td>
<td>ELUSOL</td>
<td>Elution solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>KM4000RE</td>
<td>RECSOL</td>
<td>Reconstitution solution</td>
<td>25 ml</td>
</tr>
</tbody>
</table>

The UPLC separation column (KM4000RP), the pure substances for instrument tuning, as well the µElution plates (KM4000PL) and the Oasis®HLB, 1cc (10 mg) cartridges (KM4000CK) can be ordered separately from us. The complete Kit as well as all individual components can be ordered separately. Please ask for our single component price list.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- µElution 96 well plate (e.g. Oasis®HLB, 30 µm) / Oasis®HLB, 1cc (10 mg) cartridges
- Glass vials; LC-MS/MS-suitable
- Precision pipettors and disposable tips to deliver 10-1000 µl
- Vacuum station for µElution plates / 1 ml cartridges
- Vortex-Mixer
- LC-MS/MS equipment
- RP-C18 column, e.g. XSelect CSH C18 (2,1 x 50 mm), 2,5 µm
- Methanol p.a.
- Ultra pure water*

* AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25°C (≥ 18.2 MΩ cm).
6. PREPARATION AND STORAGE OF REAGENTS
The test reagents are stable until the expiry date when stored at 2–8°C; calibrators (CAL 1 and CAL 2) and controls (CTRL 1 and CTRL 2) at -20°C.

**WARNING:** Do not repeatedly freeze and thaw the -20°C components.

*Mobile phases*
Before use, a 0.1% activation reagent (ACTSOL) must be added to the mobile phases (MOPHA A and MOPHA B), e.g.

500 ml MOPHA + 500 µl ACTSOL

The prepared solutions can be used within 2 weeks. For this reason, it is recommended to prepare only the desired amount necessary for each assay.

**WARNING:** The activation reagent (ACTSOL) must be added under the fume hood. All vials to be used must be absolutely clean, detergent-free and preferably made of a LC-MS/MS suitable glass.

*Calibrators, controls and internal standard*
Dissolve calibrators (CAL 1, CAL 2) in 500 µl and controls (CTRL 1, CTRL 2) in 250 µl of reconstitution solution (RECSOL).

Dissolve internal standard (INT STD) in 2 ml of reconstitution solution (RECSOL). The prepared solutions can be used within 1 week.

7. SAMPLE PREPARATION
Use serum as sample material for the assay.

The quality controls should be analyzed with each run.

Prior to use in the assay, allow all samples and reagents to come to room temperature (18–26°C). Mix well samples and reagents before use.

*Preparation of the 96-well µElution plate / 1 ml cartridges*
Conditioning of the wells / cartridges needed with 200 µl methanol and subsequent equilibration with 200 µl ultra pure water.
8. TEST PROCEDURE

<table>
<thead>
<tr>
<th>Step</th>
<th>Instructions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Pipet in the prepared wells / cartridges: <strong>200 µl</strong> sample, calibrator (CAL 1 und CAL 2) or control (CTRL 1, CTRL 2) + <strong>100 µl</strong> internal standard (INT STD), evacuate</td>
</tr>
<tr>
<td>2.</td>
<td><strong>200 µl</strong> wash solution 1 (WASH 1), evacuate</td>
</tr>
<tr>
<td>3.</td>
<td><strong>200 µl</strong> wash solution 2 (WASH 2), evacuate</td>
</tr>
<tr>
<td>4.</td>
<td><strong>200 µl</strong> wash solution 1 (WASH 1), evacuate</td>
</tr>
<tr>
<td>5.</td>
<td>Afterwards, elute the wells / cartridges with <strong>100 µl</strong> elution solution (ELU-SOL)</td>
</tr>
<tr>
<td>6.</td>
<td>Dilute the eluate 1:1 with wash solution 1 (WASH 1), e.g. 75 µl eluate + 75 µl WASH 1</td>
</tr>
</tbody>
</table>

Inject **50 µl** of diluted solution into the LC-MS/MS system

9. CHROMATOGRAPHIC CONDITIONS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column material</td>
<td>e.g. XSelect CSH C18 (2,1 x 50 mm), 2,5 µm</td>
</tr>
<tr>
<td>Column dimension</td>
<td>2.1 x 50 mm</td>
</tr>
<tr>
<td>Flow</td>
<td>0.4 ml/min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>35 °C</td>
</tr>
<tr>
<td>Inject volume</td>
<td>50 µl</td>
</tr>
<tr>
<td>Run time</td>
<td>6 min</td>
</tr>
<tr>
<td>Gradient</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>% A</td>
</tr>
<tr>
<td>0 min</td>
<td>90</td>
</tr>
<tr>
<td>1,5 min</td>
<td>90</td>
</tr>
<tr>
<td>3,0 min</td>
<td>5</td>
</tr>
<tr>
<td>4,0 min</td>
<td>5</td>
</tr>
<tr>
<td>5,0 min</td>
<td>90</td>
</tr>
<tr>
<td>6,0 min</td>
<td>90</td>
</tr>
</tbody>
</table>

We recommend to use a guard column/filter to extend column’s life.
After the analysis, the separation column should be washed with ~20 ml of 50 % me-
thanol. The column can be stored in 50% methanol.

10. MS/MS-METHOD (LISTED AS AN EXAMPLE FOR A WATERS QUATTRO PREMIER XE TANDEM MASS SPECTROMETER)

Mode: MRM
Polarity: ESI+
Capillary (kV): 4
Cone (V): var.
Extracor (V): 4
RF Lens (V): 0
Source temperature (°C): 130
Desolvation Temperature (°C): 500
Cone Gas Flow (l/h): 300
Desolvation Gas Flow (l/h): 950
Collision Gas Flow (ml/min): 0.25

_MRM transitions (m/z)_

**Hepcidin-25 (Molecular weight: 2789.4 g/mol)**

698.41 > 644.54 Cone voltage: 37 Collision energy: 23
698.41 > 354.09 Cone voltage: 37 Collision energy: 33

The detected precursor ion corresponds to the 4-fold charged mass.

**Internal Standard (Calcitonin Gene Related Peptide human; Molecular weight: 3789.31 g/mol)**

758.91 > 718.83 Cone voltage: 40 Collision energy: 25
758.91 > 689.5 Cone voltage: 40 Collision energy: 25

The detected precursor ion corresponds to the 5-fold charged mass.

11. CALCULATION

The linear regression can be used as model for evaluation of the results, whereby the internal standard should be considered. The two calibrator concentration points are connected by a straight line. The samples can be calculated using the obtained line.
12. EXAMPLES OF CHROMATOGRAMS

Blank

Calibrator

Sample
13. QUALITY CONTROL
Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control sample are outside the acceptable limits.

Reference values
Preliminary reference range (serum): ≤ 4 nmol/l (11,15 ng/ml)
(Thomas et al., 2011)
We recommend each laboratory to establish its own reference range.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay (n = 4)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hepcidin 25 [ng/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63.8</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Inter-Assay (n = 10)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hepcidin 25 [ng/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68.7</td>
<td>7.3</td>
</tr>
<tr>
<td>2</td>
<td>124.5</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Detection limit
Detection limit Hepcidin 25: 1 ng/ml
It should be noted that the determination of the detection limit depends not only on the application method but also on the instrument.

15. DISPOSAL
Mobile phase (MOPHA), elution solution (ELUSOL), activation reagent (ACTSOL) and wash solution 2 (WASH 2) must be disposed as non-halogenated solvents.
16. PRECAUTIONS

- All reagents in the kit package are for in vitro diagnostic use only.
- Human material used in the kit components was tested and found to be negative for HIV, hepatitis B and hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.

17. TECHNICAL HINTS

- Do not mix different lot numbers of any kit component.
- Control samples should be analyzed with each run.
- Reagents should not be used beyond the expiration date shown on the kit label.
- The assay should always be performed according the enclosed manual.

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- All reagents in the kit package are for in vitro diagnostic use only.
- The guidelines for medical laboratories should be followed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from wrong use.
- Warranty claims and complaints in respect of deficiencies must be lodged within 14 days after receipt of the product. The product shall be send to Immundiagnostik AG together with a written complaint.

19. REFERENCES

2. Ganz, T., 2003. Hepcidin, a key regulator of iron metabolism and mediator of ane-


**Used symbols:**

- **Temperature limitation**
- **Catalogue Number**
- **In Vitro Diagnostic Medical Device**
- **To be used with**
- **Manufacturer**
- **Contains sufficient for <n> tests**
- **Lot number**
- **Use by**

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