Enzymatic Vitamin B6 Assay--96-well plate format

The A/C Enzymatic Vitamin B₆ Assay is intended for the quantitative in vitro diagnostic determination of pyridoxal 5'-phosphate (PLP, vitamin B₆) in EDTA-Plasma. The device will be used to monitor PLP concentrations in plasma for aid in diagnosis of vitamin B₆ deficiency. The A/C Enzymatic Vitamin B₆ Assay is for in vitro diagnostic use only.

For IN VITRO DIAGNOSTIC USE ONLY

FDA 510k #: 111260

CAT NO. AC-B₆-100
96 Tests

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A/C Enzymatic Vitamin B₆ Assay

INTENDED USE

The A/C Diagnostics Enzymatic B₆ Assay is intended for the quantitative determination of vitamin B₆ (pyridoxal 5’-phosphate) in plasma.

For In Vitro Diagnostic use only.

INTRODUCTION

Plasma Vitamin B6 levels are high in infants but decrease with age. Deficiency usually occurs in association with other nutrient deficiencies. The dependence upon exogenous sources to maintain adequate levels of Vitamin B6 in the body makes it clinically desirable to measure pyridoxal 5’-phosphate, the biologically active form of this Vitamin, in plasma in a simple and efficient way as an indicator of Vitamin B6 status (1-4).

The A/C Enzymatic Vitamin B₆ Kit is designed for measurement of pyridoxal 5’-phosphate in human EDTA-plasma.

PRINCIPLE OF THE ASSAY

The Enzymatic Vitamin B₆ Assay is based on PLP-dependent recombinant homocysteine-α,γ-lyase (rHCYase), which is prepared in the apo-enzyme form by stripping off the cofactor PLP (vitamin B₆). The restoration of enzymatic activity by reconstitution of the holoenzyme depends on the amount of PLP in the plasma bound to apo-enzyme (Reaction 1) and produces hydrogen sulfide (H₂S). H₂S combines with N,N – dibutyl phenylene diamine (DBPDA), which then forms a chromophore (Reaction 2). The absorbance of this compound is read at 660 – 680 nm. (5-7).

Reaction 1,

\[
\text{apo-rHCYase} \xrightarrow{\text{L-Homocysteine}} \text{α - ketobutyrate + PLP} \xrightarrow{\text{H₂S} + \text{NH₃}}
\]

Reaction 2,

\[
\text{DBPDA + H₂S} \xrightarrow{\text{K₃Fe(CN)₆, HCl, phenothiazine} -5’ -ium chloride} \text{3, 7-Bis (dibutylamino)}
\]

REAGENTS SUPPLIED AND PREPARATION

Reagent Kit, 80 Samples Tests in duplicate

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
<th>Reconstitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding Buffer</td>
<td>1 vial, 50 ml.</td>
<td>See preparation of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Working Binding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Buffer:</td>
</tr>
<tr>
<td>Assay Buffer</td>
<td>1 vial, 40 ml.</td>
<td>See preparation of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Working assay buffer:</td>
</tr>
<tr>
<td>Apo-enzyme Lyoph. powder</td>
<td>1 vial</td>
<td>Ready to use</td>
</tr>
<tr>
<td>DI-homocysteine Powder</td>
<td>1 vial</td>
<td>Storage at -20ºC.</td>
</tr>
<tr>
<td>Chromogen I</td>
<td>1 vial, 7 ml.</td>
<td>Ready to use</td>
</tr>
<tr>
<td>Chromogen II</td>
<td>1 vial, 5 ml.</td>
<td>Ready to use</td>
</tr>
<tr>
<td>Controls Low/High levels</td>
<td>2 vials,</td>
<td>Ready to use</td>
</tr>
<tr>
<td></td>
<td>120 µl/vial</td>
<td></td>
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<tr>
<td>Calibrators</td>
<td>6 vials,</td>
<td>Ready to use</td>
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<tr>
<td></td>
<td>120 µl/vial</td>
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</table>

SPECIMEN COLLECTION AND STORAGE

The assay is formulated for use with human EDTA-plasma samples. A minimum volume of 0.5 ml of blood is recommended for duplicate determinations. Draw blood into an EDTA venipuncture tube. Centrifuge for 15 minutes at 1000 x g and 2 – 8ºC immediately after collection. Avoid exposure to light. After centrifugation, collect the plasma in suitable tubes, store at 2 - 8ºC and it is stable for a week, or store at – 20ºC or less if not assayed immediately. PLP in plasma will remain stable for up to 1 year if stored at -20ºC or less and protected from light. Avoid repeated freeze-thaw cycles.

KIT STORAGE INSTRUCTIONS

1. The kit should be stored at 2-8ºC and should not be frozen.
2. The apo-enzyme and DL-homocysteine must be stored at – 20ºC.
3. The expiry date of the kit is shown the outer label.

WARNING AND PRECAUTIONS

1. The product is for IN VITRO DIAGNOSTIC USE.
2. The product should only be used by appropriately trained personnel.
3. This product contains human sourced and/or potentially infectious components. Donor units of components sourced from human plasma have been tested and found to be nonreactive for HBsAg, HIV-1 RNA or HIV-1 Ag, anti-HCV and anti-HIV-1/HIV-2. Therefore, all human blood products, including patient samples, should be considered potentially infectious. Handling and disposal should be in
accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.

**MATERIALS REQUIRED BUT NOT PROVIDED**

1. 20 µl, 100 µl and 1000 µl precision pipettes and 5-50 µl and 20-200 µl multi-pipettes with disposable tips for 200 µl.
2. 96-well plates (recommend purchase from Corning Inc. 96 well cell culture cluster lot # 3596).
3. 96-well microtiter plate reader with filter wavelength between 660 and 680 nm (maximum absorbance at 675 nm).
4. Plate incubator set at 37°C and the accurate temperature uniformity offers high reliability (Uniformity <0.6°C across whole plate).
5. Microtiter plate shaker

**PROCEDURAL NOTES:**

Test Before you start
Protect calibrators, controls and plasma samples from exposure to direct light the entire assay.

1. Prior to testing, plasma samples in freezer should be moved to refrigerator (2-8°C) for thawing one day before testing. For accurate results, EDTA-plasma should be free of fibrin, lipemic material, and other particulate matter and avoid hemolytic plasma. All plasma samples should be spun down by high-speed centrifugation before testing. Centrifuged specimens with a lipid layer on the top must be transferred to secondary tube. Care must be taken to transfer only the clarified specimen without the lipemic material.
2. For each test, the calibrators, controls and plasma samples should be run in duplicate. The addition of all reagents in the assay must be consistent. It is suggested that, pipetting should be in the same order from well to well at the same rate and do not make bubbles. Check software and reader requirements for the correct calibrators/controls configurations.
3. Reconstitution of reagents:
   a. Apo- Enzyme solution: A vial of apo-enzyme is dissolved in 0.75 ml binding buffer and vortex the vials for 30 seconds. The solution is stable for one day on ice.
   b. DL-Homocysteine solution: A vial of DL-homocysteine powder is dissolved in 1.0 ml Assay Buffer and vortex the vials for 30 seconds. The solution is stable for one day on ice.

**ASSAY PRECEDURE**

1) Pipet 10 µl calibrators, controls and samples to the reaction wells of microtiter plate.

Use multiple pipettes. Add 150 µl working binding buffer to each well, mix gently (10 seconds) with a microtiter plate shaker and incubate at 37°C for 60 minutes.

2) Working Assay Buffer also should be made plate by plate, because the buffer is prepared for immediate use. The DL-homocysteine solution is transferred 0.5 ml to 12 ml of Assay Buffer for each plate, mixed well. Add 100 µl working assay buffer to each well of the reaction plate, mix gently (10 seconds) with a microtiter plate shaker and incubate at 37°C for 20 minutes with the plate cover.

3) Add 25 µl Chromogen RI, change pipette tips and add 15 µl Chromogen RII, mix gently (10 seconds) with a microtiter plate shaker and incubate at 37°C for 10 minutes.

4) Read the OD at 675 nm (or at 660-680 nm) in a microtiter plate reader.

**96-well plate Layout**

<table>
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<tr>
<th>C0</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>L1</th>
<th>H1</th>
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<tr>
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<td>S4</td>
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**CALCULATION OF RESULTS**

If a 96-well plate absorbance reader with built in data calculation program is used, refer to the manual for the plate reader and create a linear regression program using the concentrations stated on the labels of each of the calibrators.
For manual evaluation, a calculation curve is constructed linear regression plot by linear-linear graph paper, and plotting the average net absorbance values obtained for each the Calibrator against the corresponding concentration (nmol/L). The unknown vitamin B6 concentrations can then be read from the calibration curve using the average net absorbance values of each sample.

For automatic or manual calculation of A/C Enzymatic Vitamin B6 Assay results refer to following procedures:

1. Calculate the mean absorbance value for each sample in duplicate including calibrators, controls and samples, and calculate the average net OD for each calibrators, controls and samples by subtracting the average Calibrator 0 OD (Blank OD).

   Average Net OD = Average OD – Average Calibrator 0 OD (Blank OD)

2. Construct a calibration curve by plotting the average net OD obtained for each calibrator against its concentration of PLP in nmol/L to linear regression.

3. Use the mean net OD values for each specimen to determine the corresponding concentrations of PLP on the horizontal or X axis.

EXAMPLE OF CALIBRATION CURVE

Results of a typical standard run with 96-well plate reader at 675nm shown in the Y axis against PLP concentrations shown in the X axis. This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each laboratory must provide its own data and standard curve in each run.

Table 1; Example of results

<table>
<thead>
<tr>
<th>PLP nmol/L</th>
<th>Average OD675nm</th>
<th>Net OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1785</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>0.198</td>
<td>0.0195</td>
</tr>
<tr>
<td>25.0</td>
<td>0.2125</td>
<td>0.034</td>
</tr>
<tr>
<td>50.0</td>
<td>0.2525</td>
<td>0.074</td>
</tr>
<tr>
<td>100.0</td>
<td>0.3325</td>
<td>0.154</td>
</tr>
<tr>
<td>200.0</td>
<td>0.4495</td>
<td>0.271</td>
</tr>
</tbody>
</table>

Fig. 1. Example of a calibration curve

QUALITY CONTROL

The kit consists six Calibrators and two Controls, two controls are assayed in each run together with the six calibrators and EDTA-plasma sample.

1. Quality control
   In order for an assay to be valid, all the following criteria must be met:
   - Calibrators and two controls must be included in each run.
   - The values obtained for the Low and High controls should be in the range specified on the its assigned range.
   - Both or one of the values of the Low and High Controls provide with the kit must be with the lot specific range indicated on the vials. The results are valid.
   - If the ranges of two Controls are not met, the results are invalid and the test should be repeated.

2. Plot calibration curve
   The calibration curve can be plotted either automatically or manually as follows by plotting the average net OD obtained for each calibrator against its concentration of PLP in nmol/L to linear regression.
   - Automatic – use appropriately validated software, and the curve fit that best fits data.
   - Manual – use linear-linear graph paper; draw a smooth curve through the points.

EXPECTED NORMAL VALUES

The expected normal range of vitamin B₆ is 20 - 120 nmol/L according to the literature cited (8-9). When the outcome of analyses was low compared with higher plasma PLP, “low” was defined as ≤ 20 nmol/L. This definition of low vitamin B₆ status was the index of vitamin B adequacy used to set current Estimated Average Requirements (EARs) and Recommended Dietary Allowances (RDAs) of vitamin B₆ (9).

LIMITATIONS AND TROUBLESHOOTING

1. The A/C Enzymatic Vitamin B₆ Assay is formulating for use with human EDTA-plasma sample (only collected in EDTA collection tube). Do not use sodium heparin.
2. The Lipemic, hemolyzed and icteric plasma should be avoided. The hemolyzed plasma specimens should not be used.
3. Since PLP is light-sensitive, calibrators, controls must be protected from light.
4. The absorbance of the C0 (blank) should be between 0.07 and 0.3 at OD675nm.
5. The procedure 2 and 3, should be immediately gently shaking for 10 seconds and cover with plate cover during incubation time avoid to H₂S is volatilized to reduce OD.
6. Make sure Chromogen RI is added first, then change tips and add Chromogen RII.
7. If temperature of the plate incubator is inaccuracy (Uniformity of well to well is >0.6°C), leave edges wells of the test plate empty in order to avoid temperature effects on signal.

**PERFORMANCE CHARACTERISTICS**

1. **Sensitivity**
   The minimum detectable concentration of the A/C Enzymatic Vitamin B₆ Assay as measured by 2 SD from the mean of a zero calibrator is 9.28 nmol/L. The LoQ was defined as the lowest concentration where the CV < 20%. The A/C Enzymatic Vitamin B₆ Assay has a LoQ claim of 15.6 nmol/L.

2. **Measurement Range:**
   The measurement range of the A/C Enzymatic Vitamin B₆ Assay is 15.6 to 192 nmol/L.

3. **Precision**
   Precision was determined as described in NCCLS Protocol EP5-T2. A three different PLP levels plasma panel was assayed in duplicate, and two runs per day for 20 days. Representative data follow.

   **Table 2: Precision**

<table>
<thead>
<tr>
<th>Samples levels</th>
<th>Mean Conc. Value (nmol/L)</th>
<th>SD</th>
<th>%CV</th>
<th>SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOW</td>
<td>26.6</td>
<td>2.05</td>
<td>7.5</td>
<td>3.22</td>
<td>12.1</td>
</tr>
<tr>
<td>Medium</td>
<td>53.1</td>
<td>2.98</td>
<td>5.6</td>
<td>5.77</td>
<td>10.9</td>
</tr>
<tr>
<td>High</td>
<td>111.2</td>
<td>7.22</td>
<td>6.5</td>
<td>10.83</td>
<td>9.7</td>
</tr>
</tbody>
</table>

4. **Recovery Study**
   Recovery was determined by adding known quantities of PLP to various plasma samples and assayed in duplicate. The mean recovery was 99± 5%.

5. **Specificity**
   Cross-reactivity was tested for compounds whose prrdoxal, pyridoxamine, pyridoxine and pyridoamine – phosphate usage may potentially interfere with the A/C Enzymatic Vitamin B₆ Assay. 1,000.0 nmol/L pyridoxal, pyridoxamine, pyridoxine and pyridoamine – phosphate were added to a plasma sample with 50 nmol/L PLP and then assayed (n = 4 replicates). These concentrations are more than twenty-fold the physiological concentrations. The following compounds do not cross-react with apoenzyme in the A/C Vitamin B₆ Assay. Data from this study are summarized in the following Table 3.

   **Table 3: Specificity**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Final Concentration (nmol/L)</th>
<th>% Cross-reactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyridoxal</td>
<td>1,000.0</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>pyridoxamine</td>
<td>1,000.0</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>pyridoxine</td>
<td>1,000.0</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>pyridoxamine-phosphate</td>
<td>1,000.0</td>
<td>&lt; 3.2</td>
</tr>
</tbody>
</table>

6. **Linearity**
   The linearity study was performed according the EP6-A CLSI evaluation protocol. Pooled human plasma sample with respectively a high level PLP (vitamin B₆) were diluted with calibrator 0 to make six intermediate levels. Each mixture measured eight times. The range of samples tested was from 7.85 – 251.3 nmol/L. The mean observed values were compared to the expected values. The results were plotted as a least-squares linear regression of the expected concentration versus the observed concentration. The resulting regression equation was: Y (Expected) = 0.9999X (mean Observed) + 0.000056, r = 0.999.

   **Fig. 2: Dilution Linearity**

7. **Method Comparison**
   The A/C Enzymatic Vitamin B₆ Assay was compared to an ALPCO Vitamin B₆ REA assay. The study was performed using 50 EDTA-plasma samples which were split. Each sample was analyzed in duplicate with both methods. The results from the linear regression analysis summarized below:
8. Interference of the Assay

Various potential interferents were added to two EDTA-plasma samples containing measured PLP concentrations of 32.5 nmol/L and 115 nmol/L. The interferents were as follows: Intralipid (Sigma) was added two levels PLP plasma samples to produce lipid concentration 500 and 1000 mg/dL; hemoglobin was spiked in the samples to produce hemoglobin concentration 250 mg/dL and 500 mg/dL; also the two plasma samples were spiked with bilirubin to produce 10.0 mg/dL and 20.0 mg/dL, and compared to the same sample without added intralipid, hemoglobin and bilirubin. The samples were analyzed in quadruplicate by the A/C Enzymatic Vitamin B6 Assay.

The results demonstrate that triglyceride (intra-lipid) and bilirubin concentrations of up to 1000 mg/dL, 20 mg/dL respectively show ≤ 10% interference and therefore, do not interfere with the measurement of vitamin B₆ by the A/C Enzymatic Vitamin B₆ Assay.