Vitamin B₁ HPLC Kit

For the determination of Vitamin B₁ (thiamin pyrophosphate) in EDTA-whole blood

Valid from 30.01.2009

KC 2201

For Reference Purposes Only
For Research Use Only

Immundiagnostik AG
1. INTENDED USE

The Immundiagnostik Assay is intended for the quantitative determination of Vitamin B1 in EDTA-blood. This assay is designed for in vitro diagnostic use only.

2. SUMMARY AND EXPLANATION OF THE TEST

Vitamin B₁ (thiamin) is a water-soluble Vitamin, which consists of a pyrimidine- and thiazolring linked via a methylene bridge. It is sensitive against alkaline solution, oxidation and reduction.

Vitamin B₁ is produced by plants and microorganisms. It is found free, peptide bound and as phospho-esters (mono-, di- and triphospho-esters). In animals and also in humans it is necessary to be supplemented by food. The intake of thiamin in the gut is maintained by active transport and passive diffusion. The different phospho-esters are synthesized by phosphorylation and dephosphorylation. The active form in metabolism is thiamin pyrophosphate and thiamin triphosphate in brain. After dephosphorylation thiamin is secreted by the kidney.

Thiamin pyrophosphate plays an important role as a co-enzyme in carbohydrate- and amino acid metabolism. An important reaction is the oxidative carboxylation. Thiamin itself is required for stimulating nerve cells. Beside this, it stimulates the fatty acid- and cholesterol-synthesis in nervous tissues.

A classical disease for the lack of Vitamin B₁ is Beri Beri, which is known from Asians eating predominantly white rice. The symptoms are paralysis, drop in muscle mass and heart failure. Other diseases are the Wernicke-encephalopathy, the Korsakow-syndrome and several forms of the Landry`s paralysis. Also many alcoholics have a deficient thiamin status.

Applications:

- Determination of Vitamin B₁ status
- Disturbance in amino acid metabolism
- Malabsorption (alcoholism)
- Suspicion for neuritis
3. **Principle of the Test**

The first step in the determination of thiamin pyrophosphate includes the sample preparation with additional derivatisation. During the precipitation higher molecular substances are removed. After centrifugation, the supernatant is used for the derivatisation (10 min at 60°C) to obtain a fluorescent Vitamin B1 derivative. The sample is cooled, centrifuged and injected into the HPLC system.

The separation via HPLC follows an isocratic method at 30°C using a „reversed phase“ column. One run lasts 12 minutes. The quantification is performed with the delivered calibrator. The concentration is calculated via integration of the peak heights.

**Summary**

The HPLC technique provides an easy, fast and precise method for quantitative determination of vitamin B1. The kit contains all reagents necessary for sample preparation and separation in ready-to-use form except the column.

Besides many other parameters, the advantage of HPLC method lies in the simultaneous handling of many analytes in a single test. The HPLC system enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical routine determination in a quick and precise manner. Unlike immuno assays with up to six calibrators per test, a one-point calibration is mostly sufficient to calibrate the test system. It is possible to automate the sample application and calculation of the results so that even higher sample numbers of can be handled nearly without control.
### 4. MATERIAL SUPPLIED

<table>
<thead>
<tr>
<th>Cat. No</th>
<th>Content</th>
<th>Kit Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC 2201LM</td>
<td>MOPHA</td>
<td>Mobile phase</td>
<td>1000 ml</td>
</tr>
<tr>
<td>KC 2201KA</td>
<td>CAL</td>
<td>Calibrator, lyophilized</td>
<td>1 vial</td>
</tr>
<tr>
<td>KC 2201FR</td>
<td>PREC</td>
<td>Precipitating reagent</td>
<td>5 ml</td>
</tr>
<tr>
<td>KC 2201VL</td>
<td>DIL</td>
<td>Dilution solution</td>
<td>20 ml</td>
</tr>
<tr>
<td>KC 2201RB</td>
<td>REABUF</td>
<td>Reaction buffer</td>
<td>5 ml</td>
</tr>
<tr>
<td>KC 2201DL</td>
<td>DER</td>
<td>Derivatisation solution (lyoph.)</td>
<td>5.5 ml</td>
</tr>
<tr>
<td>KC 2201LC</td>
<td>SOLC</td>
<td>Solution C</td>
<td>5.5 ml</td>
</tr>
<tr>
<td>KC 2201KO</td>
<td>CTRL1</td>
<td>Control 1 and 2; 250 µl lyophilized</td>
<td>2 x 3 vials</td>
</tr>
</tbody>
</table>

HPLC column (KC 2201RP) as well as individual components can be ordered separately from Immundiagnostik. Please ask for the price list of the individual components.

### 5. MATERIAL REQUIRED BUT NOT SUPPLIED

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with Fluorescence-detector
- Reversed phase C<sub>18</sub>-column
- Heatable shaker or waterbath
- Vortex mixer
6. **PREPARATION AND STORAGE OF REAGENTS**

- Resuspend the **calibrator** (CAL), EDTA-whole blood with a defined thiamin pyrophosphate concentration, with dilution solution (volume is given on the label), aliquote and store at -20 °C. The Vitamin B1 concentration might have minor changes from lot to lot; the actual concentration is given on the label.

- Reconstitute the **controls** (CTRL1, CTRL2) in **250 µl** dilution solution (DIL).

- Resuspend the **derivatisation solution** (DER) in 5.5 ml solution C (SOLC). The dissolved derivatisation solution is stable for 6 month at 2-8 °C.

- All other test components are ready to use.

- Store test reagents at 2-8 °C, **calibrator** (CAL) and **controls** (CTRL1, CTRL2) at –20 °C. They are stable up to the expiry date which is given on the label.

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.

- The supplied reagents contain solvents like acetonitrile (mobile phase) and acid (precipitating reagent). Even diluted, they still must be handled with care. They can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.

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

8. **SPECIMEN COLLECTION AND PREPARATION**

EDTA-whole-blood is used in this assay.

Vitamin B1 is light- and temperature sensitive. Therefore, protect samples from light and cool immediately after collection.

The samples are stable at 2-8°C in the dark for 1 day. For longer storage samples should be frozen at -20°C. Do not re-freeze the samples.
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 wrong use.
- The assay should always be performed according the enclosed manual.

Sample preparation

Pipet into 1.5 ml reaction tubes:

50 µl sample (EDTA-whole blood), calibrator (CAL) or control (CTRL1, CTRL2)  
+  
150 µl dilution solution (DIL)  
+  
50 µl precipitating reagent (PREC)

Mix well. Leave the tubes for 10 minutes at 2-8°C and centrifuge afterwards at 10,000 x g for 10 minutes.

Take 150 µl supernatant  
+  
50 µl reaction buffer (REABUF)  
+  
50 µl derivatisation solution (DER), incubate for 10 minutes at 60°C on a shaker or in a water bath.  
Cool to 2-8°C. Centrifuge at 10,000 x g for 5 minutes. (The prepared supernatant is stable for 3 days at 2-8 °C).

Inject 50 µl of the supernatant for chromatography into the HPLC-system.
Chromatographic conditions

**Column material:**  Bischof Eurobond; 5 µm  
Lichrospher RP18; 5 µm  
Nucleodur Sphinx RP18; 5 µm

**Column dimension:**  125 mm x 4 mm

**Flow rate:**  0.8 - 1.2 ml/min

**Fluorescence Detection:**  Excitation: 365 nm  
Emission: 440 nm

**Temperature:**  30 °C

**Injection volume:**  50 µl

**Running time:**  12 minutes

Cartridge holder (KC2201RK) is necessary for the use of Nucleodur Sphinx RP18 cartridges. The cartridge holder can be used repeatedly.

It is recommended that a guard column (KC2201VS) is used to extend column’s life.

**10. TREATMENT OF THE COLUMN**

After the analysis, the column should be flushed with 30 ml aqua bidest. (1 ml/min) and stored in 50% methanol in aqua bidest. (ca. 30 ml, flow 0.7 ml/min). Before use, the system should be equilibrated with ca. 30 ml mobile phase (MOPHA).

**11. RESULTS**

Calculation

\[
\text{Concentration sample} = \frac{\text{Peak height sample} \times \text{Concentration of the calibrator}}{\text{Peak height calibrator}}
\]
12. LIMITATIONS

We recommend not to measure lipaemic patient samples. The measurement of serum and plasma samples is possible but not recommended, because the concentration is mostly below the detection limit.

13. QUALITY CONTROL

Expected values

**Vitamin B1 normal values:** 32 - 95 ng/ml  (mean ± 2SD)

It is recommended that each laboratory should establish its own normal range. Above mentioned values are only for orientation and may vary from other published data.
Controls
Control samples or EDTA-blood pools should be analyzed with each run of calibrators and patient samples. 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 samples are outside the acceptable limits.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

**Intra-Assay VK:**
- 3,3 % (31,2 ng/ml) [n = 6]
- 4,3 % (59,0 ng/ml) [n = 6]

**Inter-Assay VK:**
- 3,2 % (33,0 ng/ml) [n = 12]
- 4,7 % (62,3 ng/ml) [n = 12]

Linearity
- up to 250 ng/ml

Detection limit
- 0.5 ng/ml

15. DISPOSAL

The mobile phase (MOPHA) and derivatisation solution (DER) must be disposed as non-halogenated solvent. The precipitation solution (PREC) can be neutralized to neutral pH with NaOH and disposed as a salt solution.

**Important:** Reaction will produce heat, be careful!

Please refer to the appropriate national guidelines.
### 16. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible reason</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>
<tr>
<td>Baseline 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>
17. REFERENCES


18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- The test components contain organic solvents. Contact with skin or mucous membranes has to be avoided.
- All reagents in the test package are for research use only.
- Reagents should not be used beyond the expiration date shown on the kit label.
- Do not interchange different lot numbers of any kit component within the same assay.
- 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 wrong use.