Laboratory Procedure Manual

Analyte: **Total Folate**

Matrix: **Whole Blood**

Method: **Microbiologic Assay**

Method No: 4000.05

Revised:

as performed by:

Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences
National Center for Environmental Health

contact:

James L. Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

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 for data file **FOLATE_H**.

Variable name	SAS Label
LBDRFO	RBC Folate (ng/mL)
LBDRFOSI	RBC Folate (nmol/L)

1. Summary of Test Principle and Clinical Relevance

a. Clinical relevance

Folate belongs to the group of water-soluble B vitamins that occur naturally in food. It is required in cellular one carbon metabolism and hematopoiesis. Prolonged folate deficiency leads to megaloblastic anemia. Low folate status has been shown to increase the risk of women of childbearing age to have an offspring with neural tube defects. Low folate status also increases plasma homocysteine levels, a potential risk factor for cardiovascular disease, in the general population. Potential roles of folate and other B vitamins in modulating the risk for diseases (e.g., heart disease, cancer, and cognitive impairment) are currently being studied.

The measurement of total folate (TFOL) provides information on the folate status of the individual. Serum folate is an indicator of short-term status, while red blood cell (RBC) folate is an indicator of long-term status.

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. **[1]** and Molloy et al. **[2]** 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 **[1]** and is used to quantitatively measure serum and RBC folate in human specimens. The method is relatively easy to perform, reliable, and considerably less costly than chromatographic or commercial kit assays.

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 microplate reader. The assay is calibrated with 5-methyltetrahydrofolic acid (5MeTHF). If seven plates are processed in a run, 132 patient specimens can be analyzed.

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. During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

b. The raw data file from the microplate reader is collected using the instrument software and stored on the CDC network. The results file (including analyte , sample IDs, replicate count, replicate variation, sample dilution factor, instrument results, final results, data file name, acquisition time, etc) is imported into a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See "**4000_SOP Computerization and Data System Management**" for a step-by-step description of data transfer, review, and approval.

c. For NHANES, data is transmitted electronically on a regular basis (approximately weekly for 3-week turnaround analytes). 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. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

d. The experiment files (raw data from plate reader) and the result files (Power Export files) are stored on the DLS network and are automatically backed up nightly by ITSO support staff.

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 µL) with 10 parts of 1 g/dL (1%) ascorbic acid solution (1 mL), corresponding to a 1/11 dilution, and freezing the hemolysate promptly, which keeps the folate in the reduced state.

c. A minimum of 400 µL of serum and 500 µL of whole blood hemolysate is needed to do a proper dilution when using automated pipetting. Serum is typically diluted 1/100 (20 µL of serum is added to 1980 µL of 0.5 g/dL sodium ascorbate). Whole blood hemolysate is typically diluted at 1/140 (15 µL of hemolysate is added to 2085 µL 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 hemolysate 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 hemolysis 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 **[4]**. 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 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 Cie, AG, IM Laternenacker 5, Schaffhausen 8200 [CH] Switzerland)
- 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) Sodium ascorbate solution (0.5 g/dL) for diluting samples and standard

   Two and half grams of sodium ascorbate is dissolved in 500 mL of deionized water. This solution should be freshly prepared before each assay.

2) *L. casei* assay medium inoculated with microorganism (folate free)

   To prepare 200 mL of assay medium: add 14.1 g of Folic Acid Casei Medium, 6 mg of chloramphenicol (or 2 mL of a 3 mg/mL chloramphenicol stock solution) and 60 µL of Tween-80 to 200 mL of ultrapure water, heat to boil for 2-3 min with stirring. Cool down to ~37°C, then add 30 mg of manganese sulfate (or 1 mL of a 30 mg/mL manganese sulfate stock solution) and 150 mg of ascorbic acid (or 1 mL of a 150 mg/mL ascorbic acid stock solution), 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 600-700 µL into 200 mL of assay medium, keep stirring slowly. About 25 mL of assay medium is needed per microplate.

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

   Dissolve 15 g of ascorbic acid in 100 mL of deionized water. Aliquot 1 mL/vial and store at -70°C. Add 1 mL into 200 mL of Folic Acid Casei medium. Make fresh stock solution every 6 months.

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

   Dissolve 3.6 g of manganese sulfate in 120 mL of deionized water, stir thoroughly (~20 min). Aliquot 1 mL/vial and store at -70°C. Add 1 mL into 200 mL of Folic Acid Casei medium. Make fresh stock solution every 6 months.

5) Chloramphenicol stock solution (3 mg/mL)

   Dissolve 600 mg of chloramphenicol in 4 mL of ethanol and then make up to 200 mL with deionized water, aliquot 1 mL into cryovials and keep at -70°C freezer. Add 2 mL of stock solution into 200 mL of Folic Acid Casei medium. Make fresh stock solution every 6 months.

6) *L. casei* growth medium (with folate to create new inoculum)

   To prepare 200 mL of growth medium: Add 9.4 g of Folic Acid Casei Medium, 100 mg of ascorbic acid, 40 mg of chloramphenicol, 300 µL of folic acid stock solution (100 ng/mL), 40 µL of Tween-80 to 200 mL of deionized water. Heat to boil for 2-3 min. Cool down to ~37°C and sterilize the medium by either autoclaving or filtering through a 0.2 µm filter. Aliquot 20 mL into sterile 50-mL tubes and keep at -20°C.

7) Ascorbic acid (1 g/dL) for hemolysis of whole blood samples

   Dissolve 1 g of ascorbic acid in 100 mL of deionized water. Prepare fresh solution before each use.

8) Blocking solution for color control

   Sodium azide is used as a blocking solution. Five µL 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 µL /control well).

c. Standards Preparation

The concentrations of the folate stock solution is calculated using molar absorptivity. Information on the absorption maximum, absorption coefficient, and formula to calculate the concentration for the stock solution is provided in **Appendix 1.**

1) 5MeTHF stock solution I (~200 µg/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: 5-methylTHF 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 µg/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°C in 2-mL labeled cryovials.

3) 5MeTHF stock solution III (1 µmol/L): Dilute 459 µL of stock II (100 ug/mL) with 0.5% degassed ascorbic acid in a 100-mL volumetric flask. Aliquot 500 µL into labeled cryovial and freeze the aliquots at -70°C. Prepare a fresh stock solution III every 6 months.

d. Preparation of Quality Control Materials

Three levels of serum or whole blood hemolysate (whole blood diluted 1/11 with 1% ascorbic acid solution) quality control pools are used as bench QCs in every run to detect any problems with the microbiologic assay. 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.

Pool specimens 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 µL 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 µL, 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 cyrovials (1 mL/vial) and store at -70°C.

f. Equipment

- Precision Incubator 6842: 37°C (Fisher, Pittsburgh, PA)
- PowerWave microplate reader and GEN5 software (Bio-Tek Instruments, Inc, Winooski, VT, USA)
- JANUS™ Automated Workstation (PerkinElmer, MA, USA)
- Vortex – Genie 2 (VWR, Suwanee, GA)
- Orbitron Rotator I (VWR # 56261-508, Suwanee, GA)
- Eppendorf adjustable pipettes (1-200 µL and 100-1000 µL); Eppendorf Repeater Plus pipette; 8-channel pipetter (Rainin)
- Stirring Hotplate (Fisher # 11-100-16SH, 11-100-49SH, Suwanee, GA and Thermolyne SP46925)
- Digiflex CX (Titertek #0603310, Huntsville, AL)

g. Other Materials

- Ultrapure water (18.2 M-Ohm from an AquaSolutions 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 µL 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 vendor)
- Beakers (50 mL, 400 mL, 600 mL, any vendor)
- 96-well microplate (Nunc, 12-565-361, Waltham, MA)
- Microplate sealing membrane (MP Biomedicals Linbro, #7640205, 100/pack) or aluminum foil sealing membrane (CIC NC9327419, Vienna, VA)
- Pipetter solution basin (VWR, # 21007-970, Suwanee, GA)

7. Calibration and Calibration Verification Procedures

This assay uses an aqueous calibration curve with 5MeTHF (Merck Cie [Eprova]) as calibrator. Calibrators are carried through the entire sample processing procedure. Calculation of folate concentrations in QCs and unknown patient samples is based on results obtained from a daily 11-point calibration curve (0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 0.60, 0.80, 1.0 nmol/L) using a polynomial regression (third degree) since the growth response of the microorganism is not linear.

Calibration verification is conducted at least twice a year using international reference materials. For details, see **4000_SOP Calibration and Calibration Verification FOL MA**.

The National Institute of Standards and Technology (NIST) has 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. 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). We routinely use this SRM material at least twice a year during 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 calibration verification and any time we experience assay problems.

We participate in the UK NEQAS Haematinics survey external proficiency testing program twice a year. Details can be found in the proficiency testing form.

Method figures of merit are presented in **Appendix 2**.

Results from a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied are presented in **Appendix 3**.

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 **[5]**. 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, however overall the microbiologic assay gives results that are 10-25% higher than LC-MS/MS results **[6]**.

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 hemolysate samples, serum or whole blood hemolysate quality control samples, 5MeTHF stock solution III (1 µmol/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 1485 µL of 0.5 g/dL sodium ascorbate into labeled 75x12-mm glass tube using a Digiflex, and then the JANUS Automated Workstation adds 15 µL 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 hemolysed whole blood QC and unknown samples (whole blood diluted 1:11) at 1:140 add 2085 µL of 0.5 g/dL sodium ascorbate into each 75x12-mm labeled glass tube using a Digiflex, and then Packard Multiprobe adds 15 µL of whole blood

hemolysate into each tube. After diluting is completed, cover the tubes with plastic wrap and vortex all the tubes together thoroughly to mix.

3) To prepare the standard working solution, dilute 5MeTHF stock solution III (1 µmol/L) at 1:5 by adding 100 µL of stock III to 400 µL of 0.5 g/dL sodium ascorbate. Make a further 1/200 dilution (250 µL 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 nmo/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 2 different dilutions (2 replicates/dilution) and 8 replicates for each standard concentration. Fully automatic pipetting is performed by the JANUS Automated Workstation operated by the WinPrep software. The total pipetting time is ~1.5 hours for each run (5 plates). The total time for each assay is ~5 hours. A total of 22 unknown patient samples can be analyzed per sample plate (if 7 plates are run in an assay).

1) Plate #1 - 5MeTHF calibration plate

   a) Add 200 µL of folic acid casei medium inoculated with *L.casei* into each well of 96-well microplate (see calibration plate - Table 1).

   b) Add a different amount of 0.5 g/dL sodium ascorbate into each column of the 96-well plate (see calibration plate -Table 2).

   c) Add a different amount of 5MeTHF working standard solution into each well (see calibration plate -Table 3). The calibration curve is made up of 11 different concentration points. The total standard volume is 100 µL in each well.

   d) Add 5 µL 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°C for 42-45 hours.

Calibration Plate -Table 1: Add 200 µL *L.casei* assay medium inoculated with microorganism

#1	1	2	3	4	5	6	7	8	9	10	11	12
A	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
B	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
C	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
D	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
E	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
F	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
G	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
H	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL

Calibration Plate - Table 2: Add 0.5 g/dL sodium ascorbate

#1	1	2	3	4	5	6	7	8	9	10	11	12
A	100ul	100ul	95ul	90ul	85ul	80ul	70ul	60ul	50ul	40ul	20ul	0ul
B	100ul	100ul	95ul	90ul	8ul	80ul	70ul	60ul	50ul	40ul	20ul	0ul

C	100ul	100ul	05ul	90ul	85ul	80ul	70ul	60ul	50ul	40ul	20ul	0ul
D	100ul	100ul	95ul	90ul	85ul	80ul	70ul	60ul	50ul	40ul	20ul	0ul
E	100ul	100ul	95ul	90ul	85ul	80ul	70ul	60ul	50ul	40ul	20ul	0ul
F	100ul	100ul	95ul	90ul	85u	80ul	70ul	60ul	50ul	40ul	20ul	0ul
G	100ul	100ul	95ul	90ul	85ul	80ul	70ul	60ul	50ul	40ul	20ul	0ul
H	100ul	100ul	95ul	90ul	85ul	80ul	70ul	60ul	50ul	40ul	20ul	0ul

Calibration Plate - Table 3: Add 5MeTHF working solution (1 nmol/L)

#1	1	2	3	4	5	6	7	8	9	10	11	12
A	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul
B	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul
C	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul
D	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul
E	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul
F	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul
G	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul
H	0ul	0ul	5ul	10ul	15ul	20ul	30ul	40ul	50ul	60ul	80ul	100ul

2) <u>Plate #2 to #7: Serum QCs and serum unknown samples</u>

a) Add 200 µL of folic acid casei medium inoculated with *L.casei* into each well (see Serum Plate - Table 4)

b) Add 50 µL of 0.5 g/dL sodium ascorbate into the wells that will have 50 µL diluted sample; don't add sodium ascorbate into the wells that will have 100 µL diluted sample (see Serum Plate -Table 5).

c) Add 50 and 100 µL of the 1/100 diluted serum QCs and unknown samples to the corresponding wells (see Serum Plate -Table 6). Total sample volume is 100 µL in each well. A total of 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°C for 42-45 hours

Serum Plate – Table 4: Add 200 µL *L.casei* assay medium inoculated with microorganism

#2	1	2	3	4	5	6	7	8	9	10	11	12
	QC low 1		**Sample 2**		**Sample 6**		**Sample 10**		**Sample 14**		**Sample 18**	
A	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
B	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	QC med 1		**Sample 3**		**Sample 7**		**Sample 11**		**Sample 15**		**Sample 19**	
C	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
D	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	QC high 1		**Sample 4**		**Sample 8**		**Sample 12**		**Sample 16**		**Sample 20**	
E	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
F	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	Sample 1		**Sample 5**		**Sample 9**		**Sample 13**		**Sample 17**		**Sample 21-blank**	
G	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
H	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL

Serum Plate – Table 5: Add 0.5 g/dL sodium ascorbate

#2	1	2	3	4	5	6	7	8	9	10	11	12
	QC low 1		**Sample 2**		**Sample 6**		**Sample 10**		**Sample 14**		**Sample 18**	
A	50uL		50uL		50uL		50uL		50uL		50uL	
B	50uL		50uL		50uL		50uL		50uL		50uL	
	QC med 1		**Sample 3**		**Sample 7**		**Sample 11**		**Sample 15**		**Sample 19**	
C	50uL		50uL		50uL		50uL		50uL		50uL	
D	50uL		50uL		50uL		50uL		50uL		50uL	
	QC high 1		**Sample 4**		**Sample 8**		**Sample 12**		**Sample 16**		**Sample 20**	
E	50uL		50uL		50uL		50uL		50uL		50uL	
F	50uL		50uL		50uL		50uL		50uL		50uL	
	Sample 1		**Sample 5**		**Sample 9**		**Sample 13**		**Sample 17**		**Sample 21-blank**	
G	50uL		50uL		50uL		50uL		50uL		50uL	
H	50uL		50uL		50uL		50uL		50uL		50uL	

Serum Plate – Table 6: Add diluted serum QC and unknown samples

	1	2	3	4	5	6	7	8	9	10	11	12
	QC low 1		**Sample 2**		**Sample 6**		**Sample 10**		**Sample 14**		**Sample 18**	
A	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
B	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
	QC med 1		**Sample 3**		**Sample 7**		**Sample 11**		**Sample 15**		**Sample 19**	
C	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
D	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
	QC high 1		**Sample 4**		**Sample 8**		**Sample 12**		**Sample 16**		**Sample 20**	
E	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
F	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
	Sample 1		**Sample 5**		**Sample 9**		**Sample 13**		**Sample 17**		**Sample 21-blank**	
G	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
H	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL

3) Plate #2 to #7: 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 hemolysate 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 µL of folic acid casei medium inoculated with *L.casei* into each well.

b) Add either 50 (for QCs and unknowns) or 100 µL (for blank) of 0.5 g/dL sodium ascorbate into wells using an 8-channel pipette (see Plate whole blood – Table 5).

c) Add 50 µL and 100 µL of the 1/140 diluted hemolysate QCs and unknown samples to the corresponding wells (see Plate whole blood-Table 6).

d) Add 5 µL 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°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 – Table 5: Add 200 µL *L.casei* assay medium inoculated with microorganism

	1	2	3	4	5	6	7	8	9	10	11	12
	WB QC low 1			**Sample 2**			**Sample 6**			**Sample 10**		
A	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
B	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	WB QC med 1			**Sample 3**			**Sample 7**			**Sample 11**		
C	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
D	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	WB QC high 1			**Sample 4**			**Sample 8**			**Sample 12**		
E	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
F	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	Sample 1			**Sample 5**			**Sample 9**			**Sample 13-blank**		
G	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
H	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL

Whole blood sample plate – Table 6: Add 0.5% sodium ascorbate

	1	2	3	4	5	6	7	8	9	10	11	12
	WB QC low 1			**Sample 2**			**Sample 6**			**Sample 10**		
A	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
B												
	WB QC med 1			**Sample 3**			**Sample 7**			**Sample 11**		
C	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
D												
	WB QC high 1			**Sample 4**			**Sample 8**			**Sample 12**		
E	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
F												
	Sample 1			**Sample 5**			**Sample 9**			**Sample 13-blank**		
G	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
H												

Whole blood sample plate – Table 6: Add diluted whole blood hemolysates

	1	2	3	4	5	6	7	8	9	10	11	12
	WB QC low 1			**Sample 2**			**Sample 6**			**Sample 10**		
A	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
B	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL
	WB QC med 1			**Sample 3**			**Sample 7**			**Sample 11**		
C	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
D	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL
	WB QC high 1			**Sample 4**			**Sample 8**			**Sample 12**		
E	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
F	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL
	Sample 1			**Sample 5**			**Sample 9**			**Sample 13-blank**		
G	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
H	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL

d. Instrument & Software Setup for PowerWave Microplate Reader

The Bio-Tek PowerWave microplate reader uses the GEN5 software. Templates of various folate MA protocols (for different number of plates) have been saved and can be used to efficiently create an experiment file (saved by date) by entering the sample IDs and dilution factors. This should be done as soon as the assay setup is finished and the plates are put into the incubator.

Table 7. 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 has 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 (see Table 8).

Table 8. Dilution factors for serum and whole blood hemolysate samples

Sample		Dilution factor (100 uL added into plate)	Sodium Ascorbate (uL)	Serum or whole blood hemolysate (uL)	Final volume (uL)	Comment code	Dilution factor in GEN5 software
Serum	Routine Dilution	100	1485	15	1500	none	100 w/ 100 uL 200 w/ 50 uL
	GEN5 raw concentration ~ < 0.05 nmol/L	50	735	15	750	115	50 w/ 100 uL 100 w/ 50 uL
		30	435	15	450	115	25 w/ 100 uL 50 w/ 50 uL
	GEN5 raw concentration > 1.00 nmol/L	200	1485	Predilute at 1/2, then add 15 uL	1500	97	200 w/ 100 uL 400 w/ 50 uL
		400	1485	Predilute at 1/4, then add 15 uL	1500	97	400 w/ 100 uL 800 w/ 50 uL
Whole blood hemolysate (1/11 diluted WB)	Routine Dilution	140	2085	15	2100	none	1540 w/ 100 uL 3080 w/ 50 uL
	GEN5 raw concentration ~ < 0.05 nmol/L	70	1035	15	1050	115	770 w/ 100 uL 1540 w/ 50 uL
		50	735	15	750	115	385 w/ 100 uL 770 w/ 50 uL
	GEN5 raw concentration > 1.00 nmol/L	280	2085	Predilute at 1/2, then add 15 uL	2100	97	3080 w/ 100 uL 6160 w/ 50 uL
		560	2085	Predilute at 1/4, then add 15 uL	2100	97	6160 w/ 100 uL 12320 w/ 50 uL

e. Collection of data and calculation of results

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

Remove all plates from the incubator after 42-45 hours, and let them cool to room temperature while mixing them thoroughly by inverting them for at least 1 min or using a rotator. Once the incubation time is completed, plates should be read promptly. However, if that is not possible, plates can be placed in the refrigerator for up to 2 days and reading can be delayed. Plates have to be brought up to room temperature and mixed thoroughly prior to reading. 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 to dissipate. The same procedure is used for every plate before collecting data. Do not take off the sealing membranes from more than 1 plate at a time. If plates sit for too long on the bench prior to reading the OD, there might be some uneven settlement of the organisms leading to inaccurate results.

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 wells that have obvious leaking, dark color, less volume, or where the OD is greatly different from the average. Analyst checks OD readings at each concentration point and logs the ODs in the calibrator OD tracking file. The calibration curve represents the following concentrations: 0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.5, 0.6, 0.8 and 1.0 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 examines the results in detail as described above (including blank contamination, leaking wells, dark color wells, volumes in wells). A review is also performed for number of replicates, CV of replicates, dilution factors, formulas, results >high cal, results <cutoff value, and unusual results to determine which samples require repeating and which samples require special comment codes (i.e., hemolyzed sample, no ascorbic acid solution added to whole blood, potential antibiotic interference). Appropriate notes are made.

Four replicates are run for each patient sample.

- If the CV of the 4 replicates is ≤15%, report the result
- 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 with code 26 and repeat the sample.
- If there is no clear outlier (ie, 6, 8, 8, 10), leave all 4 replicates in. Don't report the result and use code 26. Repeat the sample.
- Do not report results if <3 replicates and use code 26. 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.

<u>Calculation of RBC Folate Concentration:</u>
Whole blood hemolysate 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 a Hct value is not available for a patient sample or QC sample, a Hct of 40% can be assumed for calculation. If a serum folate value is not available, a value of 18 nmol/L can be 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.

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

f. Special Procedure Notes – CDC Modification

This method is based on the method described by O'Broin et al. **[1]** and Molloy et al. **[2]**.

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 µL addition of diluted sample] = 10 nmol/L

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

Serum samples with a raw concentration in assay less than 0.05 nmol/L or greater than 1 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 hemolysate * 1/140 dilution of the hemolysate), 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 raw concentration in assay less than 0.05 nmol/L or greater than 1 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 hemolysate * 1/40 dilution of the hemolysate) resulting in an LOD of 44 nmol/L. Assuming a hematocrit of 40%, this would correspond to a RBC folate concentration of 110 nmol/L RBC:

0.05 nmol/L * 440 * 2 [to ensure 4 valid results from the 50 and 100 µL 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 can be inserted into the mix of patient specimens. These QC specimens are generally prepared at two levels that would be encountered in 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 processed from three serum and/or whole blood hemolysate pools, which represent low, medium and high folate levels (~25, ~40, and ~60 nmol/L for serum folate and ~300, ~400, and ~600 nmol//L for whole blood folate), approximating the 25th, 50th, and 75th percentile in the U.S. population. The QC specimens are processed in the same manner as patient samples and analyzed in 4 replicates each at the front and back of each run. Each sample plate should contain at least one QC specimen.

The QC results are checked after each run using of a multi-rule quality control program **[7]**. 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 to generate Shewhart QC charts. For this assay, the mean of the 4 replicates represents one QC result. Thus, there are two QC results (front and back QC) that are evaluated.

(1) If all three QC run means are within 2 Sm limits and individual results are within 2 Si limits, accept the run ("in control")

(2) If one of the three QC run means is outside a 2 Sm limit, reject run if ("out of control"):
- (a) 1 3S Rule—Run mean is outside a 3 Sm limit or
- (b) 2 2S Rule—Two or more of the three run means are outside the same 2 Sm limit or
- (c) 10 Xbar Rule—Current and previous nine run means are on the same side of the characterization mean

(3) If one of the six QC individual results is outside a 2 Si limit, reject run if ("out of control"):

- (a) Outlier—One individual result is beyond the characterization mean ±4 Si or
- (b) R 4S Rule—Two or more of the within-run ranges in the same run exceed 4 Sw (i.e. 95 percent range limit)

Si = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).
Sm = Standard deviation of the run means (the limits are shown on the chart).
Sw = Within-run standard deviation (the limits are not shown on the chart).

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 are stored in a LIMS database. For runs that are not imported into the database (i.e., R&D, troubleshooting, research-type runs), QC results are stored electronically in the analyte-specific folder on the DLS network. A hardcopy of the QC results from each run is also maintained by the analyst.

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.

13. Reference Ranges (Normal Values)

Clinical reference ranges reported for serum folate are 11-36 nmol/L with the microbiologic assay and 7-36 with the chemiluminescence assay **[8]**. Clinical reference ranges reported for RBC folate are 317-1422 nmol/L with the CPB radioassay **[8]**.

The newest post-fortification reference ranges for the U.S. population generated with the microbiologic assay for NHANES 2005-2010 are shown below **[9]**. Pfeiffer et al. also reported microbiologic assay-equivalent reference ranges for pre-fortification (NHANES 1988-1994) and

early post-fortification (NHANES 1999-2004) periods, as well as reference ranges by population subgroups for all three time periods **[9]**.

Serum folate: 12.7-104 nmol/L (2.5^{th} -97.5^{th} percentile; *n* = 23,528; NHANES 2005-2010)

RBC folate: 505-2,490 nmol/L (2.5^{th} -97.5^{th} percentile; *n* = 23,528; NHANES 2005-2010)

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. 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. Emails sent concerning abnormal results are maintained by the supervisor for the duration of the study. 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 generally 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)

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, generally through electronic mail or via ftp site.

For NHANES 1999+, all data are reported electronically weekly to Westat who then transfer the results 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 LIMS database is used to keep records and track specimens for NHANES 1999+. If analyses are performed for smaller, non-NHANES studies, records may be kept in Excel files on the DLS network.

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.

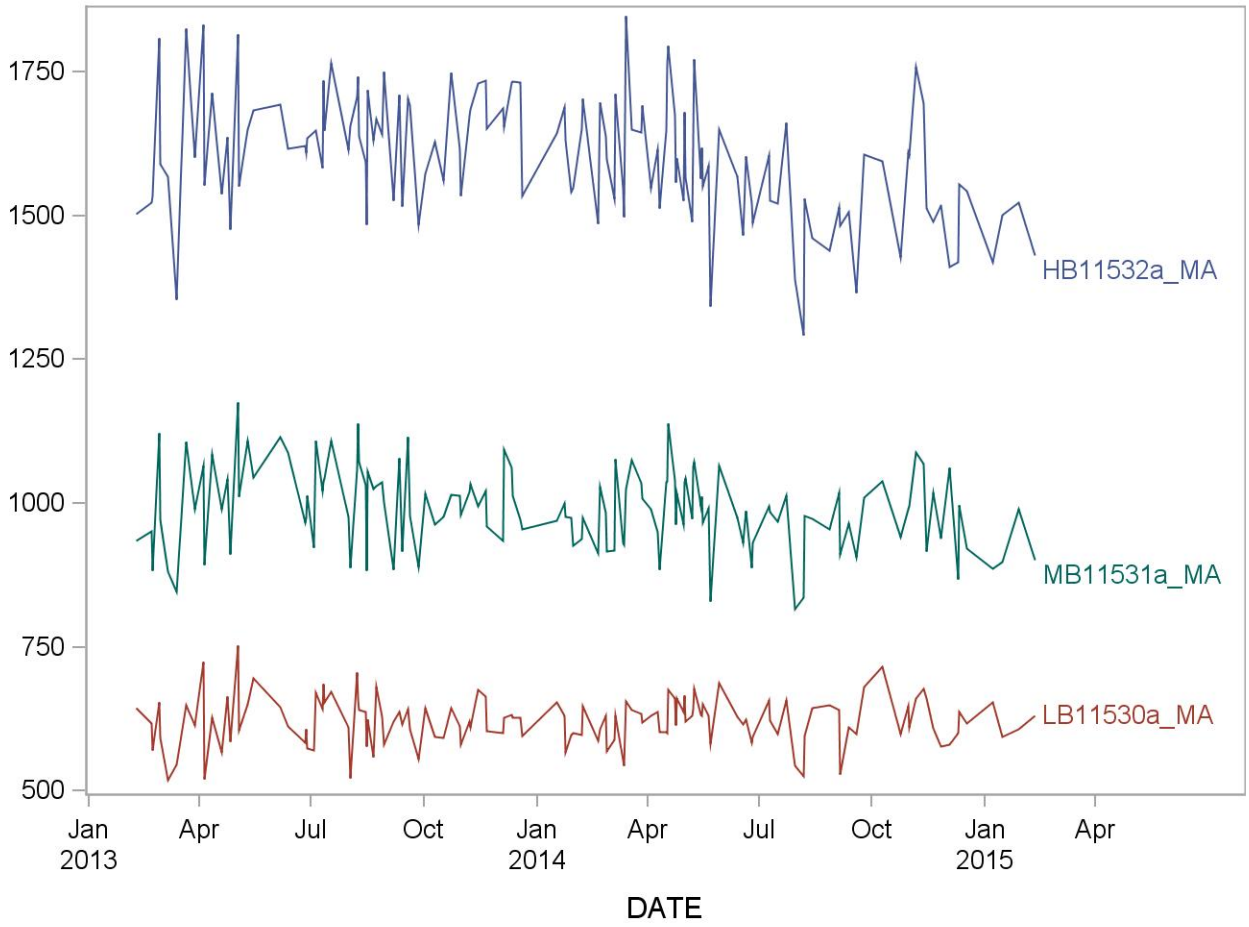
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the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

See following pages.

Summary Statistics and QC Chart for RBC folate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HB11532a_MA	142	08FEB13	11FEB15	1600.5	107.9	6.7
LB11530a_MA	143	08FEB13	11FEB15	620.8	41.4	6.7
MB11531a_MA	143	08FEB13	11FEB15	990.5	70.0	7.1



References

1. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. J Clin Pathol 1992;45:344-7.
2. AM Molloy, JM Scott. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol 1997;281:43-53.
3. Fazili Z, Pfeiffer CM, Zhang M, Jain R. Erythrocyte folate extraction and quantitative determination by LC/MS/MS: Comparison of results with microbiologic assay. Clin Chem 2005;51(12):2318-25.
4. Mastropaolo W, Wilson MA. Effect of light on serum B12 and folate stability. Clin Chem 1993;39(5):913.
5. Fazili Z, Pfeiffer CM, Zhang M. Comparison of serum folate species analyzed by LC-MS/MS with total folate measured by microbiologic assay and Bio-Rad radioassay. Clin Chem 2007;53:781-4.
6. Fazili Z, Pfeiffer CM, Zhang M, Ram JB, Koontz D. Influence of 5, 10-methylene-tetrahydrofolate reductase polymorphism on whole blood folate measured by LC-MS/MS, microbiologic assay and BioRad assay. Clin Chem 2008;54:197-201.
7. Caudill SP, Schleicher RL, Pirkle JL. 2008. Multi-rule quality control for the age-related eye disease study. Stat Med 27:4094-4106.
8. Tietz NW. Clinical Guide to Laboratory Tests. Third Edition. 1995. WB Saunders Company.
9. Pfeiffer CM, Hughes JP, Lacher DA, Bailey RL, Berry RJ, Zhang M, Yetley EA, Rader JI, Sempos CT, Johnson CL. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988-2010. J Nutr. 2012;142:886-893.
10. Pfeiffer CM, Zhang M, Lacher DA, Molloy AM, Tamura T, Yetley EA, Picciano M-F, Johnson CL. Comparison of serum and red blood cell folate microbiologic assays for national population surveys. J Nutr. 2011;141:1402-9.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of:

- Mindy Zhang, MD for developing and validating this method and preparing the manuscript for this chapter.
- Christine M. Pfeiffer, Ph.D. for the assistance and advice provided.

Appendix 1 – Formulas to calculate the concentration of the folate stock solution

$$Conc.(\,ppm\ or\ \mu g/mL) = [Absorbance\ (cm-1) \times dilution \times 1000 \times MW\ (gmol-1)]/\,\varepsilon \max (Lmol-1\ cm-1)$$

Or

$$Conc.\ (\mu mol/L) = [Absorbance\ (cm-1) \times dilution \times 1000 \times 1000]/\,\varepsilon \max (Lmol-1 cm-1)$$

€ max = molar extinction coefficient
Conc. = concentration

Example – 5-Methyltetrahydrofolic acid (5-MeTHF)

Abs	0.661
Dilution	10
€ max	31,700
MW	459.4

Concentration (ppm or µg/mL) = 0.661 x 10 x 1,000 x 459.4 / 31700 = **95.79**

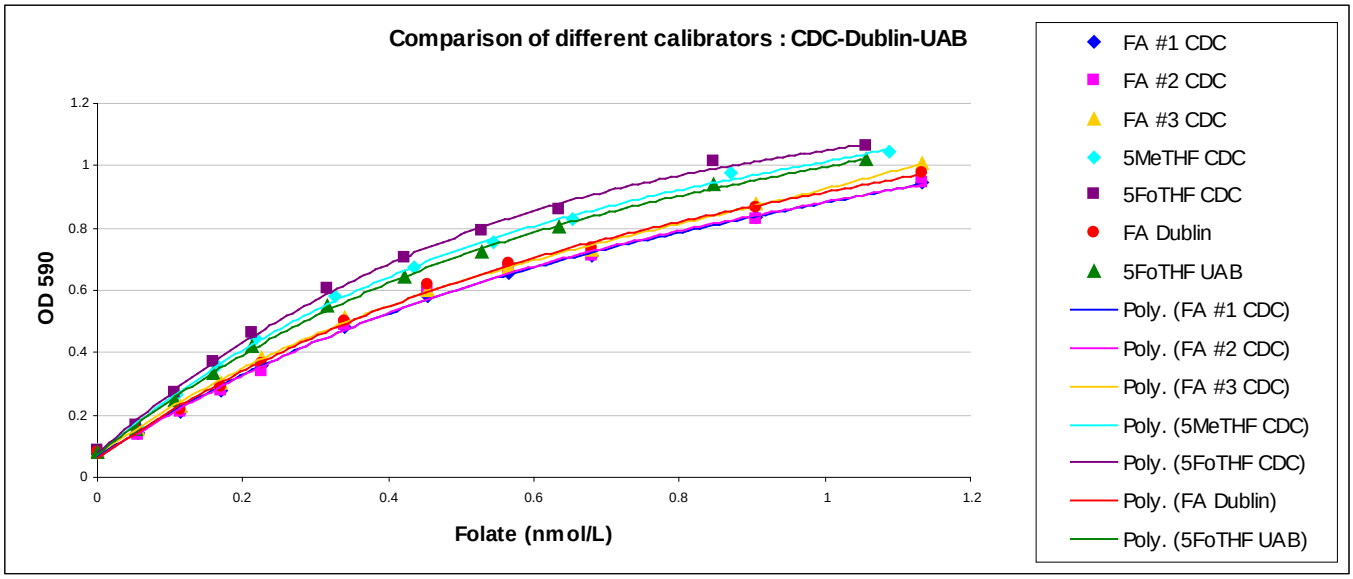
Concentration (µmol/L) = 0.661 x 10 x 1,000 x 1,000 / 31700 = **208.52**

Appendix 2 – Method Figures of Merit

Response of *L. casei* to different folate calibrators

As part of our assay validation, we investigated the response of the *L. casei* microorganism to different calibrators. We observed slight differences, with reduced forms (5MeTHF and 5FoTHF) giving a slightly higher response than folic acid (see Fig. 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 ~25% lower values than calibration with folic acid. This effect has been documented in a paper that describes data from 3 different microbiologic assays applied to a subset of NHANES 2007-2008 **[10]**.

Fig. 1: Response of *L. casei* to different folate calibrators



Spiking recovery

When we spiked serum samples (mean (SD), *n*=3 days), we obtained satisfactory recoveries for most folate species **[5]:** 5-methylTHF 88% (9%), 5-formylTHF 120% (9%), and 5,10-methenylTHF 101% (7%). The recovery for folic acid was 69% (3%) and for THF 36% (10%). Since we calibrate the microbiologic assay with 5-methylTHF and this compound produces a slightly higher response curve than folic acid, we expect to under-recover folic acid. The lower recovery of THF is probably due to oxidative loss of THF during the 42 hours of incubation and could potentially be improved by increasing the ascorbic acid concentration of the medium.

Our recovery experiments with whole blood samples **[6]** also showed satisfactory recovery [mean (SD), *n*=3 days] for 5-methylTHF [97% (11%)], 5-formylTHF [124% (7%)], and 5,10-methenylTHF [107% (13%)] and under-recovery for THF [46.4% (8%)]. These results compared well with the recoveries found in serum samples.

Dilution linearity

Dilution experiments for serum and whole blood samples showed good dilution linearity when serum samples were diluted between 1/25 and 1/200 ($y=0.9933x+0.2156$, $R^2=0.9987$, n=3 subjects) and whole

blood hemolysate samples were diluted between 1/70 and 1/280 (y=1.0159x-4.8306, R^2=1, n=2 subjects).

Precision

Long-term quality control CVs for serum folate were 5.9%-10% for NHANES 2007–2008 and 4.7%-8.5% for NHANES 2009–2010. For RBC folate, CVs were 8.0%-14% for NHANES 2007–2008 and 7.5%-8.2% for NHANES 2009–2010.

Limit of detection

Because the microbiologic assay is very sensitive, samples are always pre-diluted prior to the analysis. An LOD can therefore not be determined by serial dilution and extrapolation. The LOD is dependent on the dilution factor. We use the lowest point of the calibration curve (0.05*100 = 5 nmo/L) divided by 3 to define the LOD (~1.7 nmol/L).

Appendix 3 – Ruggedness Testing

This method has 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 below. Please refer to the DLS Policies and Procedures Manual for further information on ruggedness testing.

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:

	Lower level	Method specifies	Higher level a	Higher level b	Higher level c
Reading time (min)	0.5	1	2	3	4
Mean serum folate (nmol/L)[1]	20.3	18.6	20.6	20.4	19.8
Difference from 1 min for serum	9%		11%	10%	6%
Mean WB folate (nmol/L)[1]		354	370	364	364
Difference from 1 min for WB			5%	3%	3%

[1]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:

	Method specifies	High level a	High level b
Reading time point	Directly after ~45 h incubation	After storing at 4°C for 4 days	After storing at room temperature overnight

Mean serum folate (nmol/L), N = 44	37.0	35.2	
Difference		-5%	
Mean serum folate (nmol/L), N = 92	38.6	39.4	
Difference		2%	
Mean serum folate (nmol/L), N = 4	12.6		12.2
Difference			-3%

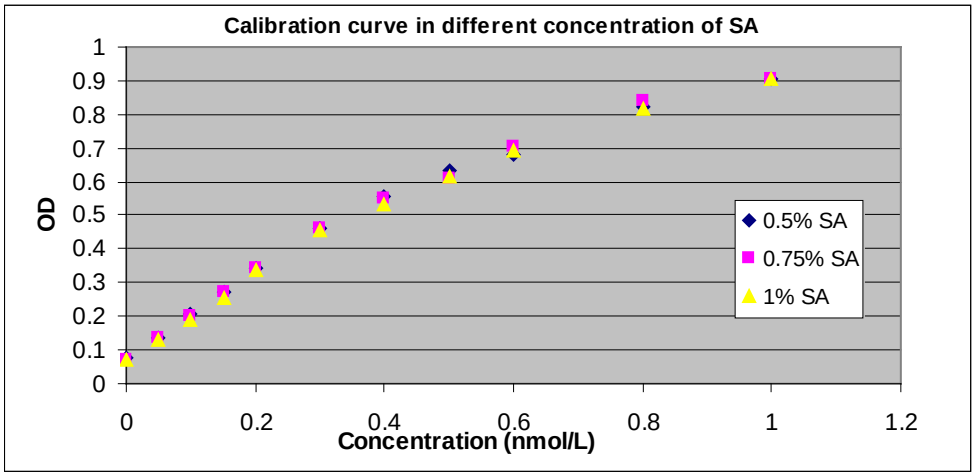
Conclusion: Delaying the plate reading does not appear to affect serum folate concentrations, as long as the plate is stored overnight at room temperature or up to 4 days at 4°C.

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:



	Method specifies	Higher level a	Higher level b
SA concentration	0.5%	0.75%	1%
Mean serum folate (nmol/L), N = 13	40.3	40.1	43.1
Difference		0%	9%
Mean WB folate (nmol/L), N = 16	448	428	427
Difference		-4%	-5%

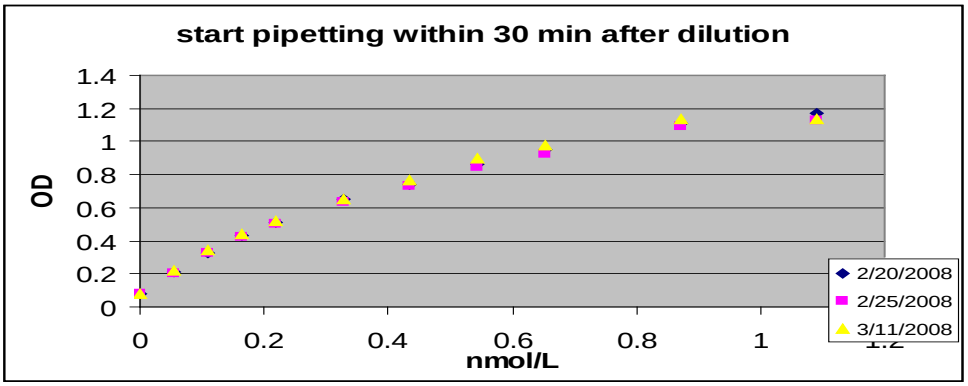
Conclusion: Increasing the sodium ascorbate concentration from 0.5% to up to 1% does not appear to provide a protective effect to serum and WB folate concentrations, or to the 5-methyltetrahydrofolate standard in the calibration curve.

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:

Serum	Shorter Incubation Time	Mean Diff (SD)	Longer Incubation Time	Mean Diff (SD)
4/28/2008 N=6	24h vs. 47h	3.0% (10.5%)		
4/29/2008 N=5	24h vs. 48h	-5.2% (11.5%)		
4/29/2008 N=10	24h vs. 48h	-8.1% (9.2%)		
9/8/2008 N=46	24h vs. 47h	7.4% (12.9%)	50h vs. 46h	-1.9% (7.3%)
9/23/2008 N=6	24h vs. 42h	4.2% (13.4%)	49h vs. 42h	-16.7% (7.9%)
11/19/2008 N=45	24h vs. 42h	3.3% (14.0%)	49h vs. 42h	-3.5% (7.4%)

WB	Shorter Incubation Time	Mean Diff (SD)	Longer Incubation Time	Mean Diff (SD)
4/28/2008 N=6	24h vs. 47h	1.6% (12.2%)		
8/27/2008 N=46			51h vs. 46h	-8.9% (7.6%)
9/2/2008 N=113			49 h vs. 47h	-6.5% (5.5%)
9/2/2008 N=17	24h vs. 41h	39.2% (16.6%)	48h vs. 41h	5.6% (6.9%)
9/3/2008 N=91			49h vs. 45h	-8.5% (7.4%)
1/14/2009 N=23	24h vs. 45h	-9.7% (13.4%)	48h vs. 45h	1.3% (5.7%)

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).