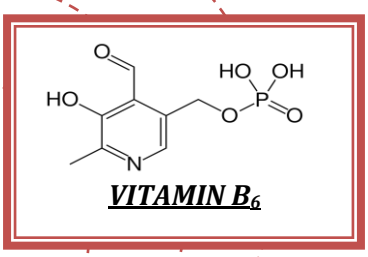
A/C DIAGNOSTICS



VITAMIN B_6

FDA 510k #: 111260

Enzymatic Vitamin B6 Assay--96-well plate format

The A/C Enzymatic Vitamin B_6 Assay is intended for the quantitative in vitro diagnostic determination of pyridoxal 5'-phosphate (PLP, vitamin B_6) in EDTA-Plasma. The device will be used to monitor PLP concentrations in plasma for aid in diagnosis of vitamin B_6 deficiency. The A/C Enzymatic Vitamin B_6 Assay is for in vitro diagnostic use only.

For IN VITRO DIAGNOSTIC USE ONLY

FDA 510k #: 111260

CAT NO. AC-B_6-100
96 Tests

A/C DIAGNOSTICS LLC.
7917 OSTROW STREET
SAN DIEGO, CA92111
Phone: (858) 654-2555
Fax: (858) 268-4175
Email: all@anticancer.com

A/C Enzymatic Vitamin B_6 Assay

INTENDED USE

The A/C Diagnostics Enzymatic B_6 Assay is intended for the quantitative determination of vitamin B_6 (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 B_6 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 B_6). 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 (H_2S). H_2S 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,

$$\text{L-Homocysteine} \xrightarrow{\text{apo-rHCYase}} \alpha\text{ - ketobutyrate} + \text{PLP} + H_2S + NH_3$$

Reaction 2,

$$\text{DBPDA} + H_2S \xrightarrow[\text{HCl}]{K_3Fe(CN)_6} \text{3, 7-Bis (dibutylamino) phenothiazine -5' –ium chloride}$$

REAGENTS SUPPLIED AND PREPARATION

Reagent Kit, 80 Samples Tests in duplicate

Reagents	Quantity	Reconsitution
Binding Buffer	1 vial, 50 ml. Storage at 2-8°C	See preparation of Working Binding Buffer:
Assay Buffer	1 vial, 40ml. Storage at 2-8°C	See preparation of Working assay buffer
Apo-enzyme Lyoph. powder	1 vial Storage at -20°C.	Ready to use
Dl-homocysteine Powder	1 vial. Storage at -20°C.	Ready to use
Chromogen I	1 vial, 7 ml.	Ready to use
Chromogen II	1 vial, 5 ml	Ready to use
Controls Low/High levels	2 vials, 120 µl/vial	Ready to use
Calibrators	6 vials, 120 µl/vial	Ready to use

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 – 8°C immediately after collection. Avoid exposure to light. After centrifugation, collect the plasma in suitable tubes, store at 2 -8°C and it is stable for a week, or store at – 20°C or less if not assayed immediately. PLP in plasma will remain stable for up to 1 year if stored at -20°C or less and protected from light. Avoid repeated freeze-thaw cycles.

KIT STORAGE INSTRUCTIONS

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

WARNING AND PRECUATIONS

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 675nm)
4. Plate incubator set at 37^{o}C and the accurate temperature uniformity offers high reliability (Uniformity <0.6 ℃ 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^{o}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-Homcysteine 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.

Working Binding Buffer should be made plate by plate, because the buffer is prepared for immediate use. Transfer 0.37 ml of apo-Enzyme solution to 18 ml of Binding Buffer for each plate, and mix well.

Use multiple pipettes. Add 150 µl working binding buffer to each well, mix shortly (10 seconds) with a microtiter plate shaker and incubate at 37^{o}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^{o}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^{o}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

C0	C0	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
C1	C1	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C2	C2	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C3	C3	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
C4	C4	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
C5	C5	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
L	L	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

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)

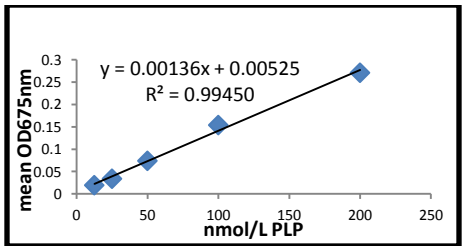
2. Construct a calibration curve by plotting the average net OD obtained for each calibrator against its concentration of PLP in nmol/L to linear regression.
3. Use the mean net OD values for each specimen to determine the corresponding concentrations of PLP on the horizontal or X axis.

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

PLP nmol/L	Average $OD675_{nm}$	Net OD
0	0.1785	0
12.5	0.198	0.0195
25.0	0.2125	0.034
50.0	0.2525	0.074
100.0	0.3325	0.154
200.0	0.4495	0.271

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 B_6 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/. This definition of low vitamin B_6 status was the index of vitamin B adequacy used to set current Estimated Average Requirements (EARs) and Recommended Dietary Allowances (RDAs) of vitamin B_6 (9).

LIMITATIONS AND TROUBLESHOOTING

1. The A/C Enzymatic Vitamin B_6 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 H_2S 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 B_6 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 $\leq$ 20%. The A/C Enzymatic Vitamin B_6 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

Samples levels	Mean Conc. Value (nmol/L)	Within Run N=80		Total Run N=80	
		SD	%CV	SD	%CV
LOW	26.6	2.05	7.5	3.22	12.1
Medium	53.1	2.98	5.6	5.77	10.9
High	111.2	7.22	6.5	10.83	9.7

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$\pm$ 5%.

5. **Specificity**

Cross-reactivity was tested for compounds whose prridoxal, 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 apo-enzyme in the A/C Vitamin B_6 Assay. Data from this study are summarized in the following Table 3.

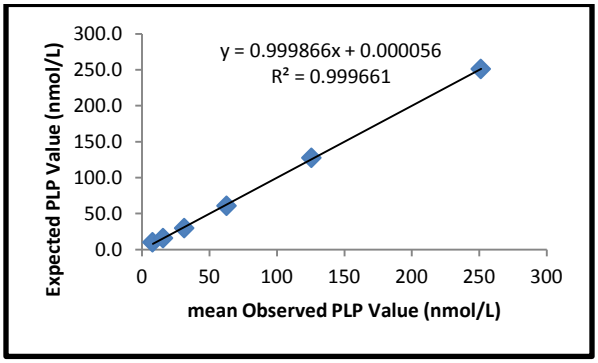
Table 3; Specificity

Compounds	Final Concentration (nmol/L)	% Cross-reactivity*
pyridoxal,	1,000.0	< 0.5
pyridosamine	1,000.0	< 0.5
pyridoxine	1,000.0	< 0.5
pyridoxamine-phosphate	1,000.0	< 3.2

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 B_6) 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) = 0.999X (mean Observed) + 0.000056, r = 0.999.

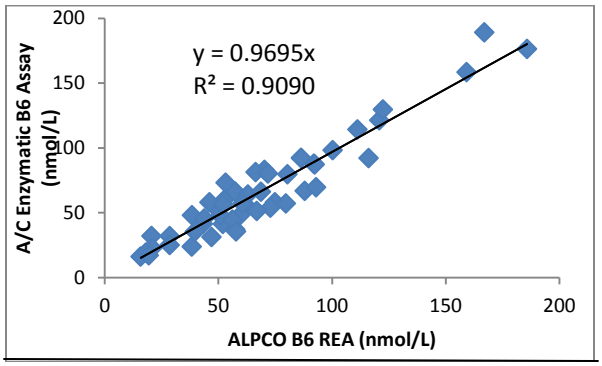
Fig. 2; Dilution Linearity



7. Method Comparison

The A/C Enzymatic Vitamin B_6 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:

Linear regression analysis: $y = 0.969x$ ($R^2 = 0.909$).
Fig. 3. Linear regression between A/C Enzymatic Vitamin B_6 Assay and ALPCO Vitamin B_6 REA.



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: Interalipid (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 interalipid, hemoglobin and bilirubin. The samples were analyzed in quadruplicate by the A/C Enzymatic Vitamin B6 Assay,

Table 4; Evaluation of Interference

Interfering Substances		Interference %*	
		Sample 1 32.5 nmol/L PLP	Sample 2 115.3 nmol/L PLP
Intralipid mg/dL	1000	8.2	7.5
	500	5.2	3.5
Hemoglobin mg/dL	500	12.2	10.9
	250	6.3	6.0
Bilirubin mg/dL	20	3.4	3.2
	10	1.3	1.1

*(Spiked sample Conc. – Non-spiked sample Conc.) / Non-spiked sample Conc. x 100 = % Interference.

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 B_6 by the A/C Enzymatic Vitamin B_6 Assay.

Interference studies showed that hemoglobin concentrations (> 500 mg/L) reduced the recovery of PLP in plasma by more than 10% bias. In any event, Hemoglobin concentrations above 250 mg/dL cause interference > 10%.
The labeling states that hemolyzed plasma specimens should not be used.

REFERENCES/LITERATURES

1. Rodwell, V. W. Metabolism of vitamin B6. In Metabolic Pathways. D. M. Greenberg, editor. Academic Press, Inc., New York. 3rd edition. 4: 411.
2. Chang, AJ., Huang, YC., Hsiao, LJ., Lee, YC., and Hsuen, SY. Determination of Vitamin B-6 Estimated Average Requirment and Recommended Dietary Allowance for Children Aged 7-12 Years Using Vitamin B-6 Intake, Nutritional Status and Anthropometry. *J Nutr. 2002 Oct;132(10):3130-4.*
3. Joosten E, Van den A, Riezler R, Naurath HJ, Lindenbaum J, and Stabler SP, et al. Metabolic evidence that deficiencies of vitamin B12 cobalamin, folate and vitamin B6 occur commonly in elderly people. *American Journal of Clinical Nutrition 1993;58:468-76.*
4. Morris MS, Picciano MF, Jacques PF, Selhub J. Plasma pyridoxal 5'-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. *Am J Clin Nutr 87, 1446-1454, (2008).*
5. Han, Q, et al. High expression, purification, and properties of recombinant homocysteine α, γ-lyase. *Protein Expr Purif* **14**, 267-274 (1998).
6. Han, Q., Xu, M., Tang, L., Tan, XZ., and Hoffman, RM. Homogeneous, non-radioactive, enzymatic assay for plasma pyridoxal 5' – phosphate. *Clin Chem 2002; 49: 1560-64.*
7. Han, Q. and Hoffman, RM. Nonradioactive enzymatic assay for plasma and serum vitamin B_6. *Nature Protocols/V. 3, NO. 12/1815—19 (2008).*
8. National Committee for Clinical Laboratory Standards. Evaluation of precision performance of clinical chemistry devices; tentative guideline – second edition. *NCCLS Document EP5-T2. Wayne, PA: NCCLS, 1992*.
9. Food and Nutrition Board, Institute of Medicine. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin, and choline. A report of the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on folate, Other B Vitamins, and Choline and Subcommittee on Upper Reference Levels of Nutrients. Washington, DC: National Academy Press, 1998.