Catalog Number:  A1C31-H100
1000 Tests
For Research Use Only. Not for use in diagnostic procedures.

EAGLE BIOSCIENCES, INC.
20A Northwest Blvd., Suite 112, Nashua, NH  03063
Phone: 866-419-2019    Fax: 617-419-1110
www.EagleBio.com
1. Intended purpose

The Eagle Biosciences HbA1c HPLC Assay Kit is intended for the quantitative determination of HbA1c in whole blood. The HbA1c HPLC Assay Kit is for research use only and should not be used for diagnostic procedures.

2. Introduction

One of the major sources of energy for the body is carbohydrates. During digestion they are cleaved to monosaccharides. Insulin, a hormone produced in the pancreas, is controlling the transport of glucose into the cells. Diabetes mellitus is a metabolic disturbance of the sugar metabolism, characterized by high blood glucose levels. Due to a lack of or ineffective insulin, glucose cannot be taken up into the cells, remaining in the blood. There are four different forms of diabetes described:

- Type 1 is characterized by an absolute insulin deficiency, caused by a destruction of the pancreatic beta cells. This type is observed in young patients.
- Type 2 Diabetes describes all forms of diabetes with relative insulin deficiency which can be caused by insulin resistance or secretory defects. It is observed mainly in elderly patients.
- Type 3 diabetes is a rare form. It can result from a genetic defect of the beta cells or in insulin action, a disease of the pancreas or hormone disorders. It can be caused by chemicals, drugs or by infections.
- Type 4 is gestational diabetes.

Untreated diabetes leads to micro and macro vascular complications. Nephropathy, neuropathy, retinopathy and cardio vascular diseases (heart attack and stroke) are described. The DPP study (Diabetes Prevention Program of the American Diabetes Association) clearly documented that the occurrence of type 2 diabetes can be prevented or significantly retarded with changes in life style and by treatment with anti-diabetic drugs.

Many studies mentioned the importance of a continuous monitoring of the blood glucose level and hemoglobin A1c. Hemoglobin is glycosylated by a non-enzymatic reaction depending on the amount of glucose in the blood. It reflects the average blood glucose level over a prolonged period of time (1-3 months).

The Eagle Biosciences HbA1c HPLC Assay Kit makes it possible to determine HbA1c in an easy, fast and precise way. The HbA1c HPLC Assay Kit includes all reagents in ready to use form for preparation and separation of the samples with exception of the controls (IC4050ko) which can be supplied by Eagle Biosciences. Beside the complete test kit it is possible to order all components separately. Please request our single component price list.
3. Warnings and precautions

- All reagents of this HbA1c HPLC Assay Kit are strictly intended for research use only.
- Test kit and column are concerted. Using alternative columns might cause in insufficient separation, resulting in false high results. The given test characteristics might not be fulfilled.
- Do not interchange the HbA1c HPLC Assay Kit components from different lots.
- Calibrator and controls contain human plasma. It was tested and found negative for HBsAg, anti-HIV-1/2, and anti-HCV. No test can guarantee the absence of HBsAg or HIV, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
- Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
- Reagents should not be used beyond the expiration date shown on kit label.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

4. Materials Provided

<table>
<thead>
<tr>
<th>Article no.</th>
<th>Component</th>
<th>Designation</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC4050la</td>
<td>ELU A</td>
<td>Mobile phase A</td>
<td>1 x 5000 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 x 2500 ml</td>
</tr>
<tr>
<td>IC4050lb</td>
<td>ELU B</td>
<td>Mobile phase B</td>
<td>1 x 3750 ml</td>
</tr>
<tr>
<td>IC4050ka</td>
<td>CAL</td>
<td>Calibrator, (lyoph. 1 ml)</td>
<td>3 vials</td>
</tr>
<tr>
<td>IC4050he</td>
<td>HEM</td>
<td>Hemolysis reagent</td>
<td>1 x 1000 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 x 250 ml</td>
</tr>
<tr>
<td>IC4050rp</td>
<td>COLUMN</td>
<td>HPLC column HbA1c</td>
<td>1 Column</td>
</tr>
</tbody>
</table>
5. Additional special equipment

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with UV-detector
- Heatable shaker or water bath
- Vortex mixer

6. Reagent preparation

- Reconstitute the **calibrator (CAL)** in 1 ml deionized water, divide the calibrator in several portions and store them at -20 °C. Avoid repeated freeze-thaw circles. The content of HbA1c might have minor changes from lot to lot.
- All other test reagents of the HbA1c HPLC Assay Kit are stable at 2-8 °C, up to the date of expiry stated on the label.

7. Specimen

- EDTA blood could be used in this test system.
- The samples are stable in the dark at 2-8°C for 1 week. For longer storage samples should be frozen at -20 °C.

8. Procedure

**Principle of the method**

For the determination of HbA1c a lysis of the blood cells is performed first. The samples are incubated at 37 °C to eliminate the unstable aldimine form. After centrifugation the supernatant is injected into the HPLC system. The gradient separation via HPLC at 30°C lasts 5 minutes. The chromatograms are recorded by an UV-detector. The quantification is performed with the delivered blood calibrator; the concentration is calculated via integration of the peak heights respectively areas.
**Sample preparation**

1. Pipette into 1.5 ml reaction tubes:
   
   $5 \mu$l sample  
   
   +  
   
   $1250 \mu$l HEM

2. Mix well. Leave the tubes for **30 minutes at 37°C** and centrifuge afterwards at $10,000g$ for 2 minutes.

3. CAL and CTRL are ready to use after reconstitution.

4. Inject **20 µl** of the supernatant for chromatography into the HPLC-system. The supernatant is stable in the dark for at least 24 hours at 2-8°C.

**Chromatographic settings**

- **Column material:** Cation exchanger, 3 µm
- **Column dimension:** 35 mm x 4,6 mm
- **Flow rate:** 1,5 ml/min
- **UV-detection:** 415 nm
- **Injection volume:** 20 µl
- **Running time:** 5 min
- **Temperature:** 30 °C

**Gradient:**  
- Step 1: 0 min (4.5 % B)  
- Step 2: 0.8 min (4.5 % B)  
- Step 3: 0.9 min (100 % B)  
- Step 4: 1.4 min (100 % B)  
- Step 5: 1.5 min (4.5 % B)  
- Step 6: 5 min (4.5 % B)

The amount of ELU B in step 1, 2, 5 and 6 can be slightly varied depending on the lot of the column.
Treatment of the HPLC column

After the analysis the column should be flushed with 20 ml deionized water (1 ml/min) and stored in 20% acetonitrile in deionized water. Before use, the system should be equilibrated with approx. 30 ml ELU A.

9. Calculation of analytical results

Calculation

The area of the HbA1c peak and the total hemoglobin are determined and expressed as % HbA1c of total hemoglobin. The samples are then multiplied with the calibration factor.

\[
\text{Calibration factor} = \frac{\text{mean value calibrator (\% HbA1c)}}{\text{determined value calibrator (\% HbA1c)}}
\]

Patient sample (\% HbA1c) = determined value (\% HbA1c) x calibration factor

Typical chromatogram
10. **Internal Quality Control**

**Reference intervals**

<table>
<thead>
<tr>
<th>% HbA1c</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 6.5</td>
<td>normal</td>
</tr>
<tr>
<td>6.5 – 7.5</td>
<td>increased</td>
</tr>
<tr>
<td>&gt; 7.5</td>
<td>high (diabetic)</td>
</tr>
</tbody>
</table>

We recommend that each laboratory develop their own normal range. The values mentioned above are only for orientation and can deviate from other published data.

11. **Validation data**

**Precision and reproducibility**

**Intra-Assay CV:** 0.9 % (4.3 % HbA1c) \[n = 6\]

**Inter-Assay CV:** 2.2 % (4.3 % HbA1c) \[n = 6\]

12. **Limitations of the method**

- **Lifetime of the erythrocytes**
  - The HbA1c value depends on the lifetime of the erythrocytes. In diseases where the lifetime of the erythrocytes is reduced (hemolytic anemia) the value of HbA1c may be decreased. On the other side diseases with an increased lifetime of the erythrocytes (polycythemia, post-splenectomy) may result in elevated HbA1c values.

- **Abnormal hemoglobin variants**
  - Samples with ß-thalassemia showing decreased HbA1c values. An increased level of HbA2 co-eluting with HbA0 leads to an increased total hemoglobin value.
  - Samples with an abnormal peak pattern should be monitored for the different hemoglobin variants carefully.
  - In literature it is described that the hemoglobin variant Okayama is co-eluting with HbA1c resulting in HbA1c values around 45%. Nevertheless HbA1c values higher than 20% are not plausible.
### 13. 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>Autosampler 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>Continue baseline is drifting</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 is not smooth</td>
<td>Pump delivers imprecise</td>
<td>Check pump, degas the system</td>
</tr>
<tr>
<td></td>
<td>Detector flowcell is dirty</td>
<td>Clean flow cell</td>
</tr>
</tbody>
</table>
14. Literature references


- The UK Prospective Diabetes Study (UKPDS) Group: Intensive Blood-Glucose Control with Sulphonylureas or Insulin Compared with Conventional Treatment and Risk of Complications in Patients with Type 2 Diabetes (UKPDS 33), Lancet 352 (1998) 837-853.

For further information about this kit, its application or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.