Laboratory Procedure Manual

Analyte: **Folate**

Matrix: **Serum/Whole Blood**

Method: **Microbiological Assay**

Method No: 4000.03

Revised:

as performed by:

Nutritional Biomarkers Branch (NBB)
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 testing the items listed in the following table:

Data file name	Variable name	SAS Label
FOLATE_F	LBDRBF	RBC folate (ng/mL)
	LBXRBFSI	RBC folate (nmol/L)
	LBDFOL	Serum folate (ng/mL)
	LBXFOLSI	Serum folate (nmol/L)

1. Summary of Test Principle and Clinical Relevance

a. Clinical relevance

Folates are a group of water-soluble vitamins that function in biological reactions to transfer one-carbon units from one compound to another and are required in cellular metabolism and hematopoiesis. The prolonged folate deficiency leads to megaloblastic anemia, causes neural tube defects in fetus and increases the level of homocysteine. It is essential to determine the levels of folic acid to establish the etiology of the anemia or neural tube defects, and determine the folate status in US population. This microbiological method is used to quantitatively measure serum and red blood cell (RBC) folate. The method is relatively easy to perform, reliable, and considerably less costly than chromatographic assays, particularly where large numbers of samples are involved.

b. Test Principle

Microbiological assays have been used for many years to estimate the concentration of folate in blood and other tissues. In the 1990s O'Broin et al. and Molloy et al. have introduced robust and reliable procedures that use microtitre plates for higher throughput and a cryopreserved antibiotic resistant microorganism to avoid having to work under aseptic conditions. The herein described procedure is an adaptation of the O'Broin et al. method. Diluted serum or whole blood hemolysate is added to an assay medium containing *Lactobacillus rhamnosus* (formerly known as *L. casei*) (NCIB 10463) and all of the nutrients necessary for the growth of *L. rhamnosus* except for folate. The inoculated medium is incubated for 45 hours at 37^{o}C. Since the growth of *L. rhamnosus* is proportional to the amount of total folate present in serum or whole blood samples, the total folate level can be assessed by measuring the turbidity of the inoculated medium at 590 nm in a PowerWave microplate reader (Bio-Tek Instrument). We calibrate the assay with 5-methyltetrahydrofolic acid (5MeTHF) from Eprova.

2. Safety Precautions

Consider all whole blood or serum specimens potentially positive for infectious agents including HIV and the hepatitis B virus. We recommend the hepatitis B vaccination series for all analysts working with whole blood and/or serum. Observe universal precautions; wear protective gloves, laboratory coats, and safety glasses during all steps of this method. Discard any residual material (blood samples and assay medium containing bacterial) by autoclaving after analysis is completed. Place disposable plastic, glass, and paper (pipette tips, vials, tubes, gloves, microplates, etc.) that contact serum or blood in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Use disposable bench diapers during sample preparation and serum/ blood handling and discard them in autoclave pan after use. Also wipe down all contaminated work surface with 10% bleach solution when work is finished. Add bleach into the leftover of folate assay medium containing *L.casei* at final concentration ~10% to kill all the organisms.

Handle acids and bases with extreme care; they are caustic and toxic. Handle organic solvents only in a well-ventilated area or, as required, under a chemical fume hood.

Reagents used in this study include those listed in Section 6. Handle acids and bases with extreme care; they are caustic and toxic. Material safety data sheets (MSDS) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs.

Bio-Tek Instrument Inc. provides safety information that should be read before operating the Power Wave Microplate Scanning Spectrophotometer. This information can be found in the Operator's Manual.

3. Computerization; Data System Management

a. The data is collected, analyzed, and stored using the GEN 5 Data Analysis software (Bio-Tek Instruments, Winooski, VT) which runs the PowerWave and PowerWave 340 microplate readers. Hardcopies of the data reports are printed out and stored. Three folders are generated: (1) *FOL MA Protocols* containing serum and whole blood MA protocols; (2) *FOL MA Experiments* containing raw data for each assay such as sample IDs, standard curve and results (OD, final folate concentration); and (3) *FOL MA Power Export* containing results for standard curve, QCs, and patient samples in Excel format. These folders are stored at *Q:\ITN\Nutrition Lab\Folate Microbiol assay\GEN 5*.

After a run is completed, the analyst inspects the plates in detail (looking for leaking wells, dark color wells, volume in wells), reviews the data in detail (number of replicates, dilution factors, formulas, coefficient of variation [CV%], too high [>high cal] or too low [< cutoff value] folate concentrations that require repeating, need for special comment codes [i.e., hemolyzed sample, no ascorbic acid solution added to whole blood]), and makes appropriate notes. A Power Export result file (containing standard curve, patient and QC data) is then generated and imported into the FrontEnds database.

Step 1 – Analyst – Import data file into FrontEnds (Access database):

Double click the ACCESS icon on desktop, password entry required

[Add Sample Results to Database] (under Batch & X-Batch)

[Import Instrument Data File] - Enter information (instrument, assay, date, time, analyst, study)

[Import] – In "select data file" window, choose A: and import file number assigned. Check that sample ID's are recognized.

[Transfer]

Step 2 – Analyst – Review run in FrontEnds:

[Run Review] (under Batch & X-Batch) – Select assay

[Show runs] – Cursor to desired run, enter sample set name and comments

[QC Results] – Review QC results for transmission errors and whether they pass the 2S limits

[Print Report] [Back]

[Sample Results] – Review patient results to assure proper information transmission, enter appropriate comment codes on flagged samples

[Set Final] results that are ready to be reported

[Set Reviewed]

[Print Report] [Back]

Step 3 – Analyst – Send email and run folder to QA Officer:

An e-mail is sent to the QA Officer including the following run information: Analysis date, Instrument, Study, Groups, File name, Batch ID, Run #, and QC Status. Noteworthy comments are included in the email. All printouts including raw data are submitted in a run folder to the QA Officer who reviews the Bench QC data via the ACCESS database as described below.

Step 4 – QA Officer – Review Bench QC via FrontEnds:

Double click the ACCESS icon on desktop, password entry required

[Export QC to SAS] (under Batch & X-Batch) – Select Assay, Date range and Controls

[Make QC Data Infile] – Save file to I: appropriate subfolder for archival

[Run SAS] – SAS will automatically open, [go], review each generated plot, print QC cover page and standard deviation plot, [Back]

[Run Review] (under Batch & X-Batch) – Select assay

[Show runs]

[Sample Results]

[Set Batch QC] – accept or reject

[Set Reviewed]

Forward email from Analyst to Team Lead specifying Bench QC status of the run.

Step 5 – Second QA Reviewer – Review Blind QC and other parameters in FrontEnds:

Double click the ACCESS icon on desktop, password entry required

[Run Review] (under Batch & X-Batch) – Select assay, then desired run

[Blind QC Results] – Review whether Blind QC results pass the 2S limits

[Print Report] [Back]

Check other parameters if applicable (i.e., background, calibration curve, repeat values, replicates, signal intensity)

[Set RQC] – accept or reject

Verify that appropriate comment codes have been applied and that final values have been set correctly

[Set Ready] – Final results will be set ready to be exported

[Set Reviewed]

Forward email from QA Officer to Supervisor specifying Blind QC status of the run and other relevant comments.

Step 6 – Supervisor – Approval and Export of Results via FrontEnds:

Double click the FrontEnds icon on desktop, password entry required

[Run Review] (under Batch & X-Batch) – Select assay, then desired run

Perform a final review

[Set Reviewed]

[Export/Report Results] (under Study Functions) – Select study, select analytes/panel, use selected panel

[Generate Pre-Export Text File] – Review file on \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\To be transmitted

[Generate Export Text File and Set Results Exported] – \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\To be transmitted

FTP file to Westat

Send Westat an email that file was transmitted

Move transmittal file from \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\To be transmitted to \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\Transmitted\Appropriate Year Folder.

For NHANES, data is transmitted electronically regularly (typically weekly) to ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.

b. Backup of data containing "Experiment files" and "Power Export files" are the responsibility of the analyst and team lead. These files are typically backed up to the CDC network on a daily basis and periodically backed up on CD or some other medium. All sample, QC, and calibration data are stored on the CDC network and are the responsibility of the analyst. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center Staff, respectively.

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.

4. Specimen Collection, Storage, and Handling Procedures

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

b. Serum folate assays are performed on fresh or frozen serum. RBC folate samples are prepared by diluting 1 part of fresh EDTA whole blood (100 uL) with 10 parts of 1 g/dL (1%) ascorbic acid solution (1 mL), corresponding to a 1/11 dilution, and freezing the lysate promptly, which keeps the folate in the reduced state.

c. A minimum of 400 uL of serum and 500 uL of whole blood lysate is needed to do a proper dilution when using automated pipetting. Serum is typically diluted 1/100 (20 uL of serum is added to 1980 uL of 0.5 g/dL sodium ascorbate). Whole blood lysate is typically diluted at 1/140 (15 uL of lysate is added to 2085 uL of 0.5 g/dL sodium ascorbate) which constitutes a 1/1540 total dilution of whole blood. The dilution factor depends on the population from which the samples are collected. The above dilution factors are appropriate for a population consuming folic acid fortified foods.

d. Serum specimens may be collected with regular red-top Vacutioners. Whole blood is collected with lavender-top Vacutainers containing EDTA as an anticoagulant. A hematocrit measurement used for the RBC folate calculations is made at the time of collection. The appropriate amount of serum or whole blood lysate 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°C until analyzed. Serum folate and RBC folate are stable for a few weeks if the specimen is frozen at -20°C before analysis. For long-term storage, specimens should be frozen at -70°C. Ascorbic acid is typically not added to the serum specimen. Less than three freeze-thaw cycles show only minimal degradation in serum samples and whole blood samples, but freeze-thaw cycles should be avoided where possible due to the sensitive nature of folates.

f. Specimens should generally arrive frozen. Refrigerated samples may be used when 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, a single freeze-thaw cycle before analysis has the same effect as incubation (3). Whole blood samples refrigerated for 48 hours before lysis give same results as samples lysed immediately after collection.

g. Diurnal variation is not a major consideration. Hemolyzed serum specimens should be interpreted with caution because they may have falsely elevated values. Folate specimens exposed to light for longer than 8 hours may undergo 10-20% degradation. Therefore, specimens intended for folate analysis should be processed under yellow light 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 \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA\DLS Policies and Procedures Manual). The protocol discusses collection and transport of specimens and the special equipment required. Samples thawed and refrozen less than three 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 serum should be transferred into a sterile Nalge cryovial labeled 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, Organisms and All Other Materials; Equipment and Instrumentation

a. Standard and Chemicals

5MeTHF (Merck Eprova, AG, IM Laternenacker 5, Schaffhausen 8200 [CH] Switzerland, www.eprova.com)
Ascorbic acid (Sigma, St. Louis, MO, USA)
Sodium ascorbate (Sigma)
Folic Acid Casei Medium (Difco)
Chloramphenicol (Sigma)
Tween-80 (Sigma)
Manganese sulfate (Sigma)
Lactobacillus Rhamnosus (formerly called Casei) (ATCC 27773 or called NCIB 10463, American Type Culture Collection, Manassas, VA, USA)
Sodium azid (Sigma)
Glycerol (Sigma)

b. Reagent Preparation

1) Ascorbic acid solution (1 g/dL) for lysis of whole blood samples

Five grams of ascorbic acid is dissolved in 500 mL ultrapure water and stored in brown bottle. This solution should be made freshly.

2) Sodium ascorbate solution (0.5 g/dL) for diluting samples and standard

One gram of sodium ascorbate is dissolved in 200 mL ultrapure water. This solution should be made freshly before each assay.

3) Blocking solution for color control

Sodium azide is used as a blocking solution. Five uL of a 3 g/dL solution are added to each control well. Alternatively, the disinfectant "Stericol" can be used at a 1/30 dilution with ultrapure water (10 uL/control well).

4) Folic Acid Casei medium inoculated with *L. casei* (folate free)

To prepare 100 mL of assay medium: Add 7.05 g of Folic Acid Casei Medium, 3 mg of chloramphenicol and 30 uL of Tween-80 to 100 mL ultrapure water, heat to boil for 2-3 min with stirring. Cool down to 37°C, then add 15 mg of manganese sulfate and 75 mg of ascorbic acid, and keep stirring until all the chemicals are dissolved. Thaw one vial of frozen *L. casei* as quickly as possible (use running water) and add 200 uL into 100 mL of assay medium, keep stirring slowly. About 25 mL of assay medium is needed for each microplate.

5) *L. casei* growth medium (with folate)

To prepare 100 mL of growth medium: Add 4.7 g of Folic Acid Casei Medium, 50 mg of ascorbic acid, 20 mg of chloramphenicol, 150 uL of folic acid stock solution (100 ng/mL), 20 uL of Tween-80 to 100 mL of ultrapure water. Heat to boil for 2-3 min. Cool down and sterilize the medium by either autoclaving or filtering through a 0.2 um filter. Aliquot 20 mL/vial into sterile 50-mL tubes and keep at -20^{o}C.

6) Ascorbic acid stock solution (150 mg/mL)

Dissolve 15 g ascorbic acid in to 100 mL water. Aliquot 1 mL/vial and store at -70^{o}C. Add 1 mL into 200 mL medium. Make fresh stock solution every 3 months.

7) Manganese sulfate stock solution (30 mg/mL)

Dissolve 3.6 g manganese sulfate in 120 mL water, stir thoroughly (~20 min). Aliquot 1 mL/vial for 200 mL of medium. Make fresh stock solution every 6 months.

8) Chloramphenicol stock solution (3 mg/mL)

Dissolve 600 mg of chloramphenicol in 2 mL of ethanol and then make up to 200 mL with ultrapure water, aliquot 1 mL into cryovial and keep at -70°C freezers. Add 2 mL of stock into 200 mL *L. casei* medium. Make fresh stock solution every 6 months.

c. Standard Preparation

1) 5MeTHF stock solution I (~200 ug/mL): Dissolve ~5 mg 5MeTHF in degassed 20 mM phosphate buffer (pH 7.2) containing 0.1% cysteine in a 25-mL volumetric flask. Vortex briefly and make up to volume to 25 mL. A small aliquot (1mL) of this stock solution is taken in a microcentrifuge vial to determine the concentration by UV spectrophotometry. Add to the remaining stock solution ascorbic acid powder to a final concentration of ~1% (0.25 g). Prepare a 1/20 dilution of the 1-mL aliquot with phosphate buffer and record absorbance at the following wavelengths against phosphate buffer as a blank on a UV/VIS spectrophotometer using scan analysis: 5CH3THF 290 and 245 nm; the ratio of absorbance at 290/245 nm is also monitored to ensure that no oxidation took place. This ratio should exceed 3.3.

2) 5MeTHF stock solution II (100 ug/mL): Based on the actual concentration of stock solution I, the solution is diluted to yield 25 mL of stock solution II. The dilution is done with 1% degassed ascorbic acid solution in a 25-mL volumetric flask. One milliliter aliquots of this stock solution II are stored at -70^{o}C in 2-mL labeled cryovials.

3) 5MeTHF stock solution III (1 umol/L): Dilute 458.93 uL of stock II (100 ug/mL) with 0.5% degassed ascorbic acid in a 100-mL volumetric flask. Aliquot 500 uL into labeled cryovial and freeze the aliquots at -70^{o}C. Prepare a fresh stock solution III every 6 months. Note: In our

case, we obtain 5MeTHF stock II solution from the folate LC-MS/MS assay and dilute ~1/200 to a stock III for the microbiological assay.

d. Preparation of Quality Control Materials

Since we use the same pools for the folate microbiologic assay and the folate LC/MS/MS assay, we prescreen potential material with the LC/MS/MS assay to determine the concentrations of the individual folate forms. We then either spike in individual folate primary standards or dilute pools with a protein diluent or PBS solution to achieve desired concentrations. This is described in detail in the folate LC/MS/MS procedure.

The serum pools are filtered through gauze to remove fibrin and particles before being dispensed. Serum (400 µL) and whole blood pools (500 uL) are aliquoted into 2.0 mL Nalge cryovials, capped and frozen. The QC pools are stored at -70°C and are stable for at least 2 years. Means plus range limits for all pools are established by analyzing the QC pools in at least 20 runs.

e. Preparation of Cryoprotected Organism

1) Add one vial of freeze-dried *L. casei* into 20 mL of organism growth medium and incubate at 37°C for 24 hours.
2) Transfer 100 – 300 uL of the 24-hour culture into another 20 mL of organism growth medium and incubate at 37°C for 24 hours.
3) Repeat Step 2 for the third 24-hour incubation.
4) Inoculate different amount of active culture in duplicates for optimization of response. Add 500 uL, 1 mL and 2 mL of Step 3 culture into 20 mL of fresh growth medium and incubate at 37°C. Measure OD at 590 nm from one of the duplicate cultures at different incubation time. Record the log growth phase (~18 - 20 hours). Mix the log phase culture by 50/50 with 80% glycerol (sterilized by autoclaving). Aliquot the mixture in sterile cryovials (1 mL/vial) and store at -70°C.

f. Equipment

Precision Incubator 2EG: 37°C (VWR, Suwanee, GA)

PowerWave 340 and PowerWave microplate readers and GEN5 software (Bio-Tek Instruments, Inc, Winooski, VT, USA)

Packard Multiprobe II – Robotic Liquid Handling System (NY, USA)

Vortex – Genie 2 (VWR, Suwanee, GA)

Orbitron Rotator I (VWR # 56261-508, Suwanee, GA)

Eppendorf adjustable pipettes (1-200 uL and 100-1000 uL); Eppendorf Repeater Plus pipette; 8-channel pipetter (Rainin)

Stirring Hotplate (Fisher # 11-500-16SH, Suwanee, GA)

Digiflex CX (Titertek #0603310, Huntsville, Alabama)

g. Other Materials

Ultrapure water (18.2 M-ohm from the Milli-Q water purification system)

Pipette tips: 1-200 μL (eppendorf, fits up to 200 μL pipettes) and 200 -1000 μL (Eppendorf, fits between 100 μL and 1000 μL pipettes), Combitip plus 5mL and Combitip plus 0.5 mL for Eppendorf Repeater Plus (Brinkmann Instruments, Inc., Westbury, NY), 250 uL 8-channel pipette tips (#GP-L250, Rainin Instrument Co., Inc., Woburn, MA)

Dehydrated alcohol, USP (Midwest Grain Products of Illinois, Pekin, IL)

Vinyl examination gloves (Travenol Laboratories, Inc., Deerfield, IL)

Biohazard autoclave bags (Curtin-Matheson Scientific, Inc., Atlanta, GA)

Bleach (10% sodium hypochlorite solution) – any vendor

12 x 75 mm Disposable glass culture tubes (Corning Glassworks, NY)

Kay-Dry paper towels and Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA)

Volumetric flask (50 mL, any vender)

Beakers (50 mL, 400 mL, 600 mL, any vender)

96-well microplate (Sarstedt Inc, #82.1581, Newtown, NC)

Microplate sealing membrane (ICN, #7640205, Costa Mesa, CA)

(MP Biomedicals Linbro 76-402-05, 100/pack, best choice)

Pipetter solution basin (VWR, # 21007-970, Suwanee, GA)

7. Calibration and Calibration Verification Procedures

We use 5MeTHF from Eprova as calibrator. A polynomial regression (third degree) calibration curve for each run is generated using 11 different concentrations of 5MeTHF (0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 0.60, 0.80, 1.0 nmol/L).

As part of our assay validation, we investigated the response of the *L. casei* microorganism to different calibrators. We observed some differences, with reduced forms (5MeTHF and 5FoTHF) giving a slightly higher response than folic acid (see Fig. 1 in **Addendum 1**). Since most of the folate in serum and whole blood is in the 5MeTHF form, we calibrate with 5MeTHF, not with folic acid, which is typically used by other labs, although some are using 5FoTHF as a calibrator. Calibration with 5MeTHF results in ~10-20% lower values than calibration with folic acid.

Results of in-house recovery studies using both various forms of folate showed almost complete recovery (100±20%) for 5MeTHF, folic acid, 5FoTHF, and 5, 10-methenyltetrahydrofolic acid. Recovery of THF was only ~50%.

We have performed in-house comparisons of the microbiologic assay, the LC/MS/MS assay, and the BioRad assay. For serum samples, there is excellent correlation between the three assays. The microbiologic assay gives slightly lower values than the LC/MS/MS (~10%), but significantly higher values than the BioRad (~40%). For whole blood samples, there is excellent correlation and agreement between the microbiologic assay and the LC/MS/MS as long as folate polyglutamates have been properly deconjugated to monoglutamates.

The National Institute of Standards and Technology (NIST) have released in 2005 a new standard reference material for homocysteine and folate in human serum, SRM 1955. This material was characterized by various methods used at NIST and the CDC. Similar good agreement for total folate was found for the three levels of SRM 1955 between the CDC LC/MS/MS and microbiologic assay (level 1: 6.0 vs. 5.6; level 2: 13 vs. 14; level 3: 41 vs. 44). Total folate concentrations obtained

by BioRad were approximately 50% lower than those by the other two methods (level 1: 4.5; level 2: 10; level 3: 25). Our lab routinely uses this SRM material at least twice a year during our calibration verification and any time we experience assay problems.

In 2006, the National Institute for Biological Standards and Control (NIBSC) issued the first WHO certified reference material (lyophilized) for folate in human serum (03/178, established 2004). The folate concentration in this material has been certified by LC/MS/MS. The NIBSC also provides a WHO certified reference material (lyophilized) for whole blood folate (95/528, established 1996). The folate concentration in this material has been determined by consensus value assignment, but is mainly representative for the microbiologic assay. Both materials are used at least twice a year during our calibration verification and any time we experience assay problems.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

a. Preliminaries

1) Before each assay, document the specimen IDs and number the specimens in an Excel file, such as sample 1, 2, 3… These numbers will be used for labeling glass tubes for dilution, plates and layouts in the GEN5 software.
2) Thaw frozen serum or whole blood lysate samples, serum or whole blood lysate quality control samples, 5MeTHF stock solution III (1 umol/L) and let them reach ambient temperature.
3) Label 75x12-mm glass tubes for unknown samples and QCs.
4) Vortex thoroughly stock standard, QCs and unknown specimens before pipetting.

b. Dilution of samples and standard

1) To dilute serum QCs and unknown samples at 1:100 add 1980 uL of 0.5 g/dL sodium ascorbate into labeled 75x12-mm glass tube using a Digiflex, and then Packard Multiprobe adds 20 uL of serum QC or unknown samples into each tube. After diluting is completed, cover the tubes with plastic wrap and vortex all the tubes together thoroughly to mix.

2) To dilute lysed whole blood QC and unknown samples (whole blood diluted 1:11) at 1:140 add 2085 uL of 0.5 g/dL sodium ascorbate into each 75x12-mm labeled glass tube using a Digiflex, and then Packard Multiprobe adds 15 uL of whole blood lysate into each tube. After diluting is completed, cover the tubes with plastic wrap and vortex all the tubes together thoroughly to mix.

   Note: *Serum samples are typically diluted at 1/100. If the concentration is ~≤10 nmol/L, the sample needs to be repeated with a dilution of 1/50 (to ensure 4 valid results from the 50 and 100 uL addition of diluted sample); if the concentration is >100 nmol/L, the sample needs to be repeated at a dilution higher than 1/100. Whole blood lysates are typically diluted at 1/140. If the whole blood concentration is ~≤154 nmol/L, the sample needs to be repeated with a dilution of 1/70; if the concentration is >1540 nmol/L, the sample needs to be repeated with a dilution higher than 1/140.*

3) To prepare the standard working solution, dilute 5MeTHF stock solution III (1 umol/L) at 1/5 by adding 100 uL of stock III to 400 uL of 0.5 g/dL sodium ascorbate. Make a further 1/200 dilution (250 uL of 1/5 diluted 5MeTHF stock solution III made up to 50 mL in a volumetric flask using 0.5 g/dL sodium ascorbate). The final concentration of working solution is 1 nmol/L.

c. Addition of standard, QCs, and unknown samples into 96-well microplates

We conduct this assay by using 4 replicates for each QC and unknown sample at two different dilutions and using 8 replicates for each standard concentration. Fully automatic pipetting is performed by the Packard Multiprobe II Liquid Handling System operated by WinPrep software. The total pipetting time is ~1.5 hours for each run (5 plates). The total time for each assay is ~5 hours.

1) Plate #1 - 5MeTHF calibration plate

   a) Add 200 uL of folic acid casei medium inoculated with *L.casei* into each well of 96-well microplate.

   b) Add a different amount of 0.5 g/dL sodium ascorbate into each column of the 96-well plate.

   c) Add a different amount of 5MeTHF working standard solution into each well. The calibration curve is made up of 11 different concentration points. The total standard volume is 100 uL in each well.

   d) Add 5 uL of 3 g/dL sodium azide to the first column for the M590 correction (used to generate the calibration curve for whole blood, but ignored when the calibration curve for serum is generated). This step is currently not used for NHANES whole blood samples because of high folate concentrations and resulting higher dilution; if whole blood samples are diluted 1:40, the color correction should be performed.

   e) Seal the calibration plate very tightly with microplate sealing membrane and invert the plate to mix. Incubate the plate with other sample plates together at 37^{o}C for 42 - 45 hours.

   Calibration Plate - Add 200 uL *L.casei* medium

   Calibration Plate - Add 0.5 g/dL sodium ascorbate

2) Plate #2 to #6: Serum QCs and serum unknown samples

   a) Add 200 uL of folic acid casei medium inoculated with *L.casei* into each well.

   b) Add 50 uL of 0.5 g/dL sodium ascorbate into the wells that will have 50 uL diluted sample; don't add sodium ascorbate into the wells that will have 100 uL diluted.

   c) Add 50 and 100 uL of the 1/100 diluted serum QCs and unknown samples to the corresponding wells. Total sample volume is 100 uL in each well. Total 24 serum samples can be analyzed per plate including QCs and 1 blank. The last sample in each plate is always a blank.

   d) Seal the plates very tightly with microplate sealing membrane and invert several times to mix.

   e) The layouts of Plate #3-#6 are the same as for plate #2; each plate must contain at least one level of QC and a blank. Incubate the plates at 37^{o}C for 42 - 45 hours.

   Serum Plate – Add 200 uL of *L.casei* medium

   Serum Plate – Add 0.5 g/dL sodium ascorbate

   Serum Plate – Add diluted serum QC and unknown samples

3) Plate #2 to #6: Whole blood QCs and whole blood unknown samples

Because NHANES samples have relatively high folate concentration, we use higher dilution factor of 1/140 for whole blood lysate samples (original protocol uses 1/40). We have found that the hemoglobin color has no effect on OD 590 after incubation, even at 1/70 dilution, therefore we decided not to use color control and the whole blood samples have exactly the same plate layout as serum samples.

For other studies where the whole blood samples have lower folate concentrations, a lower dilution has to be used (i.e., 1/40) and the color control with sodium azide is needed. The plate layouts are shown below.

a) Add 200 uL of folic acid casei medium inoculated with *L.casei* into each well.

b) Add either 50 (for QCs and unknowns) or 100 uL (for blank) of 0.5 g/dL sodium ascorbate into wells using an 8-channel pipette.

c) Add 50 uL and 100 uL of the 1/140 diluted lysate QCs and unknown samples to the corresponding wells.

d) Add 5 uL of 3 g/dL sodium azide to the third column of each sample (column 3, 6, 9, 12) to generate a color control (there won't be any growth in these wells).

e) Seal the plates tightly with microplate sealing membrane and invert several times to mix. Incubate the plates at 37^{o}C for 42-45 hours. We have found that the plates don't have to be read immediately after incubation. It is ok to place them into a refrigerator and read them later during that day or the next day.

Whole blood sample plate – Add 200 uL *L.casei* medium

Whole blood sample plate – Add 0.5% sodium ascorbate

Whole blood sample plate – Add diluted whole blood lysates

d. Instrument & Software Setup for PowerWave Microplate Reader

Folate MA protocols have been created and saved as templates. After finishing pipetting and putting the plates in incubator, the experiment files needs to be generated: select an appropriate protocol based on the number of plates, enter sample IDs and dilution factors, and save the experiment by date in Experiment file.

Parameters in GEN5

GEN5 Parameter	Setting
Reading Type	End Point
Wave Length	590 nm
Shaking Intensity	0
Shaking Duration	0
Temperature control	No
Data Interpolate	M590 or M590 Corr (if needed)
X Axis	Lin
Y Axis	Lin
Curve Fit	Polynomial Regression, Degree 3

For different plates, the layouts vary based on different samples analyzed. The calibration curve presents the following concentrations: 0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.5, 0.60, 0.8, 1.0 nmol/L. Specific dilution factors have to be used for QCs and unknown samples.

e. Collection of data and calculation of results

Turn on PowerWave microplate reader before use and let it warm up for 30 min.

Take all the plates out of the incubator after incubating at 37^{o}C for 42-45 hours, and let them cool down to room temperature while mixing them thoroughly by inverting for at least 1 min or using a rotator. If plates have been kept in the refrigerator before reading, they will have to reach room

temperature first. Each plate should be inverted again just before taking off the sealing membrane and reading the OD.

1) Collect calibration data and construct calibration curve

Take off the sealing membrane from Plate 1 and let it sit on the bench for ~1-2 min to wait for all air bubbles are gone. The same procedure is used for every plate before collecting data. Do not take off the sealing membranes from more than 1 plate at one time. There might be some uneven settlement of organisms if leaving the plates too long on the bench before reading the OD.

In GEN5 software, open the experiment, go to plate 1 screen (calibration plate), and press "Read". The reading takes ~ 1 minute. The raw OD results are automatically collected and also the calibration curve is automatically generated; save the data by clicking "Save".

After reading is complete, the analyst makes notes of the wells that have obvious leaking, dark color, less volumes, or where the OD is greatly different from the average. Analyst checks OD readings in each concentration point and logs the ODs in the calibrator OD tracking file. The calibration curve presents the following concentrations: 0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.5, 0.6, and 0.8 and1.0 nmol/L.

2) Collect sample data and calculate the results

Put each sample plate into the microplate reader after checking for bubbles. Open the corresponding plate screen, press"Read", and save the raw OD results.

After reading is complete, the analyst again examines the results in detail as described above (including blank contamination, leaking wells, dark color wells, volumes in wells).

Four replicates are run for each patient sample. If the CV of the 4 replicates is ≥15%, evaluate whether deleting one replicate that appears to be an outlier (i.e., 6, 8, 8, 9 – 6 appears to be an outlier) reduces the CV to below 10%; if it does, use the mean of the remaining 3 replicates for reporting with comment code 114; if the CV of the remaining 3 replicates is ≥10%, leave all 4 replicates in and repeat the sample. If there is no clear outlier (i.e., 6, 8, 8, 10), leave all 4 replicates in and repeat the sample. Do not report results from <3 replicates (n<3). Repeat the sample.

The calibrator range used is 0.05-1 nmol/L (raw concentration). If the raw concentration of a serum or WBL sample is <0.05 nmol/L (lowest calibrator), the GEN5 software suppresses the calculated result; the sample is repeated with less dilution. If the raw concentration is >1 nmol/L, the same applies and the sample is repeated with higher dilution.

Calculation of RBC Folate Concentration:
Whole blood lysate folate results are multiplied by 11, the dilution factor of the whole blood. The serum folate values (multiplied by 1.0 minus the hematocrit [Hct] expressed as a decimal) are subtracted, and the resulting value is divided by the Hct to yield RBC folate in nmol/L RBC. If an Hct value is not available for a patient sample or QC sample, an Hct of 40% is assumed for calculation. If a serum folate value is not available, a value of 18 nmol/L is assumed for calculation. We recommend use of the correction for serum folate level and Hct because it provides the most accurate reflection of folate body stores.

$$\text{RBC folate, nmol/L} = \frac{(\text{Whole blood lysate folate} * 11) - \text{Serum folate}\,(1 - \text{Hct}/100)}{\text{Hct}/100}$$

f. Special Procedure Notes – CDC Modification

This method is based on the method described by O'Broin et al. and Molloy et al.

9. Reportable Range of Results

The reportable range and the LOD of this assay are dependent on the dilution factor for unknown samples. For a population with good folate status, the typical dilution of serum samples is 1/100, resulting in a reportable range of 10-100 nmol/L:

0.05 nmol/L [lowest calibrator] * 100 [dilution factor] * 2 [to ensure 4 valid results from the 50 and 100 uL addition of diluted sample] = 10 nmol/L

1 nmol/L [highest calibrator] * 100 [dilution factor] = 100 nmol/L.

Serum samples with a concentration less than 10 nmol/L or greater than 100 nmol/L are repeated with lower or higher dilution, respectively.

The lowest possible dilution of serum samples is 1/20 resulting in an LOD of 2 nmol/L:

0.05 nmol/L * 20 * 2 [to ensure 4 valid results from the 50 and 100 uL addition of diluted sample] = 2 nmol/L.

A serum folate result of <7 nmol/L (3 ng/mL) is considered to represent potential folate deficiency and needs to be repeated for confirmation.

The typical dilution of whole blood samples is 1/1540 (1/11 dilution of whole blood to lysate * 1/140 dilution of the lysate), resulting in a reportable range of 154-1540 nmol/L:

0.05 nmol/L [lowest calibrator] * 1540 [dilution factor] * 2 [to ensure 4 valid results from the 50 and 100 uL addition of diluted sample] = 154 nmol/L

1 nmol/L [highest calibrator] * 1540 [dilution factor] = 1540 nmol/L.

Whole blood samples with a concentration less than 154 nmol/L or greater than 1540 nmol/L are repeated with lower or higher dilution, respectively.

The lowest possible dilution of whole blood samples is 440 (1/11 dilution of whole blood to lysate * 1/40 dilution of the lysate) resulting in an LOD of 44 nmol/L. Assuming a hematocrit of 40%, this would correspond to a RBC folate concentration of 105 nmol/L RBC:

0.05 nmol/L * 440 * 2 [to ensure 4 valid results from the 50 and 100 uL addition of diluted sample] = 44 nmol/L.

A RBC folate result of <317 nmol/L RBC (140 ng/mL RBC; 127 nmol/L whole blood assuming a hematocrit of 40%) is considered to represent potential folate deficiency and has to be repeated for confirmation.

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

Bench QC specimens are prepared from three serum and three whole blood pools, which represent low, medium and high folate levels in serum and whole blood (~6, 22 and 45 nmol/L for serum folate and ~150, 350 and 670 nmol//L for whole blood folate). These pools are prepared in the same manner as patient samples and analyzed in 4 replicates as part of each run. Each sample plate should have at least one QC sample.

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 runs and then are re-evaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, QC results from each run are stored in the FrontEnds database. A hardcopy of the QC results from each run is also kept by the analyst. In special cases where a run can't be imported into the FrontEnds database, electronic files of the QC results are stored in the analyte-specific folder on \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Data handling\Excel runs\Folate MA data. Information on QC limits over time is stored in the FrontEnds database as well as in summary files at \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Data handling\NBB QC Review.

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

1) Contamination is the most common problem with the microbiologic assay. Make sure chloramphenicol (antibiotic) is added into assay medium to stop other organisms from growing. Make sure supplies are not contaminated with folate. Preventative actions to avoid contamination are very important. Change of gloves after preparation of working standard is recommended. Also, preparation of standard stock solutions and washing of folate contaminated glassware should take place in a different place than the preparation of the daily assay.
2) Check for preparation errors of assay medium and reagents.
3) Look for sample and standard dilution errors.
4) Pay attention to pipetting steps.
5) Check the calibrations of the pipettes.
6) If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions.

12. Limitations of Method; Interfering Substances and Conditions

1) This folate microbiologic assay measures total folate in serum or whole blood. It does not provide values for different forms of folate.
2) If samples contain antibiotics, the growth of the microorganism might be inhibited. This is typically seen by a non-linear relationship for samples with increased dilution.
3) There is still debate whether some non-folate compounds might stimulate the growth of the microorganism.
4) The most common causes of imprecision are intermittently inaccurate micropipettors or pipetting errors. Stock standards and specimens should be mixed thoroughly by vortexing before pipetting.
5) Assay medium and sodium ascorbate should be made fresh daily.
6) Hemolyzed serum samples may give falsely elevated values and results should be evaluated with caution.
7) Multiple freeze/thaws cycles for extended hours at room temperature will cause some degradation of folates.
8) The entire sample preparation and calibration should be conducted in yellow sub-dued light. Exposure to strong sunlight should be avoided since it may cause folate degradation.

This method has also undergone a series of in-house **ruggedness testing** experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. A total of five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at a value substantially lower and higher than that specified in this method while holding all other experimental variables constant. **The ruggedness testing findings for this method are presented in Addendum 2.** Please refer to Chapter 21 of the 2008 DLS Policies and Procedures Manual for further information on ruggedness testing.

13. Reference Ranges (Normal Values)

Previously generated reference ranges for the U.S. population cannot be directly applied to this assay since they were produced with the BioRad assay which measures significantly lower than the microbiologic assay. Clinical text books provide some information on reference ranges with the

microbiologic assay, however those data were produced before folic acid fortification was introduced in the U.S. Therefore we don't have post-fortification reference ranges for the U.S. population with the microbiologic assay. NHANES 2007-2008 will be used to generate these reference ranges.

14. Critical Call Results ("Panic Values")

Any samples with serum folate levels <7 nmol/L (<3 ng/mL), RBC folate levels <317 nmol/L RBC (<140 ng/mL), are considered to require follow-up because of potential folate deficiency. For smaller, non-NHANES studies, abnormal values are identified to the study principal investigator. Most of these studies are epidemiological in nature.

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

While the LC/MS/MS method also determines total folate by calculating the sum of the individually measured forms, we do not use this expensive test as an alternate method for the microbiologic 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 EMAIL by the supervisor of any serum folate result that is <7 nmol/L (<3 ng/mL), RBC folate that is <317 nmol/L (<140 ng/mL). 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.

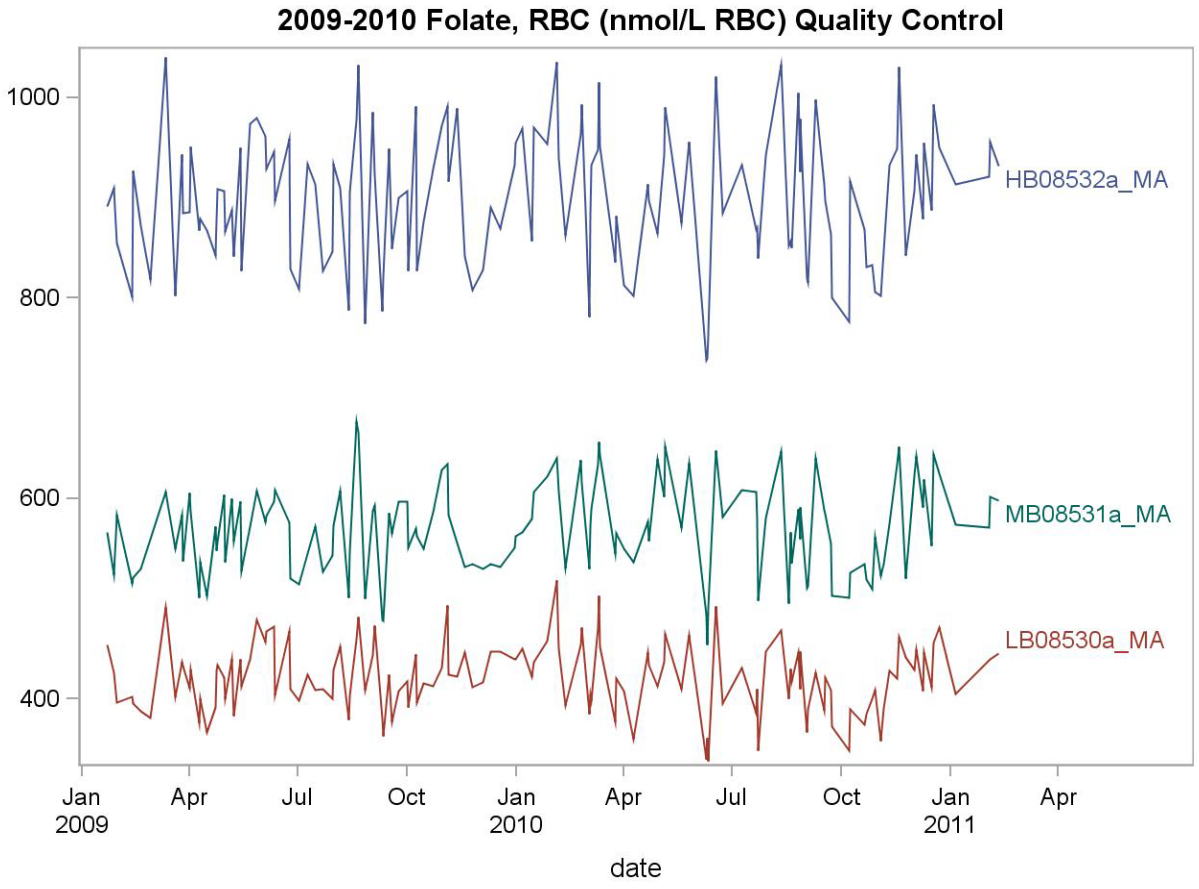
18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

The Microsoft Access database is used to keep records and track specimens for NHANES 1999+ and other studies. Records that can't be imported into the database (some R&D or troubleshooting experiments, PT results) are kept electronically at \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Data handling\Excel runs 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.

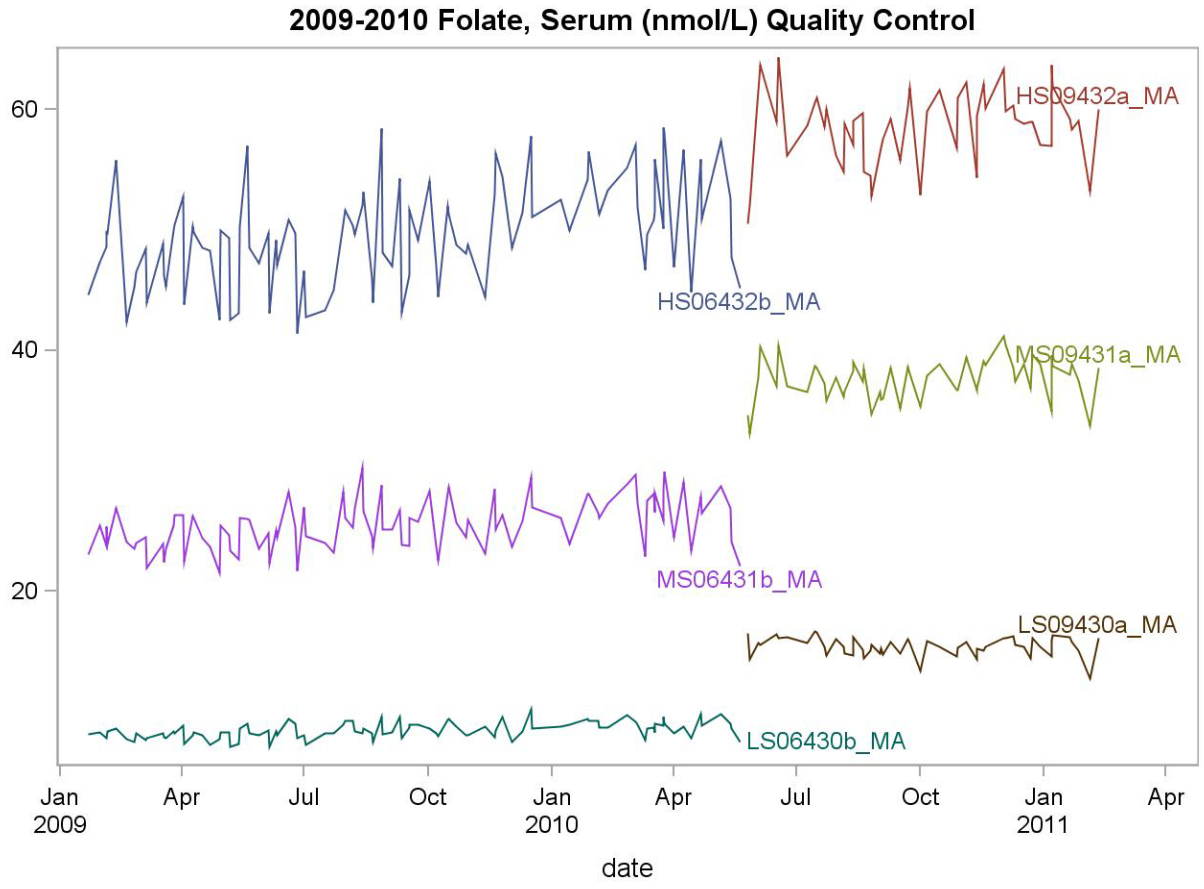
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the 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

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HB08532a_MA	142	22JAN09	10FEB11	897.8	67.8	7.5
LB08530a_MA	142	22JAN09	10FEB11	421.4	34.7	8.2
MB08531a_MA	140	22JAN09	10FEB11	570.1	45.8	8.0



Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS06432b_MA	101	22JAN09	20MAY10	49.67	4.23	8.5
LS06430b_MA	100	22JAN09	20MAY10	8.43	0.67	7.9
MS06431b_MA	101	22JAN09	20MAY10	25.56	2.08	8.1
HS09432a_MA	54	26MAY10	10FEB11	58.57	3.11	5.3
LS09430a_MA	54	26MAY10	10FEB11	15.41	0.78	5.1
MS09431a_MA	54	26MAY10	10FEB11	37.56	1.75	4.7



References

O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. J Clin Pathol 1992; 45:344-7.

AM Molloy, JM Scott? Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol 1997; 281:43-53.

Z Fazili, CM Pfeiffer, M Zhang, R Jain. Erythrocyte folate extraction and quantitative determination by LC/MS/MS: Comparison of results with microbiologic assay. Clin Chem 2005; 51(12):2318-25.

Mastropaolo W, Wilson MA. Effect of light on serum B12 and folate stability. Clin Chem 1993; 39(5):913.

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Appendices

Addendum 1:

Ruggedness Testing - Folate Microbiological Assay

1. **Turbidity reading of mixed microplates:**
   - **a. Principle:** The turbidity reading of mixed microplates has to occur as long as the microorganism is in suspension and the sample is homogenous. Standard protocol: Incubate plates at 37°C for ~45 h, mixed them with a shaker for ~15 min, then by hand inversion ~20 times; open the sealing membrane, get rid of bubbles, and then read the plates at ~1 min, as long as the microorganism is still in suspension.
   - **b. Proposal:** To vary the time window within which mixed plates are read.
   - **c. Findings:**
   - **d.** Average folate concentration of 3 QC levels, each in duplicate.

     Conclusion: Varying the reading time between 0.5 min and 4 min after plate mixing does not appear to affect serum or WB folate concentrations.

2. **Delayed turbidity reading of microplates:**
   - **a. Principle:** Microplates are usually read directly after the ~45 h incubation. In special situations, the plate reading has to be delayed and plates are stored at RT or 4°C prior to reading.
   - **b. Proposal:** To assess effects of delayed reading of plates. Plates were first read as specified in the method and then re-read after some delay.
   - **c. Findings:**

     Conclusion: Delaying the plate reading does not appear to affect serum folate concentrations, as long as the plate is stored overnight at room temperature or up to 4 days at 4°C.

3. **Sodium ascorbate concentration:**
   - **a. Principle:** Sodium ascorbate (SA) is an antioxidant. It is used at 0.5% to dilute samples and calibrators and to make up the final volume in microplates.
   - **b. Proposal:** To assess the potential protective effects of higher SA concentration.
   - **c. Findings:**

     Conclusion: Increasing the sodium ascorbate concentration from 0.5% to up to 1% does not appear to provide a protective effect to serum and WB folate concentrations, or to the 5-methyltetrahydrofolate standard in the calibration curve.

4. **Stability of diluted calibrator:**

   a. **Principle:** The 5-methyltetrahydrofolate calibrator has to be diluted before it can be pipetted into microplates. This compound is known to be sensitive to decomposition. The pipetting is therefore usually started within 30 min of calibrator preparation.

   b. **Proposal:** To vary the length of time at which the diluted calibrator is kept before starting to pipette into microplates.

   c. **Findings:** As long as pipetting is started within 30 min of preparing the diluted calibrator, calibration curves are very reproducible (see graph below). However, when the pipetting is delayed to 2 h and 4 h, the calibration curves show quite a bit of variation and are hard to interpret.

   Conclusion: The pipetting of the diluted calibrator should be started within 30 min of preparation to generate stable and reproducible calibration curves.

5. **Incubation time for microorganism growth:**

   a. **Principle:** The microorganism requires ~42-45 h of incubation at 37°C for reproducible growth.

   b. **Proposal:** To vary the incubation time and evaluate whether shorter incubation (only 1 day) or longer incubation (a few extra hours beyond the regular time) is possible.

   c. **Findings:**

   Conclusion: Incubating plates for a few extra hours beyond the regular time appears to be generally ok, but should be avoided if possible. Plates should not be incubated for only 24 h due to inconsistent results and high variability from sample to sample (as reflected in the relatively large SD).