Zinc-Protoporphyrin/Protoporphyrin HPLC Kit

For the determination of zinc-protoporphyrin/protoporphyrin in EDTA whole blood

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1. **INTENDED USE**
This HPLC application is intended for the quantitative determination of zinc-protoporphyrin and protoporphyrin in EDTA whole blood. For *in vitro* diagnostic use only.

2. **SUMMARY AND EXPLANATION OF THE TEST**
Zinc protoporphyrin (ZPP) is a metabolite formed in erythrocytes during hemoglobin synthesis. By iron deficiency during erythropoiesis, instead of incorporating a ferrous ion to form the hem precursor, zinc becomes an alternative metal ion in the protoporphyrin complex. As a result, zinc-protoporphyrin is produced instead of hemoglobin. As a consequence, in cases of iron deficiency anemia, the zinc protoporphyrin concentration in the erythrocytes is elevated.

**Indications**
- Lead poisoning
- Iron deficiency
- Sickle cell anemia
- Sideroblastic anemia
- Anemia of chronic disease
- Vanadium exposure

3. **PRINCIPLE OF THE TEST**
The first step in the determination of zinc-protoporphyrin and protoporphyrin is precipitation of the higher molecular components. After their removal by centrifugation, the supernatant is injected into the HPLC system.

The separation via HPLC follows an isocratic method at 30 °C using a reversed phase column. One run lasts 10 minutes. The chromatograms are recorded by a fluorescence detector. The quantification is performed with the delivered calibrator; the concentration is calculated via integration of the peak areas/heights by the external standard method.

Parallel measurements of the hemoglobin concentration are required, because the zinc-protoporphyrin concentration is related to the hemoglobin concentration.

**Summary**
This HPLC technique provides an easy, fast and precise method for quantitative determination of zinc-protoporphyrin and protoporphyrin. Except the column, the kit contains all reagents necessary for sample preparation and separation in ready-to-use form.
As for many other parameters, the advantage of HPLC analytics is the simultaneous handling of many analytes in a single test. The HPLC complete system enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical chemical routines quickly and precisely. Mostly, a one-point calibration is sufficient for calibrating the test system – unlike immunoassays with up to 6 calibrators per test. It is possible to automate the sample application and calculation of the results so that even higher number of samples can be handled nearly without control. With short test series, the one-point calibration is much more economic than 6-point calibration for immunoassays.

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>KC2700LM</td>
<td>MOPHA</td>
<td>Mobile phase</td>
<td>1000 ml</td>
</tr>
<tr>
<td>KC2700KA</td>
<td>CAL</td>
<td>Calibrator (500 µl lyoph.; see specification data sheet for concentration)</td>
<td>5 vials</td>
</tr>
<tr>
<td>KC2700IS</td>
<td>INT STD</td>
<td>Internal Standard (2 ml lyoph.)</td>
<td>4 x 1 vials</td>
</tr>
<tr>
<td>KC2700RE</td>
<td>RECSOL</td>
<td>Reconstitution solution</td>
<td>15 ml</td>
</tr>
<tr>
<td>KC2700FR</td>
<td>PREC</td>
<td>Precipitation reagent</td>
<td>150 ml</td>
</tr>
<tr>
<td>KC2700KO</td>
<td>CTRL 1 CTRL 2</td>
<td>Control 1 and 2 (250 µl lyoph.; see specification data sheet for concentration)</td>
<td>2 x 3 vials</td>
</tr>
</tbody>
</table>

The HPLC column (KC2700RP) as well as individual components can be ordered separately from us. Please ask for the price list of the individual components.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- 1.5 ml reaction tubes (e.g. Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with fluorescence detector
- Reversed phase C<sub>18</sub> column
- Vortex

* 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. **STORAGE AND PREPARATION OF REAGENTS**

- **The lyophilised calibrator** (CAL) is stable at -20°C until the expiry date stated on the label. Before use, the CAL has to be reconstituted with **500 µl reconstitution solution** (RECSOL). The concentration of zinc-protoporphyrin slightly changes from lot to lot, the exact concentration is stated on the specification data sheet. **Calibrator** (reconstituted CAL) is **not stable and cannot be stored**.

- **The lyophilised controls 1 and 2** (CTRL 1 and CTRL 2) are stable at -20°C until the expiry date stated on the label. Before use, they have to be reconstituted with each **250 ml reconstitution solution** (RECSOL). The concentration of zinc-protoporphyrin slightly changes from lot to lot, the exact concentration is stated on the specification data sheet. **Controls** (reconstituted CTRL 1 and 2) are **not stable and cannot be stored**.

- **The lyophilised internal standard** (INT STD) is stable at -20°C until the expiry date stated on the label. Before use, the INT STD has to be reconstituted with **2 ml precipitation reagent** (PREC). **Internal standard** (reconstituted INT STD) is **not stable and cannot be stored**.

- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at 2–8°C.

7. **PRECAUTIONS**

- Human materials used in kit components were 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.

- Reagents should not be used beyond the expiration date stated on kit label.

8. **SPECIMEN COLLECTION AND PREPARATION**

EDTA whole blood is suited for this test. Before analysis, the erythrocytes are lysed by freezing and thawing in order to release zinc-protoporphyrin and protoporphyrin. Zinc-protoporphyrin as well as protoporphyrin are light sensitive. Samples should be transported lightproof.
9. ASSAY PROCEDURE

Procedural notes

- Quality control guidelines should be observed.
- 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 incorrect use.
- The assay should always be performed according the enclosed manual.
- Use mobile phase (MOPHA) for needle wash in order to avoid sample carryover.

Test procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipet</td>
<td>200 µl sample, calibrator or control 1 or 2 into a 2 ml reaction tube.</td>
</tr>
<tr>
<td>Add</td>
<td>each 10 µl internal standard.</td>
</tr>
<tr>
<td>Add</td>
<td>each 1.2 ml precipitation reagent (PREC) and mix well (vortex for at least 15 s).</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>for 10 min at 10,000 g and take the supernatant.</td>
</tr>
<tr>
<td>Inject</td>
<td>100 µl supernatant into the HPLC system.</td>
</tr>
</tbody>
</table>

Chromatographic conditions

- Column material: Bischoff Prontosil 120-5-C18 ace EPS; 5 µm
- Column dimension: 125 mm × 4 mm
- Flow rate: 1.0 ml/min
- Excitation: 417 nm
- Emission: 635 nm
- Temperature: 30 °C
- Injection volume: 100 µl
- Running time: 10 min

It is recommended to use a guard column to extend column life.
10. TREATMENT OF THE COLUMN

After analysis, the column should be flushed with 30 ml ultra pure water (1 ml/min) and stored in 50% methanol in water (~ 30 ml, flow 0.5 ml/min). Before use, the system should be equilibrated with ~ 30 ml mobile phase (MOPHA).

11. RESULTS

Calculation

\[
\frac{\text{Peak height sample} \times \text{calibrator concentration}*}{\text{Peak height internal standard of the sample}} \times F = \text{sample concentration}
\]

\[
F = \frac{\text{Peak height interal standard of the calibrator}}{\text{Peak height calibrator}}
\]

* see specification data sheet

Tip: Alternatively, the peak area instead of the peak height can be used for quantification.

Typical chromatogram
12. QUALITY CONTROL

Reference range
Zinc-Protoporphyrin: < 40 µmol/mol haem

We recommend each laboratory to establish its own reference range. The values mentioned above are only for orientation and may deviate from other published data.

Controls
Control samples should be analysed 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.

13. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay CV
Zinc-Protoporphyrin: 3.03% (349 nmol/l) [n = 10]
Protoporphyrin: 5.54% (198 nmol/l) [n = 10]

Inter-Assay CV
Zinc-Protoporphyrin: 9.28% (310 nmol/l) [n = 8]
Protoporphyrin: 9.57% (79 nmol/l) [n = 7]

14. DISPOSAL
The mobile phase (MOPHA) and precipitation reagent (PREC) must be disposed as non-halogenated solvents.
Please refer to the appropriate national guidelines.
## 15. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No signal</td>
<td>No or defect connection to evaluation system</td>
<td>Check signal cord and connection</td>
</tr>
<tr>
<td></td>
<td>Detector lamp is altered</td>
<td>Change lamp</td>
</tr>
<tr>
<td>No peaks</td>
<td>Injector is congested</td>
<td>Check injector</td>
</tr>
<tr>
<td>Double peaks</td>
<td>Dead volume in fittings and / or column</td>
<td>Renew fittings and / or column</td>
</tr>
<tr>
<td>Contaminating peaks</td>
<td>Injector dirty</td>
<td>Clean injector</td>
</tr>
<tr>
<td></td>
<td>Contamination at the head of the column</td>
<td>Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase</td>
</tr>
<tr>
<td></td>
<td>Air in the system</td>
<td>Degas pump</td>
</tr>
<tr>
<td></td>
<td>Auto sampler vials contaminated</td>
<td>Use new vials or clean them with methanol</td>
</tr>
<tr>
<td>Broad peaks, tailing</td>
<td>Precolumn / column exhausted</td>
<td>Use new precolumn / column</td>
</tr>
<tr>
<td>Variable retention times</td>
<td>Drift in temperature</td>
<td>Use a column oven</td>
</tr>
<tr>
<td></td>
<td>Pump delivers imprecise</td>
<td>Check pump, degas the system</td>
</tr>
<tr>
<td></td>
<td>System is not in steady state yet</td>
<td>Rinse system mobile phase for 15 min</td>
</tr>
<tr>
<td>Baseline is drifting</td>
<td>Detector lamp did not reach working temperature yet</td>
<td>Wait</td>
</tr>
<tr>
<td></td>
<td>Detector lamp is too old</td>
<td>Renew lamp</td>
</tr>
<tr>
<td></td>
<td>System is not in steady state yet</td>
<td>Rinse system mobile phase for 15 min</td>
</tr>
<tr>
<td></td>
<td>Pump delivers imprecise</td>
<td>Check pump, degas the system</td>
</tr>
</tbody>
</table>
### 16. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- The test components contain organic solvents. Contact with skin or mucous membranes must be avoided.
- All reagents in the kit package are for *in vitro* diagnostic use only.
- Reagents should not be used beyond the expiration date stated on kit label.
- Do not interchange different lot numbers of any kit component within the same assay.
- 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 incorrect use.
- Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to AG along with a written complaint.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline is not smooth</td>
<td>Pump delivers imprecise</td>
<td>Check pump, degas the system</td>
</tr>
<tr>
<td></td>
<td>Detector flow cell is dirty</td>
<td>Clean flow cell</td>
</tr>
</tbody>
</table>

Zinc-protoporphyrin/protoporphyrin
**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**
- **Attention**

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