Laboratory Procedure Manual

Analyte: **Folate/Vitamin B12**

Matrix: **Serum and Whole Blood**

Method: **Bio-Rad Laboratories' "Quantaphase II Folate/Vitamin B12" Radioassay Kit**

as performed by: *Inorganic Toxicology and Nutrition Branch*
Division of Laboratory Sciences
National Center for Environmental Health

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Important Information for Users

CDC 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
lab06_c	LBXRBF	Folate, RBC (ng/mL RBC)
	LBDRBFSI	Folate, RBC (nmol/L RBC)
	LBXB12	Vitamin B12, serum (pg/mL)
	LBDB12SI	Vitamin B12, serum (pmol/L)
	LBXFOL	Folate, serum (ng/mL)
	LBDFOLSI	Folate, serum (nmol/L)

1. Summary of Test Principle and Clinical Relevance

A. Clinical relevance

Folic acid is required in cellular metabolism and hematopoiesis, and prolonged folic acid deficiency leads to megaloblastic anemia. Vitamin B12 is an essential cofactor in intermediary metabolism and is required for the biosynthesis of RNA and DNA. Since a deficiency of either vitamin may be the cause of megaloblastic anemia, it is essential to determine the levels of both vitamin B12 and folic acid to establish the etiology of the anemia. Untreated vitamin B12 deficiency may lead to severe anemia and potentially irreversible nervous system degeneration.

B. Test principle

Both vitamins are measured by using the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay kit (1). The assay is performed by combining serum or a whole blood hemolysate sample with ^{125}I-folate and ^{57}Co-vitamin B12 in a solution containing dithiothreitol (DTT) and cyanide. The mixture is boiled to inactivate endogenous folate-binding proteins and to convert the various forms of vitamin B12 to cyanocobalamin. The reduced folate and its analogs are stabilized by DTT during the heating. The mixture is cooled and then combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins. The addition of these substances adjusts and buffers the pH of the reaction mixture to 9.2. The reaction mixture is then incubated for 1 hour at room temperature.

During incubation, the endogenous and labeled folate and B12 compete for the limited number of binding sites on the basis of their relative concentrations. The reaction mixtures are then centrifuged and decanted. Labeled and unlabeled folate and vitamin B12, binding to immobilized binding proteins, are concentrated in the bottom of the tube in the form of a pellet. The unbound folate and B12 in the supernatant are discarded, and the radioactivity associated with the pellet is counted. Standard curves are prepared by using the pre-calibrated folate/B12 standards in a human serum albumin base. The concentration of the folate and vitamin B12 in the patient serum or folate in a patient's whole blood is calculated from the standard curve.

In the erythrocyte folate procedure, the sample is first diluted 1:11 with a solution of 1 g/dL ascorbic acid in water and either incubated for 90 min prior to assay or frozen immediately for later assay. The 90-min incubation or the freeze-thaw is necessary for hemolysis of the red blood cells; either allows the endogenous folate conjugates to hydrolyze the conjugated pterylpolyglutamates prior to assay. The sample is further diluted 1:2 with a protein diluent (human serum albumin), resulting in a matrix similar to that of the standards and serum samples.

2. Safety Precautions

The folate assay employs ^{125}I and ^{57}Co as tracers, and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the CDC Radiation Safety Manual. Any laboratory using radioimmunoassay (RIA) kits must hold a current NRC Certificate of Registration. In addition, all personnel must successfully complete the CDC training course, Radiation Safety in the Laboratory, or demonstrate equivalent instruction. All radioactive waste and contaminated material must be disposed of according to radiation safety guidelines.

Treat all serum specimens as potentially positive for infectious agents including HIV and hepatitis B. Observe Universal Precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method because of both infectious and radioactive contamination hazards. We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a labeled plastic autoclave bag for disposal.

Dithiothreitol, a primary reagent for this assay, is toxic. Avoid contact with eyes, skin, and clothing. Wash thoroughly after using. Wash immediately with plenty of water if exposed.

Material safety data sheets (MSDSs) for all chemicals contained in the kit are available in the MSDS section of the "Working Safely With Hazardous Chemicals" notebook located in the laboratory. MSDSs for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs.

3. Computerization; Data System Management

A. Calculation of serum folate (SFOL), vitamin B12, and whole blood folate (WBCF) values is accomplished with the software on the Packard Cobra gamma counter. SFOL, B12, and WBCF results are manually entered into a Microsoft Excel spreadsheet that calculates red blood cell folate (RBCF) based on the hematocrit. After a run is complete and any additional corrections by the analyst are made, the Excel result file (containing the patient data as well as the QC data) is electronically transferred to the appropriate analyte-specific subfolder in Q: /ITN/Nutrition Lab/Import into Access on the NCEH/DLS Local Area Network (LAN). The analyst also gives a hardcopy of the result file to the reviewing supervisor. After the reviewing supervisor approves the final values for release by checking off the bench and blind QC values and signing the hardcopy, he/she sends an email to the computer support staff that the data has been released to be imported into the NHANES 1999+ database that is located in Microsoft Access; the computer support staff imports the data into the NHANES 1999+ database by using a macro. Data entry is verified by the computer support staff and the supervisor. Data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.

B. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a week onto a floppy disk or a CD-ROM using a CD writer.

C. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

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

A. We recommend that specimen donors fast prior to specimen collection, but fasting is not required.

B. Serum folate and vitamin B12 assays are performed on fresh or frozen serum. RBC folate samples are prepared by diluting EDTA-whole blood 1:11 with 1 g/dL ascorbic acid and freezing the solution promptly, which keeps the folate in the reduced state.

C. A 400-µL serum sample is required for serum folate and vitamin B12 assays. A 400 µL solution consisting of a 100 µL whole blood specimen diluted with 1.0 mL of 1 g/dL ascorbic acid is necessary for the red cell folate assay. At assay time, 100 µL of this mixture is added to 100 µL of protein diluent in each of 2 tubes in order to provide the necessary final 1:22 dilution of the original sample for the red cell folate assay.

D. Serum specimens may be collected with regular red-top Vacutainers. Whole blood is collected with lavender-top Vacutainers that contain 1.5% K_3EDTA as an anticoagulant. A hematocrit measurement used for the red cell folate calculations is made at the time of collection. The appropriate amount of serum or whole blood/ascorbic acid solution is dispensed into a Nalge cryovial or other plastic screw-capped vial labeled with the participant's ID.

E. Specimens collected in the field should be frozen and then shipped on dry ice by overnight mail. Once received, they should be stored at –20 ºC until analyzed. Serum folate and vitamin B12 are fairly stable if the serum is frozen at –20 to –70 ºC before analysis. Ascorbic acid should not be added to the serum specimen because it will invalidate the B12 assay. Freeze-thaw cycles will cause degradation of the folate. Whole blood folate is especially sensitive to freeze-thaw degradation.

F. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was collected. Some methods call for a 90-min incubation to hemolyze the red cells and allow the endogenous folate conjugates to hydrolyze the conjugated

pteroylpolyglutamates to pteroylmonoglutamates prior to the assay for RBC folate. However, we have found that if the blood is diluted 1:11 with 1 g/dL ascorbic acid to keep the folate in the reduced state and the hemolysate is frozen promptly in the NHANES field vans, a single freeze-thaw cycle before analysis has the same effect as incubation (2).

G. Diurnal variation is not a major consideration. Hemolyzed serum specimens should not be used because they may have falsely high values. A recent article in Clinical Chemistry suggests that while serum vitamin B12 is light stable, serum folate specimens exposed to light for longer than 8 hours may have undergone 10-20% degradation (3). Therefore, specimens intended for folate analysis should be processed and stored frozen promptly if analysis is not to be performed within 8 hours of collection.

H. Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q: /ITN/Nutrition Laboratory/CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, plasma should be transported and stored at no more than –20°C. Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalge cryovial labelled with the participant's ID.

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. Reagent Preparation

(1) Working tracer reagent

Reconstitute the DTT with 10 mL deionized water. Agitate gently to dissolve, and let stand 5 min. Transfer the entire contents of the DTT vial into the appropriate tracer bottle. Cap and mix by inversion. Store at 2–8°C for 30 days.

(2) Red cell folate diluent

Add 5 mL deionized water to each vial needed. Allow to stand for 30 min. The solution will be stable for 1 month at –20°C. Two vials are required to prepare duplicate assay tubes for every 50 hemolysates.

(3) 1 g/dL Ascorbic acid solution

Add 1.0 g L-ascorbic to 100 mL deionized water and mix well to dissolve. Prepare fresh daily when needed for red cell hemolysates.

(4) Lyphochek levels I, II, III, and anemia control

Rehydrate each vial of Levels I-III with 5.0 mL deionized water and rehydrate the anemia control with 3.0 mL water. Mix the contents gently by swirling, and let stand for 15 min. Bio-Rad states that these quality control materials may be stored up to 10 days at 2–8°C. Our usual practice is to rehydrate and pool multiple vials of the same lot of a level, mix them well, aliquot 1.0 mL into polypropylene vials, and store them at –70°C to provide us with long-term quality control pools for our studies. One vial of each level is thawed for use on the day of analysis.

During the analysis of whole blood specimens, include Lyphochek red cell controls. Add 2 mL deionized water to each vial and treat the rehydrated contents as whole blood specimens and dilute them similarly for analysis. Again, we usually prepare and pool multiple vials of each level, dispense them as 1:11 hemolysates (1 part (100 L) EDTA-whole blood with 10 parts 1% ascorbic acid (1.0 mL)), and store the vials at –70°C for long-term storage. The folate

concentrations in the materials vary from lot to lot, but they represent one deficient level, one normal-range level, and one elevated level (4).

(5) Additional higher concentration serum folate pool

Because occasional lots of the Bio-Rad Lyphochek materials do not exceed 10-11 ng/mL for serum folate concentration, and folate levels in the U.S. population are steadily increasing with supplementation and food fortification, and to ensure that pursuant to CLIA requirements we had a QC material in the higher concentration range (i.e., 11-20 ng/mL before dilution), we prepared an additional high concentration pool by collecting blood from pre-screened donors known to be supplementing. This blood was collected with anticoagulant and processed exactly as we stipulate for NHANES donors: it was allowed to clot for at least 30 minutes and no more than 60 minutes, then centrifuged and the serum was separated. Serum from multiple donors was combined and the final folate concentration was verified to be > 15 ng/mL but < 20 ng/mL (the highest standard concentration in the kit). One-mL aliquots were prepared from the filter-sterilized pooled material, and stored at –70°C. One aliquot is thawed and measured with each assay.

B. Standards Preparation

Folate/Vitamin B12 Standards
These materials (0.0, 1.0, 2.5, 5.0, 10.0 and 20.0 ng/mL folate, and 0.0, 100, 250, 500, 1000, 2000 pg/mL vitamin B12) are supplied in a liquid form as pteroylglutamic acid (PGA) and cyanocobalamin in human serum albumin, ready to be used. If the entire kit is not used in one run, store the standards at 2–8°C until the expiration date of the kit. At pH 9.2 the binding affinities of PGA and N_5-methyl-tetrahydrofolate (N_5MeTHFA), the predominant biologically active form of monoglutamic folate in the body, are equivalent. PGA, however, is far more stable and can be used as an assay standard. It is also the standard material usually used in the traditional *Lactobacillus casei* microbiological assay for folate.

C. Preparation of Quality Control Materials

As outlined previously, four levels of Lyphochek serum controls, and three red cell controls are analyzed in duplicate in each run as bench quality control materials. The controls are bought in bulk, rehydrated, mixed, re-aliquoted, and stored at –70°C. Approximate values are 1.0, 2.0, 6.0, 10.0 and 14.0 ng/mL for folate in serum; 60, 250, and 500 ng/mL in the red cell controls; and 100, 400, 700, 900 and 1600 pg/mL for vitamin B12. Bench QC pools may also be made from filter-sterilized fasting human serum that has been lyophilized or aliquoted in appropriate quantities and stored at –70°C.

For blind quality control pools, two levels (low-normal and high-normal ferritin concentrations) of blind QC pools may be prepared from pooled, filter-sterilized human serum obtained from fasting donors with elevated or decreased ferritin levels. Pool serum in acid-cleaned 20-L glass carboys. Mix well on a magnetic stirrer. Clean-filter the serum through in a sequential manner using filters of the following pore sizes, each preceded by a pre-filter: 3.00 µm, 1.20 µm, 0.80 µm, 0.65 µm, 0.45 µm, 0.30 µm, and 0.22 µm.

Through the use of sterile technique under a laminar-flow hood, dispense the serum in 1-mL aliquots with a Micromedic Digiflex dispenser into 2.0 mL Nalge cryovials. (A similar process, but without filter-sterilization, is used for the RBC folate samples.) Cap and label the vials with NHANES bar-coded labels that have been specially prepared for the QC pools. Store the pools at –70°C at the CDC CASPIR Specimen Repository in Lawrenceville where they will be inserted randomly into the NHANES runs. Select 20 vials of each level for pool characterization.

D. Other Materials

(1) "Quantaphase II Folate or Folate/B12" radioassay kit (cat. no. 191-1046), 200-test size (Bio-Rad Laboratories).

(2) "Lyphochek" 3-level and "Lyphochek Anemia Control" lyophilized human serum quality control materials. Also "Lyphochek Red Cell Controls", levels I, II, and III (ECS Division, Bio-Rad Laboratories, Anaheim, CA).

(3) Disposable 12- x 75-mm polypropylene tubes (American Scientific Products, McGaw Park, IL).

(4) L-ascorbic acid, ACS certified (Fisher Scientific Co., Fairlawn, NJ).

(5) "FOAMRAC" foam rubber racks for holding tubes for decanting and blotting after centrifugation (Bio-Rad Laboratories).

(6) Red cell folate diluent: (human fraction V albumin solution) for diluting red cell hemolysates (Bio-Rad Laboratories).

(7) Combi-tips, 5.0- and 12.5-mL capacity (Brinkmann Instruments).

(8) Polypropylene test tube racks (Nalge Co., Rochester, NY).

E. Instrumentation

(1) Packard Cobra gamma automatic gamma counter (Model E5005, Packard Instruments, Downers Grove, IL) or ICN Model 10/600 plus gamma counter (ICN Biomedical, Costa Mesa, CA).

(2) Model J6B centrifuge (Beckman Instruments, Inc., Palo Alto, CA), or Centra-7 centrifuge (International Equipment Co., Needham Heights, MA).

(3) Packard Multiprobe II Liquid Handling System (Packard Instruments, Downers Grove, IL).

(4) Multi-tube vortexer (Thermolyne Maximix III, VWR, Marietta, GA).

(5) Gilson Pipetman pipettor, 100- and 200 L sizes (Rainin Instrument Co., Inc., Emeryville, CA).

(6) Eppendorf repeater pipettor (Brinkmann Instruments, Inc, Westbury, NY).

(7) Isotemp 220 water bath (Fisher Scientific, Norcross, GA).

7. Calibration and Calibration Verification Procedures

Results of in-house recovery studies using both forms of folate showed approximately 106% recovery for various levels of vitamin B12 added externally, 93% recovery for folate added to serum as N_5-methyl-tetrahydrofolate, and 99% recovery for PGA. External calibration may be verified with purified PGA; there is no National Institutes of Standards and Technology (NIST) standard reference material available for folate. The National Institute for Biological Specimens and Control (UK) has prepared an international vitamin B12 reference material, 320 pg/ampule, which will be used as an external B12 reference material at straight (320 pg/mL) and 1:2 dilution (160 pg/mL). The limits of detection as determined with dilutions of purified PGA and cyancobalamin standards are 0.2 ng/mL folate and 20 pg/mL vitamin B12.

Performance checks for the assay include:

- Trace binding: The CPM for the zero standard should be >35% of the CPM of the total counts. If it is <35% a failure of the microbead reagent or a procedural error may have occurred.
- Nonspecific binding: the CPM for the blank should be <6% for the CPM of the total counts.

The accuracy of the folate assay was re-verified in 1994 with purified Sigma and Merck PGA folate standards diluted at 1.0, 5.0, 10.0 and 20.0 ng/mL dilutions. The overall slope of the regression line of the expected and calculated values was 0.985, the y-intercept was 0.3, and the r^2 was 0.9902. This procedure may be used to re-verify the kit accuracy at annual intervals. A similar procedure may be followed with purified cyancobalamin standards. Additional 160 and 320 pg/mL points are possible with the NIBSC material.

A. Calibration of Instrument

The Packard Cobra gamma counter is used for data reduction. To ensure the accuracy of test results, take the following steps:

(1) Daily: Background and efficiency are run simultaneously using Packard Pico calibrators and associated software. Printout will indicate if all performance parameters are within acceptable limits. Efficiency should be at least 75% for I125 and 80% for Co57.

(2) Monthly: Normalization should be performed on a monthly basis. Printout will indicate if performance parameters are within acceptable limits.

(3) Semi-annually: Preventative maintenance through Packard Service Inspection.

B. Instructions for Calibration of Instrument

(1) Load Pico calibrators in positions 2, 6,10,14,18 and blank tubes in positions 1,5,9,13,17 for each isotope.

(2) Insert protocol 25 clip if measuring I125, clip 26 if measuring Co57.

(3) Select F2 (SC Commands), F6 (next protocol).

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A. Manual Pipetting

(1) Label 12- x 75-mm tubes in duplicate for each blank, standard, control, patient sample, and total counts. Allow all reagents and specimens to come to room temperature before use.

(2) Add 200 L of each standard, control, or patient serum to its replicate tubes. Add 200 L of zero standard to the blank tubes. If assaying for red cell folate, thaw the 1:11 diluted hemolysate at room temperature and mix well. Add 100 L of hemolysate and 100 L of red cell diluent to replicate tubes. (RBC specimens are now at the same protein levels as serum specimens in the assay.)

(3) Thoroughly resuspend the working tracer reagent and add 1.0 mL to all tubes, including the total counts tubes.

(4) Mix by vortexing each tube. Set aside total counts tubes until step (6), next section.

B. Using Packard Multiprobe Dilutor

(1) Prepare reaction tube racks. Label 12- x 75-mm tubes in duplicate for each blank, standard, control, patient sample, and total counts tube. These tubes should be arranged in the racks according to the chosen layout. The layout information is located in the software program associated with the desired analyte.

(2) Load samples and reagents as specified in the layout, and as required for either serum folate or the red cell folate procedure.

(3) "Execute" the protocol for the desired procedure. The parameters and values for each procedure are programmed into the protocol.

(4) Mix by vortexing the entire rack of tubes on the multi-tube vortexer. Set motor speed on "5." Using the on/off button, turn on for 2-3 sec; repeat five times for thorough mixing of the entire rack.

C. Procedure Following Completion of Manual or Autodilutor Steps

(1) Place the tubes in the rack, cover with aluminum foil, and place the rack in a boiling-water bath at 100 °C. Allow the bath to return to a rolling boil and incubate the tubes for a minimum of 35 min. Cool to room temp by placing the rack in a cold water bath.

(2) Thoroughly mix the bottles of microbead and blank reagents by placing them on a rocker-mixer for at least 15 min prior to use. Add 100 L of microbead reagent to each tube except the blanks. Add 100 L blank reagent to the blank tubes. Vortex all tubes.

(3) Incubate tubes at room temperature (about 21-30 °C) for 1 hour.

(4) Centrifuge all tubes for 10 min at 1500 x g to pack the solids at the bottom of the tubes. Proceed promptly to the next step.

(5) Place the tubes in the FOAMRACS and invert the tubes over a container designated for radioactive waste in order to discard the supernatant from each tube. (A large plastic funnel or dish pan is useful for collecting the liquid and channeling it into a large plastic bottle for proper disposal of the radioactive waste.) Remove the last drops of liquid by blotting the tube rims on plastic-backed absorbent paper.

(6) Place the tubes in racks and count for 1 min in the gamma counter. Record the counts.

D. Calculations

The Packard Cobra 10/600 gamma counter has full data reduction capabilities. Logit B/B_o vs $\log_{10}$ concentration is used in both counters where:

$$\text{logit}\ (B/B_o) = \text{Ln}\ ((B/B_o)/\ (1-B/B_o))$$

and B = corrected counts/min (blank subtracted) for each tube, and B_o = maximum binding.

This method results in a linearized standard curve with an inverse relationship of levels of radioactivity (measured in counts per min, or CPM) to the concentration of folate or B12 in the serum or whole blood sample. Results are expressed as nanograms of folate per milliliter of serum (ng/mL) or as picograms of vitamin B12 per milliliter of serum (pg/mL). Red cell folate values are multiplied by 22, the dilution factor of the whole blood. The serum folate values (multiplied by 1.0 minus the hematocrit expressed as a decimal) are subtracted, and the resulting value is divided by the hematocrit to yield red cell folate in ng/mL red blood cells.

$$\text{RBC folate, ng/mL} = \frac{\text{(whole blood folate X 22) - serum folate (1-hematocrit/100)}}{\text{hematocrit/100}}$$

We recommend use of the correction for serum folate level and hematocrit because it provides the most accurate reflection of folate body stores.

E. Special Procedure Notes – CDC Modifications

The CDC modifications for red cell folate specimen preparation necessitated by field lab collection have been included by Bio-Rad in the recommended kit instructions. The folate-only, vitamin B12-only, and folate/B12 combination kits are now used exactly as outlined by the manufacturer, usually in their entirety in one analytical run.

9. Reportable Range of Results

Values <2.0 ng/mL for serum folate, <2.0 ng/mL for whole blood folate, and <200 pg/mL for vitamin B12 are verified by re-assay. Values >20.0 ng/mL for serum or whole blood folate, or >2000 pg/mL for vitamin B12 are verified by re-assay after the solution has been diluted 1:2 with saline. These whole blood folate repeat values are approximately equivalent to 75 and 900 ng/mL RBC as red cell folate concentrations.

10. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Inorganic Toxicology and Nutrition Branch. These specimens are prepared at two levels so as to emulate the patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

B. Bench Quality Controls

Because of reliability and availability, three levels of Bio-Rad Lyphochek controls are currently used as bench quality control materials. These pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run. The system is declared “in control” if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared “out of control”:

- 1_{3s} Any of the three QC results are outside the 3s limit
- 2_{2s} Two of the three QC results in the run are outside the 2s limit (same side of mean)
- R_{4s} Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean
- 10_x Ten sequential QC results (across pools and across runs) are on the same side of the mean

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared “out of control” for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder on Q: /ITN/Nutrition Lab/Data handling/Import into Access. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder on Q: /ITN/Nutrition Lab/Data handling/QC Results in Excel. A hardcopy of the QC results from each run is also kept by the analyst.

Long-term estimates of NHANES 1III assay precision in measuring serum and red cell folate during calendar year 1993 are about 3-6% coefficient of variation (CV) at 3-15 ng/mL and 5-6% at 1 ng/mL. For vitamin B12, CVs are 5-7% at 300-1500 pg/mL and 5-6% at 200 pg/mL. Representative precision and accuracy of the method are reflected in long-term quality control pool results (See Tables 2 and 3in the APPENDIX).

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

The COBRA software will allow the standard curve to be edited if there is a single outlying point. If the controls are still outside of the acceptable range, declare the system "out of control" and repeat the entire run. If the "out of control" condition still exists, use a new kit and evaluate the pipetting precision and accuracy of the auto diluter.

Reassayed specimens for that analytical run after the system has been verified to be "in control," and report the results of the reassayed rather than the original values.

If the steps outlined above do not result in the correction of the "out of control" values for QC materials, consult the supervisor for other corrective actions. The ICN analyzer may be used if the Packard is non-functional.

12. Limitations of Method; Interfering Substances and Conditions

The most common causes of imprecision are intermittently inaccurate micropipettors, inadequate boiling time, and microbead reagent that was not mixed thoroughly before it was added to the solution.

Hemolyzed serum samples may give falsely elevated values. Exposure to strong sunlight for more than eight hours may cause 10-20% serum folate degradation.

13. Reference Ranges (Normal Values)

Current proposed normal ranges for serum folate are about 2.6-12.2 ng/mL for both sexes and all ages. Values are lower in females than in males. Serum levels of 1.4-2.6 ng/mL are usually termed "indeterminate" because of an overlap between "deficient" and "normal" ranges. Serum folate values <1.4 ng/mL are usually indicative of inadequate folate intake. Elevated values are caused by supplementation.

Red cell folate values are more indicative of body stores, whereas serum levels reflect only recent dietary intake. The approximate normal range for red cell folate is 102.6-410.9 ng/mL RBC.

The expected normal range for vitamin B12 is about 165-1600 pg/mL. Concentrations of 160-200 pg/mL are considered "indeterminate", and those less than 160 pg/mL are considered deficient. Elevated B12 levels may result from over supplementation or may reflect myeloproliferative disorders.

Values from NHANES 1999+ will be used to update the U.S. national normal ranges. Results from NHANES III are published in the Series 11 Report.

Extensive evaluation by CDC, Cambridge University, and Stanford University researchers showed PGA calibrators in original Quantaphase kits to be inaccurate by an average of 32.5% across 0-20 ng/mL range. Using freshly received NHANES 1999+ specimens, CDC performed 19 comparison runs with "old" and "new" calibrators (with spectrophotometrically verified concentrations of PGA) to establish a correction factor. The equation used for the correction was:

$$\text{Log}_{10}\text{ (corrected value)} = -0.1956 + 1.0199\ \text{Log}_{10}\text{ (uncorrected value)}$$

Or, expressed in linear terms:

$$\text{corrected value} = -0.1411 + 0.6849 \times \text{(uncorrected value)}$$

Evaluations were also made with N_5MeTHFA standards. No change occurred to affect vitamin B12 results.

The currently used reference ranges for serum and red cell folate are based on a mathematical correction of previously recommended values from the supporting documentation for HANES II and reflect the approximately 32.5% average difference for standards between current versions of the Quantaphase II folate kits. All of Phase I of NHANES 1999+ was performed with the original kit; hence, data from Phase I were corrected to correspond with the data from Phase II.

14. Critical Call Results (“Panic Values”)

Any NHANES samples with serum folate levels <1.5 ng/mL, RBC folate levels <75 ng/mL RBC, or B12 levels <200 pg/mL are considered to require follow-up. Since survey data are transmitted several times weekly to Westat, abnormal reports are automatically forwarded to the NCHS survey physician for follow-up. For smaller, non-NHANES studies, abnormal values are identified to the study principal investigator. Most of these studies are epidemiological in nature, however.

15. Specimen Storage and Handling During Testing

Specimens should be allowed to warm to and be maintained at room temperature during preparation and testing.

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

There are no acceptable alternative methods of analysis for folate or vitamin B12 in the NHANES laboratory. The ICN may serve as an alternative gamma counter; no substitution is permitted for the Quantaphase II assay.

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

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX by the supervisor of any serum folate result that is <1.5 ng/ml. RBC folate that is <75ng/ml and B12 <200 pg/ml, possibly represents a significant risk for B vitamin deficiency. Copies of Faxes sent concerning abnormal results are kept in a notebook by the supervisor for the duration of the study.

Test results that are not abnormal are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically several times weekly to the Westat ISIS computer and then are transferred to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

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

The Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If plasma or serum folate/B-12 analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN.

We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

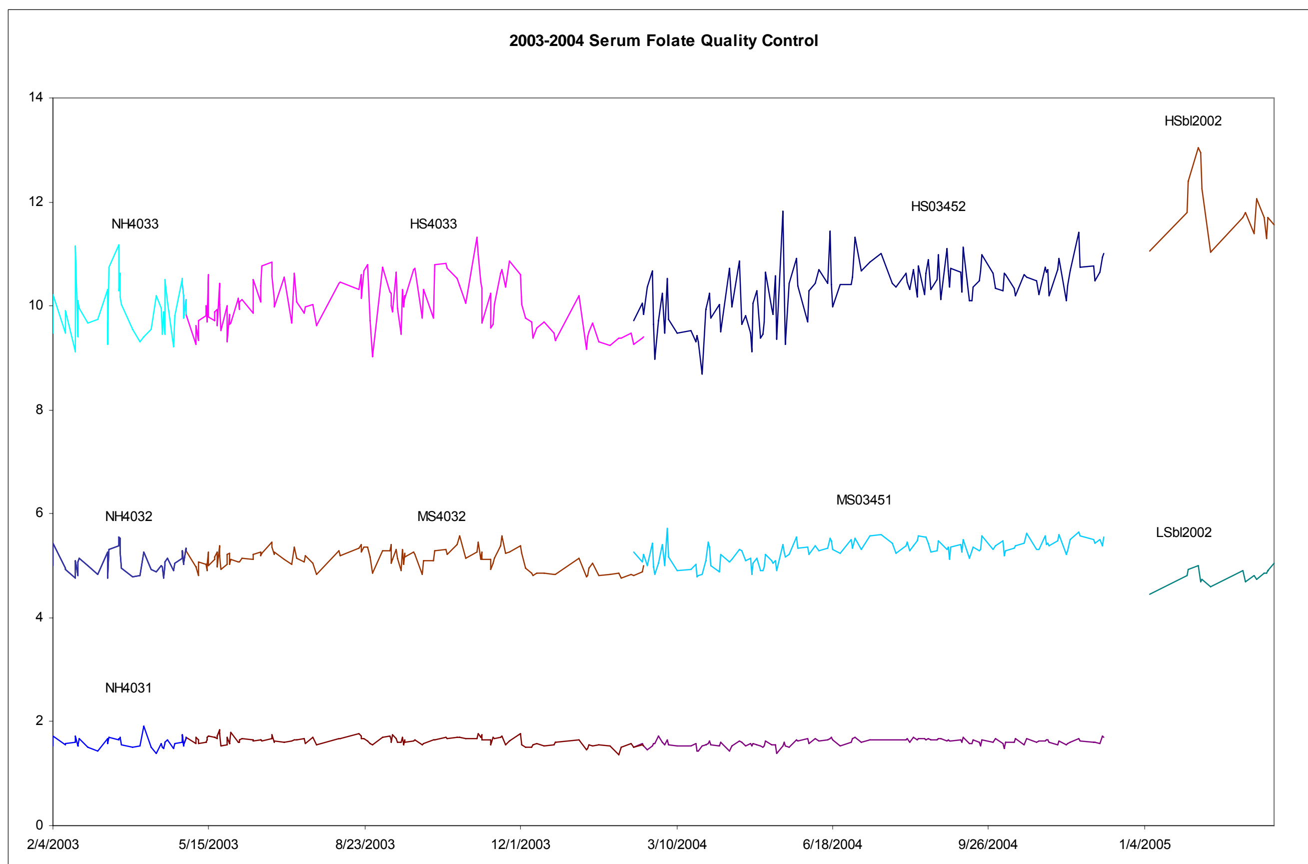
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70 °C. The specimen ID is read off of the vial by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

19. Summary Statistics and QC Graphs

A. Serum Folate

Summary Statistics for Serum Folate by Lot

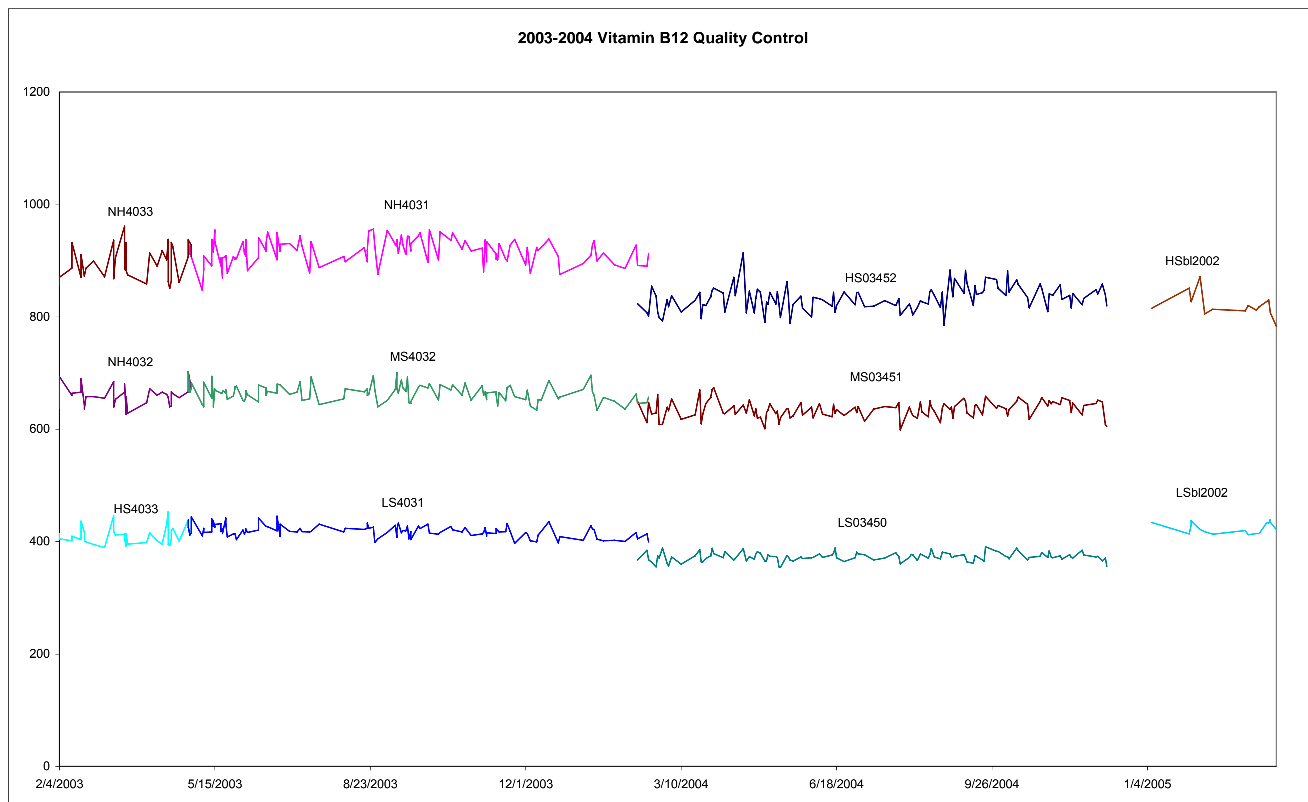
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH4031	38	2/4/2003	4/30/2003	1.599	0.101	6.3
NH4032	38	2/4/2003	4/30/2003	5.074	0.217	4.3
NH4033	38	2/4/2003	4/30/2003	9.948	0.511	5.1
LS4031	117	4/28/2003	2/18/2004	1.634	0.077	4.7
MS4032	117	4/28/2003	2/18/2004	5.123	0.189	3.7
HS4033	117	4/28/2003	2/18/2004	10.024	0.486	4.8
LS03450	118	2/11/2004	12/9/2004	1.602	0.065	4.1
MS03451	118	2/11/2004	12/9/2004	5.287	0.215	4.1
HS03452	118	2/11/2004	12/9/2004	10.305	0.550	5.3
LSbl2002	16	1/7/2005	3/28/2005	4.809	0.152	3.2
HSbl2002	15	1/7/2005	3/28/2005	11.849	0.605	5.1



B. Vitamin B_{12}

Summary Statistics for Vitamin B12 by Lot

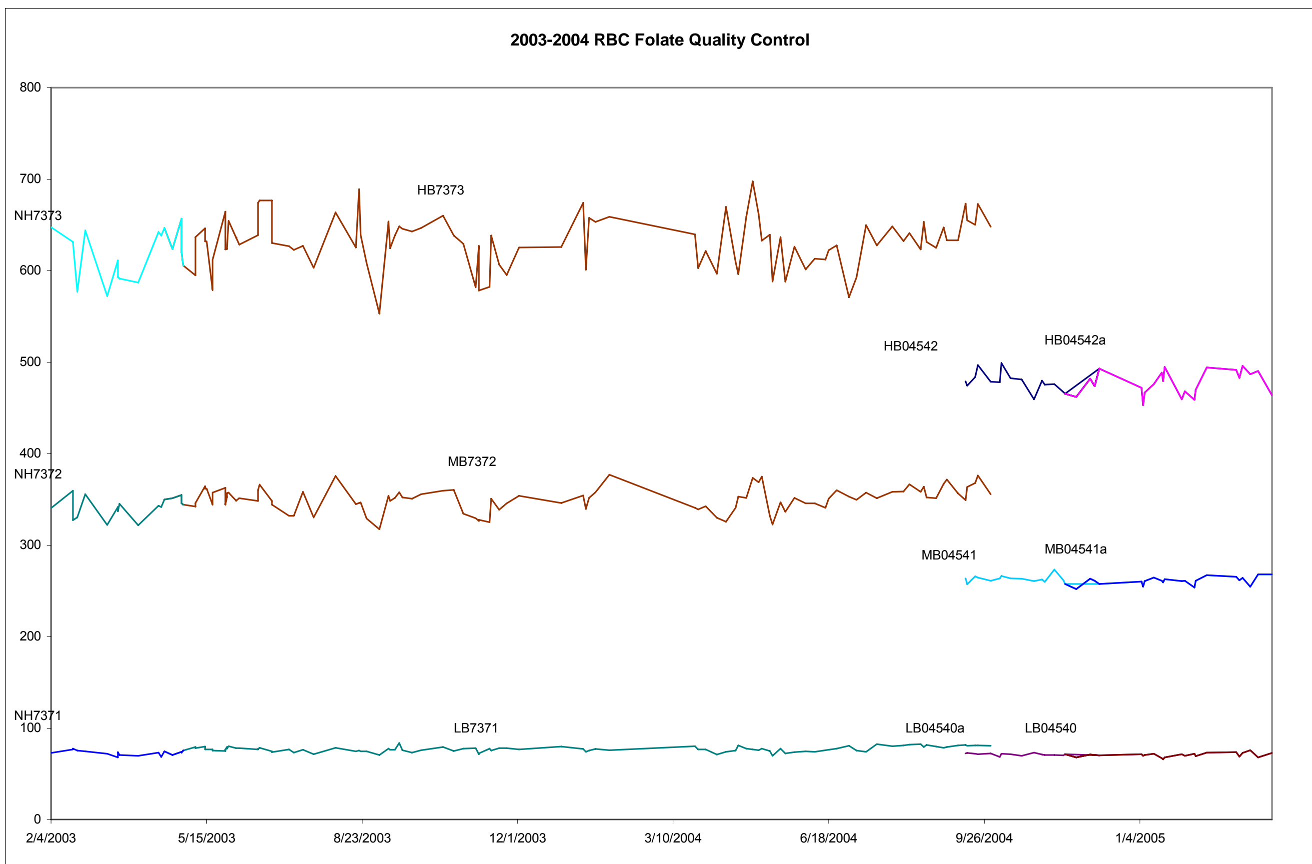
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH4031	35	2/4/2003	4/30/2003	411.9	16.8	4.1
NH4032	35	2/4/2003	4/30/2003	660.2	17.9	2.7
NH4033	35	2/4/2003	4/30/2003	896.0	29.6	3.3
LS4031	113	4/28/2003	2/18/2004	418.8	10.6	2.5
MS4032	113	4/28/2003	2/18/2004	664.6	14.8	2.2
HS4033	113	4/28/2003	2/18/2004	915.8	22.4	2.4
LS03450	117	2/11/2004	12/9/2004	373.1	7.7	2.1
MS03451	117	2/11/2004	12/9/2004	635.5	15.0	2.4
HS03452	117	2/11/2004	12/9/2004	833.2	21.9	2.6
LSbl2002	16	1/7/2005	3/28/2005	424.4	9.8	2.3
HSbl2002	14	1/7/2005	3/28/2005	820.7	21.0	2.6



C. RBC Folate

Summary Statistics for RBC Folate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH7371	18	2/4/2003	4/30/2003	73.10	2.80	3.8
NH7372	18	2/4/2003	4/30/2003	341.90	10.90	3.2
NH7373	18	2/4/2003	4/30/2003	618.00	26.20	4.2
LB7371	99	4/18/2003	9/30/2004	76.80	2.90	3.8
MB7372	99	4/18/2003	9/30/2004	350.20	12.60	3.6
HB7373	99	4/18/2003	9/30/2004	631.40	27.00	4.3
LB04540	19	9/14/2004	12/9/2004	71.00	1.40	1.9
MB04541	19	9/14/2004	12/9/2004	261.90	4.50	1.7
HB04542	19	9/14/2004	12/9/2004	478.20	10.60	2.2
LB04540a	23	11/17/2004	3/30/2005	70.50	2.30	3.3
MB04541a	23	11/17/2004	3/30/2005	260.80	4.50	1.7
HB04542a	23	11/17/2004	3/30/2005	476.80	13.40	2.8



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Tables 1-3:

Table 1
Quick Reference Summary Table of Initial Assay Preparation
(All tubes in duplicate)

Sample	Sample Size, (µL)	125I/57Co Tracer (µL)	Antibody Microbeads (µL)	Blank Reagent (µL)	RBC Prot. Diluent (µL)
Total Counts	--	1	--	--	--
Blank NSB	200	1	--	100	--
0 Standard	200	1	100	--	--
5-20 ng/mL / 100-2000 pg/mL Standards	200	1	100	--	--
Controls	200	1	100	--	--
Unknown Serum	200	1	100	--	--
Unknown RBCs	100	1	100	--	100

Table 2
Typical Vitamin B12 Pools (pg/mL) Data from NHANES III

Pool	Mean	95% Limits	99% Limits	N	Total SD	Total CV
8001	1257.7	1135.19-1380.11	1096.45- 1418.85	27	61.205	5.66
8002	797.7	711.10-848.36	689.39-870.07	27	34.33	5.30
8003	467.5	408.66-526.24	390.06-544.84	27	29.37	6.82
32AN	183.1	146.16-220.13	134.46-231.83	27	18.52	10.50

Table 3
Typical Serum and Red Cell Folate Pools (ng/mL and ng/mL RBC) Data From NHANES III

Pool	Mean	95% Limits	99% Limits	N	Total SD	Total CV
8301	11.505	10.06-12.978	9.594-13.427	41	4.90	7.02
8302	3.018	2.819-3.220	2.758-3.285	41	4.36	4.36
8302	0.949	0.800-1.101	0.750-1.142	41	4.86	7.98
33AN	0.750	0.655-0.853	0.620-0.881	40	7.53	6.11
5111	33.60	30.17-37.03	29.08-388.12	37	1.75	5.23
5112	263.20	264.94-279.49	241.80-284.64	37	9.97	3.84
5113	503.06	454.32-551.96	438.90-567.14	37	26.27	5.25