Laboratory Procedure Manual

Analyte: Pyridoxal 5'- phosphate (PLP)

Matrix: Plasma

Method: Enzymatic B_6 Assay

Method No.:

Revised:

as performed by: A/C Diagnostics
7917 Ostrow Street
San Diego, CA 92111

Contact: Dr. Robert Huffman, Director
Telephone: 858-654-2555

Important Information for Users

A/C Diagnostics periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003-2004 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
L43_c	LBXVB6	Pyridoxal 5'- phosphate (nmol/L))

1. Summary of Test Principle and Clinical Relevance

Pyridoxal 5'- phosphate (PLP) is removed from the holo-enzyme of recombinant homocysteinase by incubation with hydroxylamine to obtain apo-rHCYase. The restoration of enzymatic activity by reconstitution of holoenzyme depends on the amount of PLP bound to the Apo- rHCYase.

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

H_2S combines with 3, 7-bis (dibutyl amino) phenothiazine-5'-ium chloride (DBPDA) to form an absorbance change. The absorbance is read at 680 nm[1].

Pyridoxal 5'- phosphate (PLP) is the biologically active form of the vitamin B_6. Vitamin B_6 is involved in numerous metabolic pathways as an enzyme cofactor[2, 3, 4]. Homocysteine (HCY), a risk factor for cardiovascular and other diseases[5, 6], is converted to cysteine by PLP-dependent transsulfuration enzymes[7]. A major cause of homocysteinemia is insufficient intake of vitamin B_6, vitamin B_{12}, and folic acid, all of which are also necessary for HCY metabolism[8]. Clinical studies suggest that vitamin B_6 is independently associated with increased risk for cardiovascular disease[9, 10, 11]. Recent studies have shown that plasma PLP levels are significantly decreased in other pathological conditions, including rheumatoid arthritis[12]. High tHcy and low vitamin B_6 plasma levels are associated with an increased risk for deep venous thrombosis (DVT) independent of other established risk factors for DVT. The association of low vitamin B_6 levels with the risk for DVT is also independent of the tHCY levels [13].

2. Safety Precautions

All reagents are for in vitro diagnostic use only, not for internal and external use in humans or animals and not for use in therapeutics. All human serum specimens are pretreated with 0.5% (v/v) Triton X-100 to inactivate enveloped viruses, including the human immunodeficiency virus, which may be present.

A. Reagents Containing Human Source Material

The standard samples, low and high controls of this kit contain components of human origin. Each plasma donor unit used in the preparation of kit components should be tested by a FDA- approved method and found negative for hepatitis B surface antigen (HbsAg), Hepatitis C (HCV) antibodies and human immunodeficiency virus (HIV) antibodies. Although these methods are highly accurate, there is no guarantee that this material cannot transmit hepatitis or AIDS. All products containing human source material are handled in accordance with good laboratory practice using appropriate precautions.

B. Chromophore 3, 7- bis (dibuty amino) phenothiazine-5'-ium chloride – information:

1. Since this is a toxic compound do not ingest or inhale.
2. Avoid all contact with these materials.
3. This compound is severely irritating to skin, eyes, and the respiratory systems.
4. This compound is a possible sensitizing material.

Consider all samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses when handling all human blood products and infectious viruses. Place disposable plastic, glass, paper, and gloves that contact blood in a biohazard bag or discard

pan to be autoclaved. Autoclaved or disinfect other non- disposable material at the end of the working day.

Do not pipette by mouth. Do not eat, drink or smoke in designated work areas. Wash hands thoroughly after removal of personal protective devices used in handling specimens and kit reagents.

Material safety data sheets (MSDSs) for all reagents used in the performance of this assay are kept in the laboratory.

3. Computerization; Data System Management

Each shipment of specimens received from the NHANES mobile unit arrives with a corresponding transmittal sheet and a Send File [a comma delineated (CSV) text file] transmitted electronically (labeled boxnum.shp).

The information from the shipping file is imported into a result file. After the testing is completed, the run number, date of analysis, result, comment, analyst, and the 2.5% repeat results are entered into the result file. Data entry is checked for errors.

After the testing has also been completed, resulted, and checked, the result file is stored as a CSV file and is transmitted electronically to NHANES WESTAT. Electronic and hard copies of the files and all primary data are kept in the laboratory.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

   A. No special instructions such as fasting or special diets are required.

   B. Specimen type: whole blood with anticoagulant, draw blood into an EDTA venipuncture tube.

   C. Optimal amount of specimen required is 2-3 mL in unopened collection tube; however, minimum required sample volume is about 0.5 mL.

   D. Centrifuge for 15 minutes at 1,000-rpm, 2 –8^{o} C immediately after collection. Avoid exposure to light.

   E. After centrifugation, collect the plasma in suitable tubes and store at –20^{o} C and protect from light. Avoid repeated freeze-thaw cycles.

   F. Specimen stability has been demonstrated for at least 1 year at –20^{o} C.

   G. The criteria for unacceptable specimen are a low volume (< 0.2 mL of plasma).

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

   Not applicable for this procedure

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

   A. Reagents and standard materials.

      1. Binding Buffer
      2. Assay Buffer
      3. Apo-enzyme
      4. Chromogenic Reagent
      5. Chromogenic Reagent II

   B. Reagent Preparation

      1. Binding Buffer: 10-mmol/L citrate/phosphate buffer pH 5.1, containing 1.0 mmol/L EDTA is made as follows:

48.6 mL of 0.1 M solution of citric acid (19.21 g in 1000 mL) is add to 51.4 mL 0.2 M solution of dibasic sodium phosphate (53.65 g $Na_2HPO_4.12H_2O$ in 1000 mL) and 2.0 mL 0.5 M EDTA, diluted to a total of 1000 mL, pH is adjusted to 5.3 using 0.1 M citric acid.

2. Assay Buffer:
   20 mmol/L potassium phosphate buffer pH 8.3, containing 3.0 mmol/L DL-homocysteine; 0.5 mmol/L EDTA; 2.0% Triton X-100. 0.8 mL 1.0 M potassium phosphate buffer pH 8.3 is added to 1.2 mL of 100 mmol/L DL-homocysteine; 40 µL 0.5 M EDTA and 800 µL Triton X-100, diluted to 40 mL.

3. Apo-enzyme:

   0.2 mL stock apo-enzyme (1.0g/L protein) is dissolved in 0.8 mL 20 mmol/L potassium phosphate pH 7.6.

4. Chromogenic Reagent:

   117.2 mg DBPDA is dissolved in 10.0 mL 3.0 M HCl.

5. Chromogenic Reagent II: 49.4 mg of potassium ferricyanide dissolved in 10 mL 10-mmol/L potassium phosphate pH 7.6.

C. Instrumentation

1. Tecan Freedom EVO Robotic System (Tecan Trading AG, Switzerland)
2. Tecan Absorbance Reader(Tecan Trading AG, Switzerland)
3. iEMS Incubator/Shaker(Thermo Electron Corporation, Waltham, MA)

Standards Preparation

D. Other Materials

1. DL-homocysteine(Sigma-Aldrich, St. Louis, Missouri)
2. Dithiothreitol(Sigma-Aldrich, St. Louis, Missouri)
3. Potassium ferricyanide(Sigma-Aldrich, St. Louis, Missouri)
4. Hydroxylamine(Sigma-Aldrich, St. Louis, Missouri)
5. Potassium phosphate(Sigma-Aldrich, St. Louis, Missouri)
6. Citric acid(Sigma-Aldrich, St. Louis, Missouri)
7. EDTA(Sigma-Aldrich, St. Louis, Missouri)
8. Triton X-100(Sigma-Aldrich, St. Louis, Missouri)
9. N, N-Dibutylphenylenediamine (DBPDA) was synthesized in our laboratory (A/C Diagnostics, San Diego, CA, patent # US 6,448,446).
10. Recombinant homocysteinase was produced in our laboratory (A/C Diagnostics, San Diego, CA,[14].

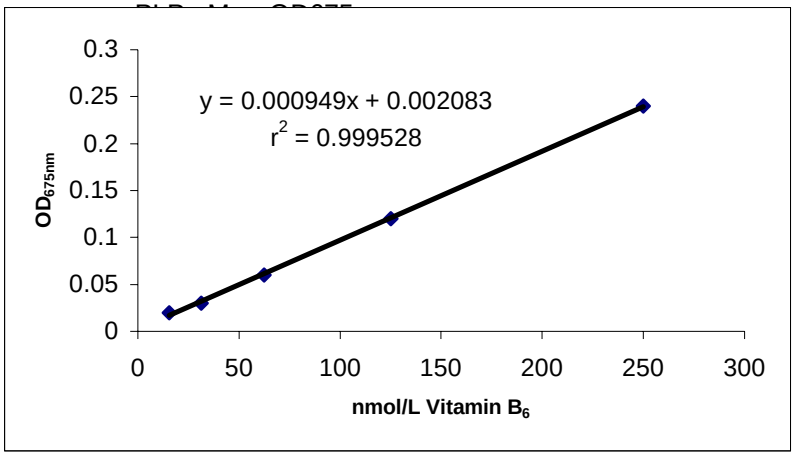
7. Calibration and Calibration Verification Procedures

Pyridoxal 5'-phosphate (ACPOS lot#A00528301) was spiked in blank solution which is a PLP-free plasma pool. The plasma pool (purchased from Intergen lot# 01E0611) was treated in two steps to produce the PLP-free plasma pool.

- The plasma pool was dialyzed in binding buffer at 4°C overnight.
- 2 % activity carbon was added, mixed and centrifuged.

A PLP spiked blank solution calibration curve (concentrations 15.625, 31.25, 62.5, 125.00 and 250.00 nmol/L) was analyzed to establish linearity (Figure 2.). The lower limit of detection (LLD) of vitamin B_6 is 10.09 nmol/L. The lower limit is defined as the concentration at two standard deviations from the mean of the Blank Solution (zero calibrator).

Figure 2. Standard curve of A/C enzymatic vitamin B_6 assay



The criteria of slope, intercept and r Square of each calibration curve are established. Control range calibration data, expiration dates of all reagents are reviewed. New calibrators are prepared as necessary.

- Slops range: 0.0005829 - 0.0012509
- Intercept: -0.0127286 - 0.0004538
- r^2 : ≥ 0.9900

The following standard operating procedures are followed:

The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The laboratory has the responsibility for ensuring that all reagents used, whether purchased or prepared by the laboratory, are appropriately reactive. The verification of reagent performance is required and must be documented. New reagents must be run in parallel with old reagents. For qualitative tests, minimum crosschecking includes retesting each new reagent lot, ensuring that the same results are obtained with previous.

All reagents must be properly labeled, as applicable and appropriate, with the following elements:

- Content and quantity, concentration or titer,
- Storage requirements,
- Date prepared or reconstituted by laboratory,

- Expiration date,

If the precision of the assay does not fall within the established limits and repetition excludes errors in technique, the following parameters are checked:

- Instruments maintenance, which includes the Tecan automatic pipetter, the incubator temperature controlling and reader filter.
- Expiration dates of reagents, buffer pH.
- Purity of water.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A. Preliminaries

1. Check reagent and system supplies, load any needed supplies onto the instrument.

2. Thaw and mix specimens. Centrifuge samples at 10,000 rpm for 10 min. prior to performing the assay.

3. Run controls to verify that the assay is performing as expected.

4. Load specimens into specimen racks, with the barcode in the open slot. Make sure there are no bubbles.

5. Protect standards, controls and plasma samples from exposure to direct light during the entire assay.

B. Tecan Freedom EVO:

1. Daily Maintenance of Tecan Freedom EVO

Instrument/ Component	Maintenance Task	Status
Liquid system	Check for leakage	
	Tighten the tubing connections	
	Flush	
	Check for air bubbles	
	After every 8 hrs of operation: -Visually check for air bubbles	
Diluters and Syringes	Tighten syringes	
	Check leakage	
Tips	Clean	
	Check for damage	
DITI-Cones	Clean	
	Check for deposits	

	Tighten	
	Adjustment Check	
At End of Day		
Instrument	Leave filled with deionized water overnight	
	Clean surface using a detergent solution	
Carriers	Clean surface using a detergent solution	
Liquid system	Check for leakages after every 8 hours of operation	
	Check all tubing connections, syringes and DiTis for leakages	
RoMa Standard, Roma long	Clean grippers using alcohol	
Operator's Name	Date	Signature

2. Running the Freedom EVO involves the following general steps:

   a. Perform daily maintenance.

   b. Start up the Freedom EVO instrument.

   c. Start up the computer system connected with the Freedom EVO.

   d. On the computer system, maker sure that the Tecan application software is installed and functioning properly.

   e. On the computer system, start up the Tecan application software.

   f. In the Tecan application, implement the required application if necessary.

   g. On the computer system, start up the Tecan application for execution.

   h. Place the required labware, e.g. carriers, racks or reagents, in the required positions on the instrument worktable.

   i. Prepare the other instrument hardware components, e.g. system liquid container, waste container or tips.

   j. In the Tecan application software, start the initialization of the instrument.

   k. Wait for the instrument initialization to terminate.

   l. In the Tecan application software, start the application.

   m. At application termination, if you plan another application run, continue with step 7 of this procedure.

n. Exit the application software

o. Switch the instrument off.

p. Perform the appropriate (e.g. daily or weekly, etc.) maintenance.

C. Tecan Absorbance Reader

The Tecan absorbance microplate reader is a 12-channel optical module that combines high-end optical performance with remarkably fast measurement time. 12 fibers guide the beam simultaneously through one row of wells while the reference channel monitors and regulates the light intensity for optimal results over the entire range of optical density. It performs single wavelength measurements 675 nm, and reports absorbance values to three decimal places.

1. Place the instrument on a clean, sturdy table or bench. It is important to keep the instrument in a clean, relatively dust free environment to insure maximum performance.

2. Connect the power cord to the back of the instrument. Before connecting the instrument to the main electrical supply, check that the AC voltage is appropriate for the instrument.

3. Turn on the power switch on the rear panel. The computer will display the version of Magellan software.

D. iEMS Incubator/Shaker

Labsystems iEMS incubator/shaker is a compact, modular instrument designed for incubation and shaking microplate-based assay. IEMS incubator/shaker provides the wide incubation temperature range from room temperature up to 40^{o} C as well as programmable timings and speed for shaking.

1. Switch the power on.

2. Give the slot (1-3). EG. Press 1, then press START.

3. Press C to return to READY for preheating and heating.

Shaking can be activated as a separate function by pressing SHAKE key:

1. Press SHAKE

2. Give the slot number (1-3). Press 1, or 2, or 3.

3. To start shaking, Press START. The shaking time and speed are the same as set in the programming phase.

E. Procedure

The addition of all reagents in the assay must be consistent. It is suggested that, pipetting should be in the same ordered from well to well, and at the same rate.

A/C Enzymatic B_6 Assay Procedure	
	100 uL of plasma sample and calibrators
Binding Buffer	150 µl
Mix (900 rpm for 10 sec.) and incubate at 37°C ± 0.5 °C.	 60 minutes
Assay Buffer	100 µl
Mix (900 rpm for 10 sec.) and incubate at 37°C ± 0.5 °C.	 20 minutes
Chromogenic Reagent I Mix immediately at 900 rpm for 10 sec.	25 µl
Chromogenic Reagent II	15 µl
Mix (900 rpm for 10 sec.) and Incubate at 37°C ± 0.5 °C.	 10 minutes
Read at OD_{675nm}	All samples are run in duplicate. ΔOD = $OD675_{nm}$ – zero calibrator OD. The PLP values are calculated accordingly to the calibration curve.

F. System Maintenance

Perform scheduled instrument maintenance (daily, weekly, and monthly) as outlined on the maintenance log. See the operator's manual for specific instructions.

G. Recording of Data

1. Analytical Results Data:

   Specimen results are entered into the assay specific results table created from the send file corresponding to the specific sample box using Excel software (Microsoft Corporation, Redmond WA). A copy of this table is printed out and checked for accuracy of data entry.

2. Quality Control Data:

   Control results are entered to the Assay Specific QC/Levey-Jennings Table using the Excel program. Compliance with the Westgard rules is evaluated. A copy of this table is printed out and checked for accuracy of data entry.

H. Special Method Notes

1. Incubation times or temperature other than those specified could cause erroneous results. Perform the assay continuously, according to the assay procedure and without interruption.

2. It is recommended to assay each standard, control and specimen in duplicate. Since conditions very from assay to assay, a complete standard curve must be generated for each set of samples to be assayed.

3. Reagents must not be used beyond their expiration dates.

4. Reagents provided with each kit lot should not be mixed with reagents from other kit lots.

6. Reportable Range of Results

Vitamin B_6 has a lower limit of detection of .05 nmol/L and is linear to at least 200 nmol/L. Recovery is approximately 100%.

7. Quality Control (QC) Procedures

The Enzymatic Vitamin B_6 assay has been applied on the Tecan-Freedom EVO Robotic analyzer. Two sources of quality control material (a low and high vitamin B_6 plasma pools from A/C Diagnostics, lot#: C004 and C005) are assayed in each run together with a reagent blank and calibrators.

Analyze two control plasmas for performance testing by obtaining a minimum of 20 measurements over at least 10 days. The mean and standard deviation of these data are calculated.

A. Intra-Assay Precision (Within-Run):

The intra-assay precision is calculated from the results of 12 pairs of values from each sample in a single run. The values are presented as nmol/L.

Sample	Mean Value	S.D.	C.V.
Low Control (LLOT#C004)	49.4	4.1	8.3%
High Control (HLOT#C005)	116.1	9.1	7.8%

B. Inter-Assay Precision (Run-to-Run):

The inter-assay precision was calculated from the results of 20 pairs of values in 20 consecutive runs (From Oct. 2, 2003 to Oct. 20, 2003). The unit values are as nmol/L.

Samples	Mean Value	S.D.	C.V.
Low Control (LLOT#C003)	49.0	6.4	13.1%
High Control (HLOTC003)	118.6	14.2	12.0%

1. Low Control (LLOT#C004)
   a. Target Value: 49.0 nmol/L
   b. Range: 36.1 –61.9 nmol/L
2. High Control (HLOT#C003)
   a. Target Value: 18.6 nmol/L
   b. Range: 90.2 –147.0 nmol/L.

8. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

Each of the two control plasmas will be analyzed once per run, providing the total control measurements per run. Control status will be judged by the rule that is defined as follows: The rule system uses the 1-2S rule as a warning rule. Warning rules display the rule that is violated, but the run is accepted and the data is evaluated more closely. The rule system uses the 2-2S rule as rejection rule. Rejection rules will indicate systematic or random errors that must be addressed. The attached diagrams illustrate use of the rules (Figure 1.).

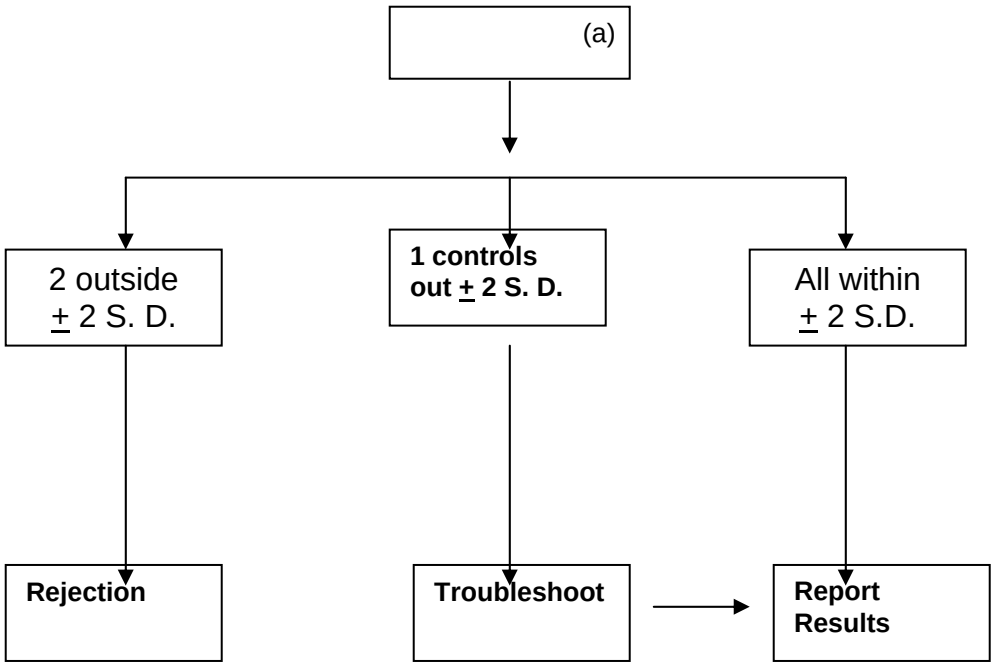
Controls must be reviewed before reporting results. It is implicit in quality control that test results will not be reported when controls do not yield acceptable results.

A. Troubleshooting

1. The run is repeated.
2. The calibration data is reviewed
3. The instruments are recalibrated
4. The reagents are reviewed to determine if they are expired.
5. A calibration verification is performed.

Every sample is measured in duplicate, and the average result is reported. If the CV between the duplicate values is greater than 20%, the data must be rejected and the sample must be tested again.

Figure 1. Two Controls Diagram



9. Limitations of Method; Interfering Substances and Conditions

   A. Since PLP is light sensitive, the calibrators and controls must be protected from light.

   B. The incubator is a high performance microplate incubator with superior temperature control to eliminate temperature gradients and edge effects.

   C. Before the assay, the samples should be centrifuged.

10. Reference Ranges (Normal Values)

    The currently accepted normal acceptance range is 20-120 nmol/L.

11. Critical Call Results (Panic Values)

    Not applicable to this procedure.

12. Specimen Storage and Handling during Testing

    Specimens should be maintained at 20–25°C during testing. After testing, the samples are stored at < –70°C.

13. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

    If the analytical system fails, we recommend that the specimens be stored at –20°C until the analytical system is restored to functionality.

14. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Not applicable to this procedure.

15. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

Standard record keeping should be used for tracking specimens. The primary results include daily test results as well as stored quality control results.

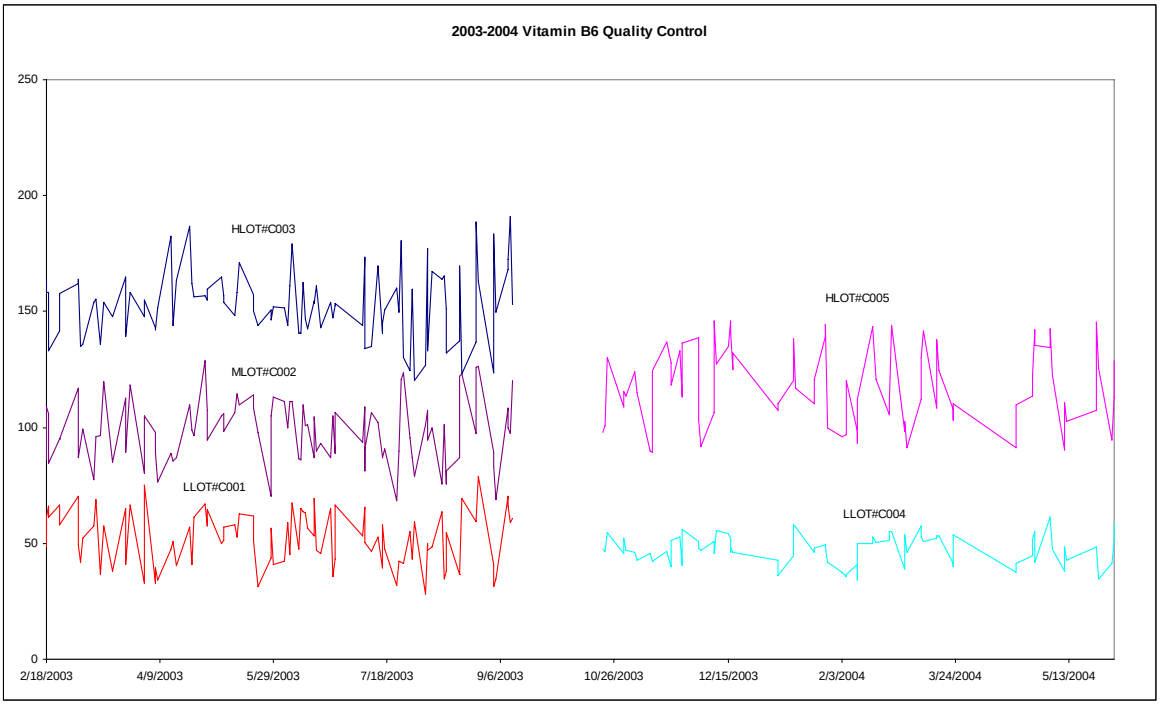
The original NHANES ship file is copied into a template Excel file and onto the hard drive of the computer. After the results are entered into the database and assay results transmitted electronically files are stored on a server that is backed up on a daily basis.

The residual serum is stored at <–70°C for up to one year after analysis; then it is returned to the NHANES Repository in Rockville, MD for long-term storage.

16. Summary Statistics and QC Graphs

Summary Statistics for Vitamin B6 by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LLOT#C001	98	2/18/2003	9/11/2003	52.20	11.81	22.6
MLOT#C002	98	2/18/2003	9/11/2003	98.39	13.77	14.0
HLOT#C003	98	2/18/2003	9/11/2003	153.06	15.03	9.8
LLOT#C004	81	10/21/2003	6/2/2004	47.61	6.37	13.4
HLOT#C005	81	10/21/2003	6/2/2004	118.56	16.91	14.3



20. References

1. Han QH, Gu MX, Tang L, Tan XZ, Tan X, Tan Y, and Hoffman RM. Homogeneous, No radioactive, Enzymatic Assay for Plasma Periodical 5-phosphate. Clinical Chemistry 48:9;1560-1564.

2. Tibet NW. Vitamins. In: Burris CA, Atwood ER, eds. Textbook of clinical chemistry, 2nd ed. Philadelphia: WB Saunders, 1994: 1300-4.
3. Tangelo, A., Sahib, J. Homocysteine and Thrombosis Disease. Blood 1997; 90, 1-11.
4. Upland, P.M. Résumé, H., Beresford, S.A.A., et al. The Controversy over Homocysteine and Cardiovascular Risk. Am J Clin Nat. 2000; 72, 324-332.

5. Schneider G, Raffia M, Pin R, Flamer Y, Lange H, Oberlin FR, et al. Decreased rate of coronary rest enosis after lowering of plasma homocysteine levels. N Engl J Med 2001; 345: 1593-600.
6. Sephardim S, Beiger A, Sahib J, Jacques PF, Rosenberg IH, D'Agostino RB, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002; 346:476-83.
7. Mansoor, M., Kistensen, O., Hervig, T., Bates, C.J., et al. Plasma Total Homocysteine Reponse to Oral Doses of Folic Acid and Pyridoxine Hydrochloride (Vitamin B6) in Healthy Individuals. Oral Doses of Vitamin B6 Reduce Concentration of Serum Folate. Scand J Clin Lab Invest 1999; 59, 139-146.
8. Rimm, E.B., Willett, W.C., Hu, F.B. et al. Folate and Vitamin B6 From Diet and Supplements in Relation to Risk of Coronary Heart Disease Among Women. JAMA, 1998; 279: 359-364.
9. Kuller, L.H., Evans, R.W. Homocysteine/Vitamins and Cardiovascular Disease. Circulation. 1998; 98,196-199.
10. Robinson K, Mayer EL, Miller DP, Green R, Van Lente F, Gupta A, et al. Hyperhomocysteinemia and low phridoxal phosphate, common and independent reversible risk factors for coronary artery disease. Circulation 1995; 92:2825-30.
11. Robinson K, Arheart K, Refsum H, Brattstrom L, Boers G, Ueland P, et al. Low circulating folate and vitamin B6 concentrations: risk factors for stroke, peripheral vascular disease, and coronary artery disease. Circulation 1998;97:437-43.
12. Friso, S., Jacques, P.F., Wilson, P.W., F. Rosenberg, I.H. and Selhub, J. Low Circulating Vitamin B6 is Associated with Elevation of the Inflammation Marker C- Reactive Protein Independently of Plasma Homocysteine Levels. Circulation 2001; 103: 2788-2791.
13. Cattaneo, M., Lombardi, R., Bucciarelli, A.L., Manrucci, P.M.. Low Plasma Levels of Vitamin B6 are Independently Associated with a Heightened Risk of Deep-Vin Thrombosis. Circulation 2001;104: 2442-2446.
14. Han Q, Lenz M, Tan Y, Xu M, Sun X, Tan XH, et al. High expression, Purification, and Properties of recombinant homocysteine α, γ-lyase. Protein Expr Purif 1998; 14:267-74.