Laboratory Procedure Manual

Analyte: **Total Folate**

Matrix: **Serum and Whole Blood**

Method: Microbiologic Assay

Method No: 4000.12

Revised: Jan 2023

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
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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.

Images are included in this document as visual aids for certain topics. They are intended to be representative images only and should not be construed as absolute references. Discrepancies between the images in this document and the actual application design are not a cause for revisions to this document.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the table below for FOLATE_L:

Data file name	Variable name	SAS Label
FOLATE_L	LBDRFO	RBC folate (ng/mL)
	LBDRFOSI	RBC folate (nmol/L)

1. Summary of Clinical Relevance and Principle

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

Microbiologic assays (MBA) 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]** 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 protein-binding 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 ~41-43 hours at 37±1°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 (5-MeTHF). If seven plates are processed in a run, 132 unknown 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) 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 an autoclave pan after use. Also wipe down all contaminated work surfaces with 10% bleach solution when work is finished. Add bleach into the leftover folate assay medium containing *L. Rhamnosus* at a final concentration ~10% to kill all the microorganisms. Add bleach to the water waste bottle to a final concentration of ~1% and discard contents in the drain.

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. Safety data sheets (SDSs) for these chemicals are readily accessible as hard copies in the lab. If needed, SDSs for these chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ossam/workplace-safety/safety-practices/chemical-safety/index.html. Additional information on hazard identification, risk evaluation, and risk mitigation for this method can be found in the method risk assessment form.

PerkinElmer and BioTek Instrument Inc. provide safety information that should be read before operating the instruments. 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 GEN5 and stored on the CDC network. The results file (including analyte, sample IDs, replicate count, replicate variation, sample dilution factor, instrument results, raw results, final results, data file name, acquisition time, analyst, notes, etc.) is imported into a STARLIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See **Appendix B_D: JA-4000-DR-01-Computerization and Data System Management** for a step-by-step description of data transfer, review, and approval.

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

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

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

(B) Serum folate 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 300 µL of serum or whole blood hemolysate is needed to do a proper dilution when using automated pipetting. Serum is typically diluted at 1/100. Whole blood hemolysate is typically diluted at 1/140 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 Vacutainers. 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 Nalgene cryovial or other plastic screw-capped vial labeled with the participant's ID.

(E) Specimens collected in the field should be kept refrigerated (+2°C to +8°C) and protected from light. After processing, specimens should be frozen and shipped on dry ice by overnight mail. Once received, they should be stored frozen during 'in-processing', which is typically completed within less than 4 hours and then stored frozen at ≤-50 °C for up to 15 business days until they are transferred to the testing laboratory. For longer storage, specimens should be stored deep frozen (-50°C to -90°C). Ascorbic acid (0.5%) is sometimes added to serum prior to storage to improve folate stability. Up to three short (3 h) freeze-thaw cycles cause only minor (~4%) folate degradation for serum and whole blood samples; however, unnecessary freeze-thaw cycles should be avoided where possible due to the sensitive nature of folates.

(F) The criteria for unacceptable specimens are insufficient sample volume for at least one analysis (<100 µL for manual pipetting), suspected contamination such as leaking, or damaged sample container. These samples are assigned an appropriate comment code and/or description and are set "no reportable" (code 98). Specimens received at ambient temperature (+15°C to +30°C) are also rejected. Refrigerated samples may be used when they are brought promptly from the site where the blood was collected.

(G) 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 at 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 (+2°C to +8°C) for 48 hours before hemolysis give the same results as samples lysed immediately after collection.

(H) 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.

(I) 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 test of interest in the specimen and it needs to be divided, the appropriate amount of blood or serum should be transferred into a Nalgene cryovial labeled with the Participant's ID; avoid cross-contamination.

(J) A series of standard comment codes are available in the STARLIMS database to identify any issues related to sample quality. These codes can be used, along with test descriptions, to document why a result was not reported (specimen rejection) or that a result should be interpreted with caution based on the sample quality.

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

Not applicable for this procedure.

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

Chemicals and other materials used in the preparation of reagents, calibrators, and quality control materials are tracked in indicated in **Appendix B_B: JA-4000-R&S-05-Reagents Tracking.** To facilitate tracking of solvents and other chemicals transferred from the original manufacturer containment into a secondary container (e.g., solvent bottle), include the expiration date provided by the manufacturer or the lot number on the secondary container.

A. Standards and Chemicals

With some exceptions, a material listed herein may be substituted with an equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed.

- 5-MeTHF (Merck Cie, AG, Schaffhausen, Switzerland)
- Ascorbic acid (Sigma, St. Louis, MO, USA)
- Sodium ascorbate (Sigma)
- Himedia Folic Acid Casei medium (M543)
- 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 azide (Sigma)
- Glycerol (Sigma)
- BioTek QC Check Solution No. 1 (PN 7120779, 25 ml; or PN 7120782)

B. Reagent Preparation

Prepare solutions, samples, and standards using 0.45 µm filtered deionized water with a resistivity of at least 18 MΩ·cm. Perform all steps involving concentrated acids, bases, and organic solvents in a chemical fume hood. Though each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare larger or smaller quantities if desired.

1) Sodium ascorbate (0.5%)

Sodium ascorbate solution (0.5 g/dL) for diluting samples and standard.
Two and a half grams of sodium ascorbate are dissolved in 500 mL of deionized water. This solution should be prepared freshly before each assay.

2) Folic Acid Casei medium inoculated with *L. Rhamnosus* (folate free)

To prepare 200 mL of assay medium: add 14.1 g of Folic Acid Casei Medium and 60 µL of Tween-80 to 200 mL of deionized water, heat to boil for 2-3 min with stirring. Cool down to ~37°C, then add 6 mg of chloramphenicol (or 2 mL of a 3 mg/mL chloramphenicol stock solution), 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 for ~5-10 min until all the chemicals are dissolved. About 15-30 minutes before adding medium into plates, thaw one vial of frozen *L. Rhamnosus* (a water bath at ambient temperature (+15°C to +30°C) can be used for quick thawing) and add 600-700 µL into 200 mL of assay medium, keep stirring slowly. About 25 mL of assay medium is needed per microplate. This medium should be prepared freshly for each assay.

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

Dissolve 15 g of ascorbic acid in 100 mL of deionized water. Aliquots of this stock solution (1 mL) are stored deep frozen (-50°C to -90°C) in 2-mL labeled cryovials. The stock solution is stable for at least 4 years. Add 1 mL into 200 mL of Folic Acid Casei medium.

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). Aliquots of this stock solution (1 mL) are stored deep frozen (-50°C to -90°C) in 2-mL labeled cryovials. The stock solution is stable for at least 4 years. Add 1 mL into 200 mL of Folic Acid Casei medium.

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. Aliquots of this stock solution (2 mL) are stored deep frozen (-50°C to -90°C) in 2-mL labeled cryovials. The stock solution is stable for at least 4 years. Add 2 mL of stock solution into 200 mL of Folic Acid Casei medium.

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

To prepare 200 mL of growth medium: Add 9.4 g of Folic Acid Casei medium, 40 µL of Tween-80 to 200 mL of deionized water. Heat to boil for 2-3 min. Cool down to ~37°C, add 40 mg of chloramphenicol, 100 mg of ascorbic acid, and 300 µL of folic acid stock solution (100 ng/mL), stir to completely dissolve, sterilize the medium by filtering through a 0.2 µm filter. Aliquots of the prepared medium (45 mL) in sterile 50-mL tube are stored frozen (-10°C to -50°C) for less than 1 year. For longer storage, the aliquots should be stored deep frozen (-50°C to -90°C), where they are stable for at least 4 years.

7) Ascorbic acid (1%)

Ascorbic acid (1 g/dL) for hemolysis of whole blood samples. Dissolve 1 g of ascorbic acid in 10 mL of deionized water. Prepare fresh solution before each use.

8) Blocking solution

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 in ultrapure water (10 µL /control well). Prepare fresh solution before each use.

For more information on reagent preparation, see **Appendix B_B: JA-4000-R&S-01-Prepare reagents and calibrator for routine assay; and JA-4000-R&S-02-Prepare reagent stock solutions.**

C. Standards Preparation

The concentrations of the folate stock solution are 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 C.**

Use Class A volumetric glassware where a volumetric flask is specified.

1) 5-MethylTHF primary stock solution (~200 µg/mL)

Prepare a primary stock solution in a volumetric flask by dissolving an accurately known mass (±0.1 mg) of the pure solid compound in degassed and filtered 20 mM phosphate buffer (pH 7.2, containing 0.1% cysteine), targeting a final concentration of ~200 µg/mL (e.g., 2 mg in 10 mL). Vortex briefly to help dissolve the contents and make up to final volume.

Remove a small aliquot (1 mL) of the primary stock solution to a microcentrifuge vial to determine its concentration by UV spectrophotometry. To the remaining stock solution add ascorbic acid powder to a final concentration of 1%. From the aliquot you removed, prepare two dilutions (e.g., 1/20 and 1/50), each in duplicate, measure the UV absorbance at the peak maximum using scan analysis against phosphate buffer as a blank, and calculate the primary stock solution concentration The ratio of absorbance at 290/245 nm can be monitored (simple reads analysis at each wavelength) to ensure that no oxidation takes place. This ratio should exceed 3.3.

Stock solution II (100 µg/mL): Based on the actual concentration of primary stock solution, the stock solution is diluted in a 25-mL volumetric flask to yield a 100 µg/mL stock solution II using 1% ascorbic acid solution (degassed & filtered). Aliquots of this stock solution II (1.2 mL) are stored deep frozen (-50°C to -90°C) in 2-mL labeled cryovials and are stable at least for a few years.

Note:
Fresh individual primary stock solutions are prepared approximately every 2 years. In-house long-term storage stability data showed that 5-methylTHF stock solution (100 µg/mL in 1% ascorbic acid and 10 µg/mL in 0.1% ascorbic acid) were stable for at least 9 years when stored deep frozen (-50°C to -90°C) **[5]**.

2) 5-MethylTHF microbiologic assay stock solution (1 µmol/L)

Based on the exact concentration determined on UV spectrophotometry, dilute 5-MethylTHF primary stock solution with 0.5% degassed ascorbic acid solution in a 100-mL volumetric flask to get final concentration 1 µmol/L. Aliquot 400 µL into labeled 2-mL cryovial (or 250 µL into 0.5-mL screw-capped vial) and store the aliquots deep frozen (-50°C to -90°C). Prepare a fresh MBA stock solution from primary stock solution every 4 years.

More information on calibrator stock preparation is provided in **Appendix B_B: JA-4000-R&S-03-Prepare calibrator stock solutions.**

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. Prescreen potential materials to determine folate concentrations, then mix materials based on their folate levels to generate low, medium, and high QC pools.

Pools are aliquoted into 2.0 mL Nalgene cryovials, capped and frozen. The QC pools are stored deep frozen (-50°C to -90°C) and are stable for at least 8 years. For more detailed information on the preparation of QC materials, homogeneity testing, and characterization refer to **SOP NBB-OC-LABOP.01.01 QC Materials**.

E. Preparation of Cryoprotected Organism

(1) Add one vial of freeze-dried *L. Rhamnosus* to 20 mL of organism growth medium and incubate at 37±1°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±1°C for 24 hours.

(3) Repeat step 2 for the third 24-hour incubation.

(4) Inoculate different amounts 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±1°C. Measure OD at 590 nm from one of the duplicate cultures at different incubation times. Record the log growth phase (~18-20 hours). Mix the log phase culture 50/50 with 80% glycerol (sterilized by filtering through 0.2 µm filter). Aliquot the mixture into sterile cryovials (1 mL/vial) and store them deep frozen (-50°C to -90°C). The inoculation is stable for at least 10 years.

(5) To test the new inoculation, set up a standard plate and a sample plate with QC samples and other samples and compare the results to those generated using the previous inoculum. The OD should be ~0.1 for the blank and ~1.0 for the highest calibrator, and the curve should have the expected polynomial 3rd degree shape.

More information on inoculation preparation from frozen inoculation is provided in **Appendix B_B: JA-4000-R&S-04-Prepare inoculation.**

F. Equipment

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.), a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., plate reader) equivalent performance must be demonstrated experimentally in accordance with DLS Policies and Procedures Manual if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type. For details, see **Appendix B_C: JA-4000-I-01-Instrument Comparison and System Verification for Janus Liquid Handler and Plate Reader.**

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

(2) JANUS™ Automated Workstation (PerkinElmer, MA, USA)

(3) Precision Incubator 6842: 37°C (Fisher, Pittsburgh, PA)

(4) Thermo Microplate Heat Sealer (Thermo Scientific, ALPS 50 V)

(5) Rotator-Genie (Scientific Industries #7400-2100)

(6) Digiflex CX (Titertek #0603310, Huntsville, AL)

(7) Stirring Hotplate (Fisher # 11-100-16SH, 11-100-49SH, Suwanee, GA and Thermolyne SP46925)

(8) Balance (Mettler Toledo, AG-104)

(9) Vortex – Genie 2 (VWR, Suwanee, GA)

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

G. Other Materials

With some exceptions, a material listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. Equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures.

(1) Deionized water (with a resistivity of at least 18 MΩ·cm from an AquaSolutions water purification system)

(2) 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 5 mL 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)

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

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

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

(6) Bleach (10% sodium hypochlorite solution) – any vendor

(7) 12 x 75 mm Disposable glass culture tubes (Corning Glassworks, NY)

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

(9) Volumetric flask (50 mL, any vendor)

(10) Beakers (50 mL, 400 mL, 600 mL, any vendor)

(11) 96-well microplate (Nunc, 12-565-361, Waltham, MA)

(12) Thermal Seal 2 Films for PCR (Thomas Scientific, TS2-100)

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

(14) Absorbance test plate for reader test (BioTek, Part #7260522E)

7. Calibration and Calibration Verification Procedures

A. Method Calibration and Calibration Verification

Calibration

This assay uses an aqueous calibration curve with 5-meTHF (Merck Cie [Eprova]) as the 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. Eight replicates are used for each calibration point and no more than 2 out of 8 replicates can be masked due to invalid wells. It is not permitted to delete any of the 11 calibration points from the calibration curve.

Calibration Verification

Since a multi-point calibration curve is included in every run, there is no additional calibration verification required. However, calibration verification using international reference materials is conducted at least every 6 months to monitor the assay performance. For details, see **Appendix B-A: JA-4000-G-01-Calibration and calibration verification**.

The National Institute of Standards and Technology (NIST) 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. 5.4; level 2: 13 vs. 14; level 3: 41 vs. 42). We routinely analyze this SRM material every 6 months during calibration verification and any time there are assay problems. The reference materials are stored deep frozen (-50°C to -90°C).

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 analyzed every 6 months during calibration verification and any time we experience assay problems. The reference materials are stored deep frozen (-50°C to -90°C).

Details about our proficiency testing (PT) activities can be found in the proficiency testing form. During 2007, we participated in the **CAP Ligand** survey; however, samples contained an additive that interfered with the growth of the microorganism. Since 2008, we participated in the **UK NEQAS Haematinics** PT program, when possible. Since shipments have to be sent overseas on dry ice, we can't receive monthly challenges but rather bundled shipments once or twice a year. Given that timely shipment of samples from international PT programs is not always feasible, we have developed an **Alternative in-house proficiency testing program** to complement participation in external PT programs; for details, see **Appendix B_A: JA-4000-G-02-Alternative In-house proficiency testing.**

Information on how the microorganism responds to various folate calibrators and how the microbiologic assay compares to other assays is presented in **Appendix D**.

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 E**.

B. Instrument Calibration

1) PerkinElmer Janus Liquid Handler

Once a year a PerkinElmer service engineer performs preventative maintenance. If necessary, calibration verification is performed as part of troubleshooting. The in-house calibration verification of the Janus Liquid handlers is scheduled every 6 months using either a yellow dye method (**see Appendix B_C: JA-4000-I-03-Janus calibration verification using yellow dye**) or gravimetrically (manufacture's method), we do not use the gravimetric method, the detailed information can be found in BioTek manual-Janus Calibration Verification Balance Test - Weigh Module WXS_PE.

2) BioTek Microplate Reader

Microplate readers are verified twice a year by measuring CAP Instrumentation survey samples and by performing a reader system test, absorbance plate test (**see Appendix B_C: JA-4000-I-05-Microplate reader calibration verification using Absorbance Plate Test**), and yellow dye liquid test **(see Appendix B_C: JA-4000-I-06-BioTek Reader yellow dye liquid test)**.

3) Pipettes and Digiflex

Pipette calibration verification is performed every 6 months. Typically, one calibration verification is done by a certified company, and one is performed by the analyst gravimetrically using a calibrated analytical balance.

4) Balances

Calibration is performed annually by a certified company. Calibration verification is performed by the analyst as needed using certified weights.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

For a quick overview on how to best manage the flow of this assay, see **Appendix F: Folate MBA Assay Procedure Steps.** The assay procedure should be performed in a room protected from strong, direct sunlight.

A. Preliminaries

(1) Thaw frozen serum or whole blood hemolysate samples, serum or whole blood hemolysate quality control samples, and the 3 frozen reagent stocks, and let them reach ambient temperature (+15°C to +30°C).

(2) Place QC samples, unknown samples, and blank vials in 4x12 Janus Liquid Handler Racks following the MBA GEN5 plate layout

(3) Label the same number of 75x12-mm glass tubes as 1, 2, 3…, and place them in 4x12 Janus Liquid Handler Racks

(4) Thoroughly mix QCs and unknown specimens on a vortex before pipetting

(5) Prepare assay medium inoculated with microorganism and 0.5% sodium ascorbate solution

B. Dilution of Samples and Calibrator

(1) To dilute serum QC and unknown samples at 1/100, add 1485 µL of 0.5 g/dL sodium ascorbate solution into each 75x12-mm glass tube using a Digiflex liquid dispenser, and then add 15 µL of serum QC or unknown sample into each tube using the JANUS Automated Workstation (**see Appendix B_C: JA-4000-I-02-Janus liquid handler operation, function checks, maintenance, and performance evaluation**). When completed, cover the tubes with plastic wrap and thoroughly mix by placing the rack with all tubes on a multi-tube shaker.

(2) To dilute hemolysed whole blood QC and unknown samples (whole blood diluted 1/11 with 1% ascorbic acid) at 1/140, add 2085 µL of 0.5 g/dL sodium ascorbate into each 75x12-mm glass tube using a Digiflex liquid dispenser, and then add 15 µL of whole blood hemolysate into each tube using the JANUS Automated Workstation. When completed, cover the tubes with plastic wrap and thoroughly mix by placing the rack with all tubes on a multi-tube shaker.

(3) Calibrator dilution (to obtain working solution I and II)

Thaw 1 vial of 5-MeTHF stock solution (1 µmol/L) for ~15 minutes before dilution

a) Dilute 5-meTHF stock solution (1 µmol/L) at 1/5 to get 200 nmol/L: transfer 100 µL of stock solution to 400 µL of 0.5 g/dL sodium ascorbate solution in a centrifuge tube, mix thoroughly.

b) Prepare working solution I: transfer 50 µL of diluted 5-MeTHF (200 nmol/L) into 0.5 g/dL sodium ascorbate solution in a 50-mL volumetric flask, make up to volume. This makes a 1/1000 dilution to get 200 pmol/L.

c) Prepare working solution II: transfer 250 µL of diluted 5-MeTHF (200 nmol/L) into 0.5 g/dL sodium ascorbate solution in a 50-mL volumetric flask, make up to volume. This makes a 1/200 dilution to get 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 2 different dilutions (2 replicates/dilution) and 8 replicates for each standard concentration. Fully automated pipetting is performed by the JANUS Automated Workstation operated by the WinPrep software. The total pipetting time is ~3 hours per run (for 7 plates). The total time for each assay is ~5 hours. A total of 23 samples including QC and unknown patient samples can be analyzed per sample plate. One blank sample is included on each plate.

1) Plate #1: 5-MeTHF calibration plate

a) Add 200 µL of Folic Acid Casei medium inoculated with *L. Rhamnosus* into each well of a 96-well microplate (see Calibration Plate - Table 1).

b) Add a different amount of 0.5 g/dL sodium ascorbate solution into each well as specified by the various columns (see Calibration Plate -Table 2)

c) Add a different amount of 5-meTHF standard working solution I and II into each well as specified by the various columns (see Calibration Plate - Table 3). The calibration curve is made up of 11 different calibration points. The total standard volume is 100 µL in each well.

d) Optional step: Add 5 µL of 3 g/dL sodium azide to the first column that is used as blank for the M590 correction. This step is used to generate the calibration curve for whole blood samples when a dilution of ≤ 1/40 is used, but it is ignored when analyzing serum samples or when higher dilution is used for whole blood samples. This step is currently not used for NHANES whole blood samples because of high folate concentrations and resulting higher dilution.

e) Seal the plate very tightly using Thermal Sealing membrane and Heat Plate Sealer (150°C for 5 seconds). Do not invert the plate. More information is provided **in Appendix B_C: JA-4000-I-07 Seal plates using a heat plate sealer.**

f) Incubate the plate together with other sample plates at 37±1°C for ~41-43 hours. Place an empty plate on the top and at the bottom of the stack of experiment plates.

Calibration Plate - Table 1: Add 200 µL *L. Rhamnosus* 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 solution

#1	1	2	3	4	5	6	7	8	9	10	11	12
A	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
B	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
C	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
D	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
E	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
F	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
G	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
H	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul

Calibration Plate - Table 3: Add 5-MeTHF working solution I and II

			working solution I				working solution II					
#1	1	2	3	4	5	6	7	8	9	10	11	12
A	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
B	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
C	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
D	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
E	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
F	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
G	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
H	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul

2) Plate #2 to #7: Serum QCs and Serum Unknown Samples

a) Add 200 µL of Folic Acid Casei medium inoculated with *L. Rhamnosus* into each well (see Sample Plate - Table 4)

b) Add 50 µL of 0.5 g/dL sodium ascorbate solution to the wells that will have 50 µL diluted sample; don't add sodium ascorbate solution to 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 Sample Plate - Table 6). The total sample volume is 100 µL in each well. A total of 23 QC and unknown samples and 1 blank sample can be analyzed per plate. The blank is always placed in the last position of the plate.

d) Seal the plates very tightly using Thermal Sealing membrane and Heat Plate Sealer (150°C for 5 seconds). Do not invert the plates.

e) The layouts of Plate #3-#7 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±1°C for ~41-43 hours.

Serum Plate – Table 4: Add 200 µL *L. Rhamnosus* 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

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

#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	

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

#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	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: WB QCs and WB Unknown Samples

a) For samples that have relatively high folate concentration, we use higher dilution factor of 1/140 for whole blood hemolysate samples. We have found that the hemoglobin color has no effect on OD 590 after incubation, even at 1/70 dilution, thus we don't use color control and the whole blood samples have exactly the same plate layout as serum samples.

b) 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.

c) Add 200 µL of Folic Acid Casei medium inoculated with *L. Rhamnosus* into each well (see Whole Blood Plate – Table 7).

d) 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 solution into the wells that will have 100 µL diluted sample (see Whole Blood Plate – Table 8).

e) Add 50 µL and 100 µL of the 1/140 diluted hemolysate QCs and unknown samples to the corresponding wells (see Whole Blood plate – Table 9).

f) 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).

g) Seal the plates very tightly using Thermal Sealing membrane and Heat Plate Sealer (150°C for 5 seconds). Do not invert the plates.

h) The layouts of Plate #3-#7 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±1°C for ~41-43 hours.

WB sample plate – Table 7: Add 200 µL *L. Rhamnosus* 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

WB sample plate – Table 8: Add 0.5% sodium ascorbate solution

	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												

WB sample plate – Table 9: Add diluted WB 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 MBA 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. The assay experiment file should be created after the assay set up is completed and the plates are put into the incubator. For detailed information, see **Appendix B_D: JA-4000-DR-06-Set Up Folate MBA Experiment in Gen5.**

Table 10: 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
X Axis	Lin
Y Axis	Lin
Curve Fit	Polynomial Regression, Degree 3
Assay Type	Multiple plate assay

For the assays with different number of plates, the layouts vary based on the number of samples analyzed. The calibration curve has the following concentrations: 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 nmol/L. Specific dilution factors have to be used for QCs and unknown samples (see Table 11).

Table 11: Dilution factors for serum and whole blood hemolysate samples

Sample		Dilution factor (100 uL added into plate)	Sodium Ascorbate (µL)	Serum or whole blood hemolysate (µL)	Final volume (µL)	Comment code	Dilution factor in GEN5 software
Serum	Routine Dilution	100	1485	15	1500	none	100 w/ 100 µL 200 w/ 50 µL
	GEN5 raw concentration ~ < 0.05 nmol/L	50	735	15	750	115	50 w/ 100 uL 100 w/ 50 µL
		30	435	15	450	115	30 w/ 100 µL 60 w/ 50 µL
	GEN5 raw concentration > 1.00 nmol/L	200	1485	Predilute at 1/2, then add 15 µL	1500	97	200 w/ 100 µL 400 w/ 50 µL
		400	1485	Predilute at 1/4, then add 15 µL	1500	97	400 w/ 100 µL 800 w/ 50 µL
Whole blood hemolysate (1/11 diluted WB)	Routine Dilution	140	2085	15	2100	none	1540 w/ 100 µL 3080 w/ 50 µL
	GEN5 raw concentration ~ < 0.05 nmol/L	70	1035	15	1050	115	770 w/ 100 µL 1540 w/ 50 µL
		50	735	15	750	115	385 w/ 100 µL 770 w/ 50 µL
	GEN5 raw concentration > 1.00 nmol/L	280	2085	Predilute at 1/2, then add 15 µL	2100	97	3080 w/ 100 µL 6160 w/ 50 µL
		560	2085	Predilute at 1/4, then add 15 µL	2100	97	6160 w/ 100 µL 12320 w/ 50 µL

E. Collection of data and calculations of results

Turn on PowerWave microplate reader ~30 min prior to use and let it warm up. More information on reader operation, function checks, maintenance, and performance evaluation is provided in **Appendix B_C: JA-4000-I-04-Microplate reader operation, function checks, maintenance, and performance evaluation.**

Remove all plates from the incubator after ~41-43 hours of incubation, let them cool for ~30 minutes to ambient temperature (+15°C to +30°C) while mixing them thoroughly by rotating on a 360-degree rotator. The mixing time is at least 15 minutes. Plates are typically read directly after mixing. However, if there is an unforeseen delay in reading, plates can be kept at ambient temperature (+15°C to +30°C) for an additional ~1-2 hours or refrigerated (+2°C to +8°C) for up to ~8 hours. Prior to reading, plates have to sit on the bench for ~1 hour to be brought up to ~20°C and they need to be mixed for at least 15 minutes on the rotator prior to reading.

(1) Collect calibration data and construct calibration curve

When the plates are ready to be read, only remove one plate at a time from the rotator and read the plates one-by-one in exactly the same way.

- Strictly control the time from stopping mixing to reading the plate to 1 minute, maximum 1.5 minutes. For example, if plate 1 is read at 1.25 minutes, read all other plates at ~1.25 min (±10 sec).
- Wipe the bottom of the plate with a tissue to remove any particles or dust.

- Gently open the sealing membrane to prevent liquid spilling and place the plate into the plate reader.
- Gently fan the air above the plate to remove micro-bubbles.
- Do not remove more than one plate from the orbital rotator and do not remove the sealing membrane from more than one plate at a time.
- If plates sit longer on the bench prior to reading the OD, uneven settlement of the organism can lead to inaccurate results.

In the 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 the calibration curve is also automatically generated; save the data.

After reading is complete, visually check the calibration plate and mask wells that have obvious contamination, particles, leaking, dark brown color, lower volume, instrument or other lab error, or outliers of the 8 replicates at any calibration point of the calibration curve. No more than 2 of the 8 replicates can be masked at any calibration point. Check OD readings at each calibration point: 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 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.

(3) After reading is complete, examine the results and mask the wells that have contamination, leaking, dark brown color, lower volume, instrument or other lab error, or 1 outlier among the 4 replicates of a sample. A review is also performed for number of replicates, CV of replicates, dilution factors, formulas, raw results > high calibrator, raw results < cutoff value (raw folate concentration <0.05 nmol/L), 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. For information on how to generate a Power Export report, see **Appendix B_D: JA-4000-DR-01-Computerization and Data System Management.** For details on data review and sample QC criteria, see **Appendix B_D: JA-4000-DR-02-STARLIMS Data Review Process and Criteria and JA-4000-DR-03_Starlims Data Review Flow Chart**

Calculation of RBC Folate Concentration:

Whole blood folate is calculated by multiplying the whole blood hemolysate folate results by 11 and the bench dilution factor used during assay sample preparation. The serum folate contribution is subtracted (by applying a factor of 1.0 minus the hematocrit [Hct] expressed as a decimal to account for the non-RBC fraction of blood). The resulting value is divided by the Hct expressed as a decimal to yield RBC folate in nmol/L RBC. If a Hct value is not available for a patient sample, a Hct of 40% can be assumed for calculation or there is no reportable RBC folate concentration. In rare cases where a serum folate value is not available, a value of 18 nmol/L can be assumed for calculation. We recommend to correction for the serum folate contribution because it provides the most accurate reflection of folate body stores.

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

F. System Maintenance

PE Janus Liquid Handler – Preventative maintenance is performed on an annual basis by a qualified service engineer. Routine maintenance should be performed as indicated in the **JA-4000-I-02-Janus liquid handler operation, function checks, maintenance, and performance evaluation** in Appendix B, section C.

BioTek Plate Reader – No preventive maintenance is required. Routine maintenance should be performed as indicated in the **JA-4000-I-04-Microplate reader operation, function checks, maintenance, and performance evaluation** in Appendix B, section C.

G. Special Procedure Notes - CDC Modifications

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

9. Reportable Range of Results (AMR – Analytical Measurement Range)

The reportable range and the LOD of this assay are dependent on the dilution factor for unknown samples. There is no known maximum acceptable dilution. When possible, avoid small volume pipetting and minimize use of serial dilutions when generating diluted samples. For a population with good folate status, the typical dilution of serum samples is 1/100, resulting in a reportable range of 10.0–100 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] = 10.0 nmol/L
- 1 nmol/L [highest calibrator] * 100 [dilution factor] = 100 nmol/L.

Serum samples with a raw concentration 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 2.0 nmol/L:

- 0.05 nmol/L * 20 * 2 [to ensure 4 valid results from the 50 and 100 µL addition of diluted sample] = 2.0 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 with lower/higher result ≤ 25%.

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 µL 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 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.
- 44 nmol/L / 40% = 110 nmol/L

A RBC folate result of <305 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.

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).

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. Alternatively, open label blind QC specimens can be used where the analyst knows that the sample is a blind QC, but they do not know what pool the sample is from. Open label blind QCs are only used if one can choose from 6 different pools and the analyte concentrations are similar to those found in patient samples. The frequency of blind QC specimens in a run is typically 1 in every 20 specimens analyzed.

The use of blind QCs is optional but encouraged. Blind QCs are used in this method as a supplementary tool to assist in monitoring accuracy, precision, and to aid in detecting errors; they are not used as part of the primary control procedure to determine if a run is out of control. For more information on blind QC review rules, see **Appendix B_D: JA-4000-DR-04-Blind QC Review Rules.**

B. Bench Quality Controls

Bench QC specimens are prepared from a minimum of two pools that represent low and high levels of serum and WB folate. This assay typically uses three serum and WB pools that represent low, medium, and high levels. Samples from these pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run (place at least 1 QC sample on each plate).

The results from these pools are checked after each run using a multi-rule quality control system **[6]** based on their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (S_w); the standard deviation associated with individual QC results (S_i); and the standard deviation associated with run mean QC results (S_m). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. These QC rules are described in the DLS Policies and Procedures Manual and a relevant selection applicable to this assay is shown below. The system is declared “in control” if all individual QC results are within 2S limits; the run is accepted. If not, then the rules shown below are applied and the run is rejected if any condition is met; the run is declared “out of control”:

(1) One QC result per pool (due to accidental loss of duplicate)

a) If one of the three QC run **individual** results is outside a 2 S_i limit, reject run if:

1. 1 3S Rule—Run result is outside a 3 Si limit or
2. 2 2S Rule—Two or more of the three run results are outside the same 2 Si limit
3. 10 X-bar Rule—Current and previous nine run results are on the same side of the characterization mean or
4. R 4S Rule—Two consecutive standardized run results differ by more than 4 Si

(2) Two QC results per pool (all three pools have duplicate results)

a) If one of the three QC run **means** is outside a 2 S_m limit, reject run if:

1. 1 3S Rule—Run mean is outside a 3 S_m limit or
2. 2 2S Rule—Two or more of the three run means are outside the same 2 S_m limit or
3. 10 X-bar Rule—Current and previous nine run means are on the same side of the characterization mean

b) If one of the six QC **individual** results is outside a 2 S_i limit, reject run if:

1. Outlier—One individual result is beyond the characterization mean ± 4 S_i or
2. R 4S Rule—Two or more of the within-run ranges in the same run exceed 4 S_w (i.e., 95 percent range limit)

Abbreviations:

S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).

S_m = Standard deviation of the run means (the limits are shown on the chart).

S_w = Within-run standard deviation (the limits are not shown on the chart).

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

The initial limits are established by analyzing pool material in 20 consecutive runs. The SAS QC program is used to monitor the QC performance over time for potential shifts, trending, or changes in assay precision. For assays performed routinely, quarterly statistics (mean, SD, CV) are calculated for each pool and compared to the characterization target values. For assays performed infrequently, statistics are calculated at least annually. As more QC data become available (covering multiple lots of reagents, multiple analysts, etc.), the initial QC limits can be reevaluated and updated. QC limits can also be reevaluated and updated as a result of a non-conforming event when the assay shows a higher than expected out of control rate and the root cause investigation does not reveal a correctable course of action to bring the assay back into control. This needs to be documented by a CAPA in STARLIMS.

While a study is in progress, QC results are stored in a STARLIMS 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. At the conclusion of studies, complete QC records are prepared and submitted as a study QC report in STARLIMS for review by the laboratory chief, branch chief, and a DLS statistician.

C. Sample QC Criteria

Sample QC is a set of criteria used to evaluate the quality of individual test results within run, and to evaluate the quality of the calibrators associated with the run. In addition to the sample QC criteria set forth in the DLS Policies and Procedures Manual that pertain to the reportable range of concentration results and calibration curves, sample QC criteria are also established for method-specific concentration and non-concentration data associated with an individual result.

The method-specific concentration and non-concentration parameters identified for sample QC evaluation, along with their associated thresholds and flagging protocols ('Pass', 'Check', 'Warn', 'Fail') are maintained and updated in the STARLIMS database, and sample QC assessment is performed and documented as part of the run review process. A sample QC result flagged as 'fail' should not be reported. A sample QC result flagged 'Warn' or 'Check" should be reviewed both by the analyst and supervisor to determine if the quality of the result is suitable for reporting. Results that are flagged during sample QC evaluation may also be assigned one of a series of standard comment codes available in the STARLIMS database to identify the nature of the sample QC flag.

For details on data review and sample QC criteria, see **Appendix B_D: JA-4000-DR-02-STARLIMS Data Review Process and Criteria.** At the minimum, the following parameters are subject to sample QC evaluation in this method. Additional parameters may also be included as needed:

- Final result is <LOD ('Warn'); result is reviewed to determine whether it is a code 37 or sample analysis needs to be repeated
- Instrument raw result is >highest calibrator ("Fail'); sample analysis is repeated with diluted sample
- Calibration curve R^2 is <0.95 ('Fail'), R^2 is 0.95 – 0.98 ("Check"), and R^2 is > 0.98 ("Pass"); run is repeated if the calibration curve fails
- Final result is null ("Fail"); sample analysis needs to be repeated

The method uses the following method specific sample QC criteria to assess data quality.

(1) CV among the 4 replicates per patient sample

a) If the CV of the 4 replicates is ≤15%, report the result

b) If the CV of the 4 replicates is >15%, evaluate whether deleting 1 replicate that appears to be an outlier (i.e., 6, 8, 8, 9 – 6 appears to be an outlier) reduces the CV to ≤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%, keep all 4 replicates with code 26 and repeat the sample

c) If there is no clear outlier (i.e., 6, 8, 8, 10), keep all 4 replicates; don't report the result and use code 26; repeat the sample

d) Do not report results if <3 replicates; use code 26 and repeat the sample

(2) Sample raw concentration

The calibration curve range used in the folate MA is 0.05-1 nmol/L (raw concentration). If the raw concentration of a sample is <0.05 nmol/L (lowest calibrator), the GEN5 software marks the result with a symbol; the sample will be repeated with less dilution in next run. If the raw concentration of a sample is >1 nmol/L, the GEN5 software doesn't calculate a result; the sample will be repeated with higher dilution in next run.

(3) Abnormal samples

Do not report results if:

a) Ascorbic acid is not added into whole blood sample, only whole blood in sample vial (should be 100 µL). Use code 64.

b) Invalid hemolysate for RBC folate incorrect lysate solution, wrong WBL color (wrong diluent used) or wrong WBL volume (> or <1.1 mL NHANES sample). Use code 156.

(4) Invalid wells after incubation

Mask wells that have obvious contamination, leaking, dark color, lower volume or if the sample was not added during sample preparation.

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

For initial steps to investigate QC failures, **see Appendix B_D: JA-4000-DR-05-QC Failure Corrective Actions.**

Additional steps are provided below as a general guideline for identifying possible problems resulting in "out of control" values for QC materials. The troubleshooting process should be done in consultation with the supervisor and may involve additional experiments beyond what are indicated below.

(A) 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 (beakers, cylinders, tubes, Digiflex, balance, pipettes, bench, lab coats, gloves, etc.) 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.

(B) Check errors in medium and reagents preparation, instrument performance (Janus, incubator and readers)

(C) Check calibration of pipettes and automated liquid handler

(D) Check errors in sample and standard dilution and dilution factors entered in Gen5

(E) Review technical skills of analysts

(F) 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

Do not report analytical results for analytes not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

(A) This folate microbiologic assay measures total folate in serum or whole blood. It does not provide values for different forms of folate.

(B) 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.

(C) There is still debate whether some non-folate compounds might stimulate the growth of the microorganism.

(D) 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.

(E) Assay medium and sodium ascorbate should be made fresh daily.

(F) Hemolyzed serum samples may give falsely elevated values and results should be evaluated with caution.

(G) Multiple freeze/thaws cycles for extended hours (>3h) at ambient temperature (+15°C to +30°C) may cause some degradation of folates.

(H) The entire sample preparation and calibration should be conducted in yellow subdued light. Exposure to strong sunlight should be avoided since it may cause folate degradation.

(I) 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 in Appendix E**. Please refer to the *DLS Policies and Procedures Manual* for further information on ruggedness testing.

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 **[7]**. Clinical reference ranges reported for RBC folate are 317-1422 nmol/L with the CPB radioassay **[7]**.

The newest post-fortification reference ranges for the U.S. population generated with the microbiologic assay for NHANES 2005-2010 are shown below **[8]**. 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 **[8]**.

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

RBC folate: 505-2,490 nmol/L (2.5th -97.5th 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) or RBC folate levels <305 nmol/L RBC (<140 ng/mL) **[9]** are considered to require follow-up because of potential folate deficiency. Since survey data are transmitted approximately weekly to Westat, abnormal reports are automatically forwarded to the NCHS survey physician for follow-up. For smaller, non-NHANES studies, abnormal values may be identified to the study principal investigator, depending on the study arrangements. 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 ambient temperature (+15°C to +30°C) during preparation and testing and then returned to deep frozen storage (-50°C to -90°C) as soon as possible.

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 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 Excel file, generally through electronic mail or via ftp site. Data are transmitted via the CLIA Director after review by the Lab Supervisor, Branch Chief, and a CDC Statistician.

For NHANES, data are 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.

For smaller studies, electronic copies of a data report are sent; a hard copy of the data report may also be sent if requested.

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

This protocol does not involve referral of specimens for testing the analytes of this method at another laboratory.

The STARLIMS 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 are retained for at least 1 year after results have been reported and may then be returned or discarded at the request of the principal investigator. Very little residual material will be available after NHANES analyses are completed; however, residual serum is retained for at least 2 years after results have been publicly released; at that point, samples with sufficient volume (>0.2 mL) are returned to NHANES and samples with insufficient volume may be 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 database and the specimens stored deep frozen (-50°C to -90°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 results are loaded into the database. The analyst is responsible for keeping records of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies. In general, these are documented using codes in STARLIMS.

19. Method Performance Documentation

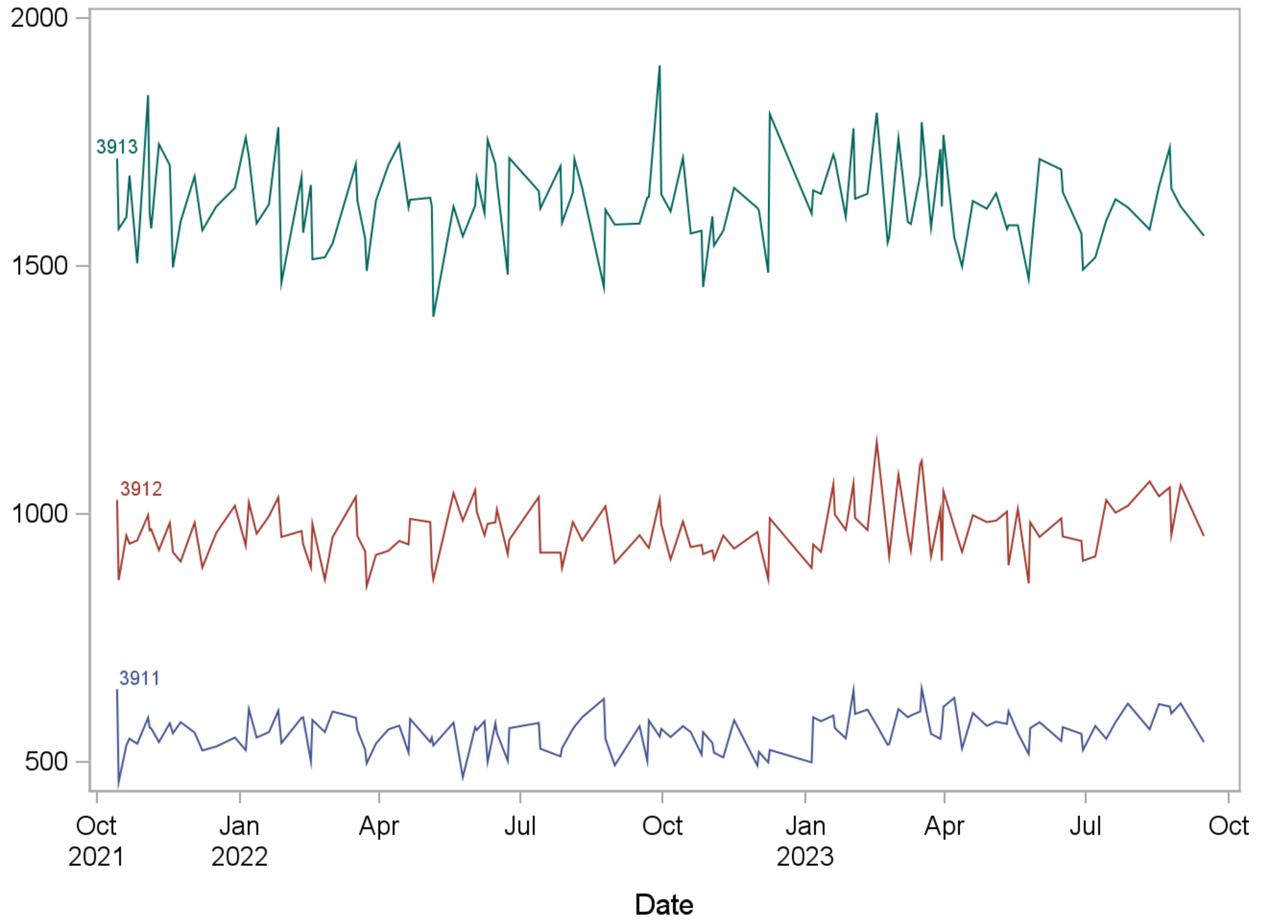
Method performance documentation for this method including accuracy, precision, sensitivity, specificity, and stability is provided in **Appendix A** of this method documentation. The approval of this procedure by the Branch Chief and CLIA Director denote that the method performance is fit for the intended use of the method.

20. Smmary Statistics and QC Graphs

Please see following page.

August 2021-August 2023 Summary Statistics and QC Chart
LBXRBF (RBC folate (ng/mL))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
3911	124	14OCT21	15SEP23	560.7	36.8	6.6
3912	123	14OCT21	15SEP23	965.6	54.9	5.7
3913	124	14OCT21	15SEP23	1627.5	87.7	5.4



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(8) 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.

(9) Institute of Medicine, Food and Nutrition Board. Dietary reference intakes: Thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington, D.C.: National Academy Press; 1998.

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Appendix A: Method Performance Documentation

Accuracy using Spike Recovery

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Serum and WB folate by microbiologic assay
Method #:	4000B and 4000S
Matrix:	Serum or WB
Units:	nmol/L
Analyte:	SFOL and WBF

		Serum sample 1: LS14620					Serum sample 2: HS14622						
			Measured concentration					Measured concentration					
	Replicate	5MeTHF Spike concentration	Day 1 (9/15/2017)	Day 2 (10/5/2017)	Mean	Recovery (%)	Spike concentration	Day 1 (9/15/2017)	Day 2 (10/5/2017)	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	18.9	18.9	18.4		0	45.3	43.8	44.0		94.1	10.5
	2		18.6	18.3				46.7	41.8				
	3		17.6	18.0				45.7	40.8				
Sample + Spike 1	1	5	22.2	23.0	23.1	94.1	5	51.8	47.5	48.8	95.9		
	2		23.5	24.5				48.7	47.8				
	3		21.2	24.2				49.7	47.3				
Sample + Spike 2	1	10	29.0	27.2	27.5	90.5	10	56.6	54.8	55.4	113.6		
	2		28.0	27.6				56.2	52.9				
	3		26.7	26.3				57.7	54.0				
Sample + Spike 3	1	20	36.8	33.471	35	84.2	20	61.1	61.7	61	86.4		
	2		37.3	34.181				66.1	59.3				
	3		36.6	33.033				62.1	57.5				

		WB Sample 1: LB14810_MA					WB Sample 2: HB14812_MA						
			Measured concentration					Measured concentration					
	Replicate	5MeTHF Spike concentration	Day 1 (1/18/2018)	Day 2 (1/25/2018)	Mean	Recovery (%)	Spike concentration	Day 1 (8/23/2017)	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1		217	201	208.1			707	602	646.8		97%	4%
	2		207	202				673	636				
	3		217	204				636	628				
Sample + Spike 1	1	22.1	454	429	446.9	98%	22.1	930	862	885.5	98%		
	2		495	398				909	851				
	3		487	419				886	875				
Sample + Spike 2	1	35.4	623	553	604.4	102%	35.4	1012	988	1033.7	99%		
	2		661	591				1093	1035				
	3		660	538				1081	993				
Sample + Spike 3	1	57.5	773	786	784	91%	57.5	1303	1236	1250	95%		
	2		804	753				1273	1140				
	3		793	794				1320	1226				

Accuracy Compared to Reference Material

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name:	Serum and WB folate by microbiologic assay
Method #:	4000B and 4000S
Matrix:	Serum or WB
Units:	nmol/L
Analyte:	SFOL and WBF
Date :	Feb-July 2017

Reference material	Replicate	Nominal value	Measured serum folate concentration (nmol/L)								Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	
NIST 1955 Level 1	1	5.4	4.2	5.1	6.0	4.1	5.0	4.92	0.62	12.50	-8.8
	2		4.3	5.5	5.4	4.5	5.1				
NIST 1955 Level 2	1	13.5	10.2	11.9	14.2	9.8	11.5	11.5	1.43	12.35	-14.5
	2		10.5	12.1	13.5	10.8	11.1				
NIST 1955 Level 3	1	42.3	39.0	41.4	41.7	33.8	37.2	38.5	2.66	6.91	-9.0
	2		36.0	41.9	39.3	37.3	37.3				

Reference material	Replicate	Nominal value	Measured serum folate concentration (nmol/L)								Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	
NIBSC 03/178	1	12.1	10.1	12.6	12.7	10.7	11.8	11.58	1.05	9.08	-4.3
	2		10.7	11.9	13.2	10.6	11.6				

Reference material	Replicate	Nominal value	Measured WB folate concentration (nmol/L)								Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	
NIBSC 95/528	1	28.327	25.6	26.5	25.3	25.6	24.3	25.81	0.96	3.72	-8.9
	2		25.6	25.1	27.8	26.6	25.7				

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Serum and WB folate by microbiologic assay
Method #:	4000B and 4000S
Matrix:	Serum or WB
Units:	nmol/L
Analyte:	SFOL & WBF

Serum quality material 1			LS14620a_MA (nmol/L)			
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	18.6	19.8	19.25	0.36	0.36	740.74
2	20.7	20.0	20.34	0.14	0.14	827.51
3	18.8	19.7	19.23	0.19	0.19	739.78
4	22.8	18.8	20.77	3.97	3.97	862.83
5	19.4	20.1	19.76	0.11	0.11	781.23
6	19.6	18.6	19.13	0.25	0.25	731.88
7	20.8	21.3	21.04	0.08	0.08	885.62
8	20.6	21.6	21.10	0.22	0.22	890.59
9	20.0	21.5	20.76	0.60	0.60	861.54
10	21.7	19.5	20.58	1.23	1.23	846.66
Grand sum	404	**Grand mean**	20.2			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	14.29	1.43	1.20	5.92		
Between Run	11.00	1.22	0.00	0.00		
Total	25.29		1.20	**5.92**		

Serum quality material 2			HS14622a_MA (nmol/L)			
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	46.8	48.8	47.8	1.1	1.1	4570
2	49.4	50.9	50.1	0.5	0.5	5028
3	49.0	51.4	50.2	1.5	1.5	5038
4	53.5	50.0	51.8	3.0	3.0	5361
5	51.5	53.9	52.7	1.5	1.5	5560
6	52.2	49.2	50.7	2.1	2.1	5142
7	54.7	53.1	53.9	0.7	0.7	5811
8	50.0	50.4	50.2	0.0	0.0	5041
9	50.1	50.8	50.4	0.1	0.1	5087
10	51.4	51.0	51.2	0.0	0.0	5243
Grand sum	1018	**Grand mean**	50.9			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	21.16	2.12	1.45	2.86		
Between Run	49.27	5.47	1.30	2.55		
Total	70.43		1.95	**3.83**		

WB quality material 1			LB14810a_MA (nmol/L)			
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	242	244	243.0	1.0	1.0	118098
2	235	218	226.5	72.3	72.3	102605
3	244	286	265.0	441.0	441.0	140450
4	257	269	263.0	36.0	36.0	138338
5	249	218	233.5	240.3	240.3	109045
6	239	273	256.0	289.0	289.0	131072
7	212	228	220.0	64.0	64.0	96800
8	275	264	269.5	30.3	30.3	145261
9	244	241	242.5	2.3	2.3	117613
10	251	241	246.0	25.0	25.0	121032
Grand sum	4930	Grand mean	246.5			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	2402.00	240.20	15.50	6.29		
Between Run	5067.00	563.00	12.70	5.15		
Total	7469.00		20.04	8.13		

WB quality material 2			HB14812a_MA (nmol/L)			
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	744	717	730.5	182.3	182.3	1067261
2	696	680	688.0	64.0	64.0	946688
3	758	813	785.5	756.3	756.3	1234021
4	747	739	743.0	16.0	16.0	1104098
5	701	649	675.0	676.0	676.0	911250
6	740	774	757.0	289.0	289.0	1146098
7	626	630	628.0	4.0	4.0	788768
8	790	761	775.5	210.3	210.3	1202801
9	723	715	719.0	16.0	16.0	1033922
10	711	784	747.5	1332.3	1332.3	1117513
Grand sum	14498	Grand mean	724.9			
	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	7092.00	709.20	26.63	3.67		
Between Run	42817.80	4757.53	44.99	6.21		
Total	49909.80		52.28	7.21		

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions

Describe condition: QC material thawed three times (3 hrs at room temperature) and re-frozen at -70°C (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: Diluted QC material in glass tubes at room temperature for 5 hrs, then pipetted into plates and incubated (9/26/17)

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: Plates were read after incubation according to protocol (initial measurement), then were kept at room temperature for ~7 hrs and read again for comparison. Reading date: serum (9/15/17); whole blood (9/11/17).

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: QC samples were stored at -70C since Aug 2014. Initial measurement: serum (4/21/15); whole blood (12/2/14). Stability was tested after 2-3 years: serum (9/1/2017); whole blood (8/11/17).

All stability sample results should be within ±15% of nominal concentration

Method name:	Serum and WB folate by microbiologic assay
Method #:	4000B and 4000S
Matrix:	Serum or WB
Units:	nmol/L
Analyte:	Total folate

Serum Quality material 1 — **LS14620_MA**

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	19.2	18.6	18.20	19.54	18.00	18.94	21.0	21.0
Replicate 2	19.3	19.3	18.03	20.47	19.41	20.57	22.5	19.9
Replicate 3	18.8	19.3	17.61	19.26	20.86	20.05	22.3	19.5
Mean	19.1	19.1	17.95	19.8	19.42	19.9	21.94	20.11
% difference from initial measurement	--	**-0.1**	--	**10.1**	--	**2.2**	--	**-8.3%**

Serum Quality material 2 — **HS14622_MA**

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	50.6	48.5	47.0	50.7	50.6	49.6	54.4	50.6
Replicate 2	51.1	46.5	47.0	52.1	51.1	51.2	47.6	53.2
Replicate 3	48.6	46.3	51.4	52.4	48.6	51.1	50.7	48.4
Mean	50.1	47.1	48.5	51.7	50.1	50.6	50.9	50.7
% difference from initial measurement	--	**-5.9**	--	**6.7**	--	**1.0**	--	**-0.3%**

WB Quality material 1 — **LB14810_MA**

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	242	283	283	229	274	262	221	274
Replicate 2	297	261	265	251	264	273	256	264
Replicate 3	251	233	259	240	248	262	258	248
Mean	263	259	269	240	262	266	245	262
% difference from initial measurement	--	**-1.6%**	--	**-10.7%**	--	**1.4%**	--	**6.9%**

WB Quality material 2 — **HB14812_MA**

	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	734	768	825	667	799	764	710	799
Replicate 2	824	688	754	756	759	745	723	759
Replicate 3	791	747	753	710	728	652	711	728
Mean	783	735	777	711	762	720	714.8	762.0
% difference from initial measurement	--	**-6.2%**	--	**-8.5%**	--	**-5.5%**	--	**6.6%**

LOD, specificity and fit for intended use

Method name:	Serum and WB folate by microbiologic assay
Method #:	4000B and 4000S
Matrix:	Serum or WB
Units:	nmol/L

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
serum folate	2*	N/A**	Yes
WB folate	44*	N/A**	Yes

* Raw folate concentration from diluted samples must be = or > lowest calibrator (0.05 nmol/L).

The limit of detection of this method depends on the dilution factor used for samples. Samples with low folate concentrations are diluted less than samples with high folate concentrations. LOD values reported in the table are for the smallest possible dilution factor (1:20 for serum samples and 1:40 for whole blood hemolysate samples).

** Growth inhibition can occur with this assay if the sample contains an antifolate (eg, methotraxate) or some antibiotics. The growth inhibition pattern can be recognized by the large difference in results from different dilutions. A repeat analysis is conducted to confirm the observed pattern and the result is set no reportable.

Appendix B: Job Aids

A. General:

1) JA-4000-G-01-Calibration and calibration verification

a) Calibration

This assay is calibrated daily by an 11-point calibration curve using 8 replicates per point. Calibration is performed with 5-methyltetrahydrofolic acid (Merck Eprova) at 0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 0.60, 0.80, 1.0 nmol/L.

Serum folate:

- The minimum detectable concentration (LOD) is 2.0 nmol/L. It is calculated using the lowest calibrator concentration 0.05 nmol/L and the lowest dilution of 1/20.
- The reportable range is 10.0 - 100 nmol/L. It is calculated using the calibration curve range (0.05 - 1 nmol/L) and regular dilution of 1/100.
- If the raw concentration <0.05 nmol/L (the lowest calibrator), manually mask the well, repeat the sample with lower dilution and use code 115.
- If the final serum result <7.0 nmol/L (critical call limit), the sample should be reanalyzed using lower dilution than 1/100 for confirmation and use code 115. It is confirmed if lower/higher result ≤25%, report the 2nd result with code 33.
- If the raw concentration >1.0 nmol/L (the highest calibrator) or the final serum result >100 nmol/L, Gen5 software automatically masks the well, need to repeat the sample with higher dilution than 1/100 and use code 97.

Whole blood folate:

- The minimum detectable concentration (LOD) is 44 nmol/L. It is calculated using the lowest calibrator concentration 0.05 nmol/L and the lowest dilution of 1/40.
- The reportable WB folate range is 154 - 1540 nmol/L. It is calculated using the calibration curve range (0.05 - 1 nmol/L) and regular dilution of 1/140.
- If the raw folate concentration is <0.05 nmol/L (the lowest calibrator), manually mask the well, repeat the sample with lower dilution and use code 115.
- If the final WB folate result <127 nmol/L, the sample should be reanalyzed using lower dilution than 1/140 for confirmation. It is confirmed if lower/higher result ≤25%, report the 2nd result with code 33.
- If the raw concentration >1.0 nmol/L (the highest calibrator) or the final WB folate result >1540 nmol/L, the sample should be reanalyzed using higher than 1/140 dilution and use code 97.

RBC folate:

- RBC folate LOD is 110 nmol/L. It is calculated using WB folate LOD and 40% Hct.
- If RBC folate <305 nmol/L (potential deficiency), repeat the WB sample with lower dilution to confirm; if confirmed with lower result / higher result ≤25%, report the 2nd result with code 33.
- Serum folate reference range: 12.7-104 nmol/L (2.5th - 97.5th percentile; n = 23,528; NHANES 2005-2010) **[8]**
- RBC folate reference range: 505-2,490 nmol/L (2.5th - 97.5th percentile; n = 23,528; NHANES 2005-2010) **[8]**

b) Calibration verification

According to the updated CLIA regulations from 2003 (see also statement of the Joint Commission on Accreditation of Healthcare Organizations), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration materials, **and** includes a low, mid, and high value, **and** is performed at least once every six months. All of these conditions are met with the calibration procedure of this assay, and therefore no additional calibration verification is required by CLIA.

Perform calibration verification whenever any of the follow occur:

- All of the reagents used for a test procedure are changed to new lot numbers, unless the laboratory can demonstrate that changing reagent lot numbers does not affect the range used to report patient test results, and control values are not adversely affected by reagent lot number changes.
- There is major preventative maintenance or replacement of critical parts that may influence the test/s performance. This includes when the laboratory sends a test system to the manufacturer for repairs. The laboratory must check the calibration of a repaired test system before resuming patients testing and reporting results.
- Control materials reflect an unusual trend or shift, or are outside of the laboratory's acceptable limits, and other means of assessing and correcting unacceptable control values fail to identify and correct the problem.
- The laboratory has determined that the test system's reportable range for patient test results should be check more frequently.

During 2007, we participated in the **College of American Pathologists (CAP) LN5 Ligand calibration verification/linearity PT challenge twice a year** however, we couldn't get valid results with the CAP samples because of assay inhibition through some sample additive.

Analysis of international standard reference material (SRM) at least twice a year can be used to monitor assay performance over time.

The **NIST SRM 1955 Homocysteine and Folate in Frozen Human Serum** (NIST SRM 1955 Serum folate_Hcy 2015-12.pdf) has been available since the fall of 2005. This is a 3-level material that approximately covers the reportable range of the assay for serum folate:

Information values for total folate		NIST SRM 1955 – Serum Folate (Target ± uncertainties_2SD)		
	Unit	Level 1	Level 2	Level 3
LC/MS/MS (CDC)	nmol/L	6.0 ± 0.4	13 ± 1	41 ± 2
Microbiologic assay (CDC)	nmol/L	5.6 ± 1.2	14 ± 3	44 ± 11
Microbiologic assay (CDC)*	nmol/L	5.4 ± 1.2	13.5 ± 3	42 ± 11
Radioassay (BioRad) (CDC)	nmol/L	4.5 ± 0.4	10 ± 1	25 ± 3

*We recalculated the target values for the microbiologic assay using the molar conversion factor for 5-MeTHF (1 ng/mL = 2.179 nmol/L) (the original certificate value used the molar conversion factor for folic acid of 2.266).

Each level is analyzed at least in duplicate as unknowns against working standards. The yearly mean of the measured concentrations should be within ±20% of the information values determined by the microbiologic assay using 2.179 conversion factor. However, no certified concentrations for total folate are available for this material and there may be method biases.

Another serum-based reference material is the **NIBSC Code 03/178 1st International Standard For Vitamin B12 and Serum Folate** (NIBSC 03-178 Serum folate and vitamin B12.pdf) available since fall 2005.

- This is a 1-level material with an assigned content of 12.1 nmol/L total folate (when reconstituted with 1.0 mL distilled/de-ionized water), made up of 9.75 nmol/L 5-MeTHF (CV 5.5%), 1.59 nmol/L 5-FormylTHF (CV 4.2%), and 0.74 nmol/L folic acid (CV 31.6%) as measured by LC-MS/MS; 12.1 nmol/L is equivalent to 5.33 ng/mL.
- Analyze reconstituted material in duplicate as unknowns against working standards. The measured concentrations should be within ±20% of the assigned content. There may be method biases.

The only reference material for whole blood folate is the **NIBSC Code 95/528 1st International Standard 1996 Whole blood folate** (NIBSC 95-528 Whole blood folate.pdf)

- This is a 1-level material with an assigned content of 13 ng/ampoule whole blood folate (consensus value by microbiologic assays and radioassays).
- Analyze reconstituted material in duplicate as unknowns against working standards. The measured concentrations should be within ±20% of the assigned content. There may be method biases.

2) JA-4000-G-02-Alternative In-house proficiency testing

a) Background

An external proficiency testing program is not available for the analysis of serum and whole blood folate species. Because of this situation, the Audit-Sample Procedure as alternative proficiency testing program as described in the guideline of the Clinical Laboratory Standards Institute (CLSI) QMS24 "Validation of Laboratory Tests When Proficiency Testing is Not Available" **[1]** was selected. Because of the lack of other laboratories performing the same type of testing, this procedure was considered the most appropriate among those described in this guideline.

b) Principle

Aliquots of a pooled specimen are stored by the laboratory and analyzed periodically across time. Periodic analysis of aliquots of audit samples assesses imprecision of the assay. The pooled specimens are blinded in a manner that the analyst does not recognize the type of pool based on the sample ID. The Audit-Sample Procedure does not evaluate accuracy (i.e., bias), nor provide interlaboratory comparison.

c) Procedure

Generation of pools

A set of proficiency testing pools for serum and whole blood folate was generated from serum and whole blood specimens acquired from U.S. blood banks. Samples were screened for their total folate concentration using the microbiologic assay in our laboratory. Based on the concentration values for total folate, samples were selected and pooled to yield values commensurate with the PT requirements for folate and other assays. At any given time, a minimum of 6 pools were available (generally 400-600 vials/pool are prepared), from which at least 3 will be used for folate PT purposes. This is commensurate with the Audit-Sample Procedure, which states that the minimum number of pools must be at least, but not limited to, 3.

Labeling/aliquoting of pools

The resulting pools were aliquoted into vials with random IDs preventing decoding by the analyst. The randomly generated 7-digit IDs are non-repeating so that each vial has a unique identifier. Each label contains the randomly generated 7-digit sample ID number, and the corresponding barcode. The 7-digit sample ID number will be decoded by the QA officer or supervisor into the pool ID at the time of result verification. The decoding program is only available to the QA officer and supervisor. All pools were aliquoted in 500-µL increments into 2-mL Nalgene cryogenic vials and are deep frozen (-50°C to -90°C) for storage.

Characterization of pools

To generate target values, the analyst must first characterize the pools by measuring 10 separate vials from each pool in duplicate across a minimum of 10 different runs to obtain the means and standard deviations.

Receipt of samples

At least 2 times per year a proficiency testing challenge will be performed as requested by the chief of the laboratory. For that, the QA officer or supervisor will randomly select 5 vials for the analyst to run. Each sample has an assigned blinded ID (e.g., "YYYY_IH4000_Ch#_01 to 05) and the label is created by the team leader preparing the samples for the PT challenge; he/she communicates the blinded sample IDs to the QA officer. The team leader also links the blinded PT challenge samples to the pool ID in the STARLIMS PT application (starting January 2020). All blinded PT samples are given to the analyst for analysis.

Analyzing PT samples

- Allow samples to come to ambient temperature (+15°C to +30°C) before testing and ensure proper mixing
- Scan in ID's with a barcode reader to avoid transcription errors
- Test PT samples as part of regular patient workload following standard operating procedures
- PT testing is performed by staff who routinely perform the testing. Rotate the performance of PT surveys among staff when there is more than one analyst performing the test regularly
- Perform the same number of replicates for PT samples as we do for routinely tested patient samples unless in some special circumstance
- Record results on the PT result form. Retain PT samples at least until results have been reviewed/approved by supervisor

  Exception for the folate microbiologic assay (test 4000): This assay has a slow turnaround (3 days from start to obtaining result) because of a ~40 h growth period. A run can only be repeated the following week. To meet a reasonable turnaround time with PT samples, these samples are measured in duplicate and the first valid result is reported. For this assay a higher proportion of samples have to be repeated than typically because of a relatively narrow calibration range, higher variation among the replicates, and sometimes imperfect plate sealing (leaking or oxidation).

Documentation, Review, and Reporting

The analyst who tests the PT samples signs and dates the attestation form to formally acknowledge that the PT samples have been handled the same way as patient samples.

The results are approved as part of the run by the level II reviewer and then given to the QA officer or supervisor who check the data to see if at least 4 of the 5 results for each analyte are within the CLIA limits (80% is considered passing for CLIA purposes). The Technical Supervisor-Lab Chief (TS-LC) reviews and signs off on the results and also signs the attestation form to attest to the routine integration of the samples into the patient workload using the routine method.

The instrument raw results, QC SAS evaluation plot, and QC failures report are uploaded for each run into STARLIMS and saved on the network share drive in method-specific folder. The in-house PT results are also imported in STARLIMS Proficiency Testing app to generate evaluation report.

The pool characterization data are stored on a restricted shared-drive location. The pool acceptability limits are determined using the characterization mean of the appropriate pool plus or minus a factor times the appropriate pool standard deviation. The value of the factor will correspond to a 0.01 two-sided significance level [± 3SD] adjusted for the number of analytes. If the results meet that criterion, no future action is required. If less than 4 of the 5 proficiency testing samples are within the CLIA limits for a given analyte, the challenge is considered as failed and appropriate corrective action to correct this problem needs to be initiated (CAPA). After correcting the problem, another set of 5 proficiency testing samples will be requested and the proficiency testing challenge must be repeated. Patient samples will be analyzed only after the proficiency testing challenge was passed. If a challenge is passed with less than 100% (e.g., 80%), a CAPA is conducted to assess whether improvements are possible.

References

[1] Clinical and Laboratory Standards Institute (CLSI). *Using Proficiency Testing and Alternative Assessment to Improve Medical Laboratory Quality*. CLSI guideline QMS24 (ISBN 1-56238-944-0 [Print]; ISBN 1-56238-945-9 [Electronic]). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, 2016.

B. Reagents & Standards:

For details on labeling requirements, see the Division of Laboratory Sciences Safety and Quality Joint Labeling Requirements.

1) JA-4000-R&S-01-Prepare reagents and calibrator for routine assay

Prepare all reagents using deionized water with a resistivity of at least 18 MΩ·cm.

a) Sodium ascorbate solution (0.5 g/dL) (for diluting samples and standard in routine assay)

Add 2.5 grams of sodium ascorbate in 500 mL of deionized water, stir to dissolve. This solution should be prepared fresh before each assay.

b) *L. Rhamnosus* assay medium inoculated with microorganism (for routine assay, folate free)

To prepare 200 mL of assay medium: add 14.1 g of Folic Acid Casei Medium 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 6 mg of chloramphenicol (2 mL of a 3 mg/mL chloramphenicol stock solution), 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. Rhamnosus* as quickly as possible (a water bath at ~20°C can be used for quick thawing) and add 600-700 µL into the 200 mL of assay medium, keep stirring slowly. About 25 mL of assay medium is needed per microplate.

c) Calibrator dilution

Prepare calibrator working solution at ~15 min before use in a yellow light hood. The calibrator may not be stable if prepared too early before use (>30 minutes). The preparation is done in a separate room from sample preparation bench for preventing contamination.

- Thaw 1 vial of frozen stock (5-MeTHF 1 µmol/L) at ambient temperature (+15°C to +30°C) for ~15 min before dilution. Protect it from light.
- Dilute 5-MeTHF stock solution (1 µmol/L) at 1/5 (200 nmol/L) by adding 100 µL of stock to 400 µL of 0.5 g/dL sodium ascorbate solution.
- Working solution I: transfer 50 µL of diluted 5-MeTHF (1/5) with 0.5 g/dL sodium ascorbate solution in a 50-mL volumetric flask and make up to volume (1/1000 dilution; 200 pmol/L).
- Working solution II: transfer 250 µL of diluted 5-MeTHF (1/5) with 0.5 g/dL sodium ascorbate solution in a 50-mL volumetric flask and make up to volume (1/200 dilution; 1 nmol/L).

2) JA-4000-R&S-02-Prepare reagent stock solutions

a) 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 deep frozen (-50°C to -90°C). Add 1 mL into 200 mL of Folic Acid Casei medium in routine assay. This solution is stable for at least 4 years.

b) 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 deep frozen (-50°C to -90°C). Add 1 mL into 200 mL of Folic Acid Casei medium in routine assay. This solution is stable for at least 4 years.

c) Chloramphenicol stock solution (3 mg/mL, for routine assay)

Dissolve 600 mg of chloramphenicol in 4 mL of ethanol and then make up to 200 mL with deionized water, aliquot 2 mL into cryovials and store deep frozen (-50°C to -90°C). Add 2 mL of stock solution into 200 mL of Folic Acid Casei medium in routine. This solution is stable for at least 4 years.

3) JA-4000-R&S-03-Prepare calibrator stock solutions

a) Example of preparing 5-MeTHF primary stock solution I (~200 µg/mL) and II (100 µg/mL):

SPECTROPHOTOMETRIC DETERMINATION OF THE CONCENTRATION OF 5-ME-THF					MetaFolin (5-methylTHF)	
					((6S)-5-CH3-H4PteGlu-Ca	
					Company	**Eprova**
Molar absorptivity coefficient (e):					**Lot#**	LMCM0357ZX
5-MeTHF	e(pH 7, 290 nm)		31,700	L/mol*cm	**Vial# (~100 mg)**	1
					Date received	November-18
Molecular weight:					**Date opened**	11/20/18
5-MeTHF	MW		459.4	g/mol	**Weight received**	~100 mg
Eprova Ca-Salt	MW		497.5	g/mol	**Weight used to date**	18.3
					Location stored	-70C/shelf 3/Yertle
					Purity	98.2%

Procedure:

Prepare a stock solution I of Folate of ~0.2 mg/mL in 20 mM phosphate buffer with 0.1% cysteine, pH 7.2, i.e., 2 mg/10 mL

This stock solution I has a molar concentration of 0.508 mM

Remove 1 mL of the stock solution for spectrophotometric determination of the concentration.

Add to the remaining 24 **mL** of stock solution 1% ascorbic acid

Dilute 20 µL of the 1 mL (ascorbic acid free) stock solution I to 1 mL in 20 mM phosphate buffer with 0.1% cysteine, pH 7.2. Prepare two replicates

Measure the absorption of the 1:50 diluted solution against 20 mM phosphate buffer with cysteine, pH 7.2 in 1 cm Quarz cuvettes at 290 nm.

Date of analysis:	**04/14/22**	
Weight of Folate:	**5.8**	mg
Volume of Buffer in stock solution I:	**25.0**	mL
Expected conc. of Folate by weight in stock solution I:	233.6	µg/mL
Expected conc. of Folate by weight in stock solution I (corrected by salt contribution):	215.7	µg/mL

Compound	/avelengt	Absorbance	Dilution	ug/mL stock solution I	% of expected	µmol/L stock sol I
5-MeTHF	289	0.587	20	170.1	78.9	
5-MeTHF	290	0.593	20	171.9	79.7	
5-MeTHF	290	0.241	50	174.6	81.0	
5-MeTHF	290	0.240	50	173.9	80.6	
Mean				**172.6**	**80.0**	**375.8**
SD				**2.0**	**0.9**	
% CV				**1.2**	**1.2**	

Formula to determine concentration spectrophotometrically:

Conc. (ug/mL) = (Abs x Dil. Factor x 1000 x MW) / Molar absorptivity coeff.

Prepare working solution (stored frozen at -70C)

1) Dilute the stock solution I (that contains the ascorbic acid) in 1% ascorbic acid to obtain a 100 µg/mL stock solution II
(1% ascorbic acid should be nitrogen flushed & filtered before use)

mL stock solution I to be used	For 100 µg/mL	for 217.6 umol	100 µg/mL = 217.6 µmol/L	For 20 µmol/L stock II
in a 25-mL volumetric flask	14.481	**14.476**	**217.6**	**2.298**
in a 10-mL volumetric flask	5.792	**5.790**		**0.919**

2) Aliquot the 100 µg/mL stock solution II into 8 cryovials with 1.1 mL each, labeled "100 µg/mL" and freeze at -70C

3) Dilute one 100 µg/mL vial (1:10) in 0.5% ascorbic acid (nitrogen flushed & filtered) to obtain a 10 µg/mL stock solution III (1.0 mL/10 mL)

To obtain 20 µmol/L stock III add **919** µL stock II & **9.081** mL 0.5% ascorbic acid (ascorbic acid should be nitrogen flushed & filtered)

4) Aliquot the 10 µg/mL stock solution III into 20 Eppendorf vials with 0.5 mL each and freeze at -70C

b) Example of diluting primary stock II (100 µg/mL) to folate MBA stock (µmol/L)

5MeTHF concentrated stock solution II : Originally prepared on 4/14/22 by folate LCMSMS team. Stock II concentration is 100 ug/mL in 1% AA.

MBA standard stock preparation date: plan to prepare in Oct 2022

0.5% ascorbic acid (AA): Prepare 800 mL of 0.5% ascorbic acid in a clean beaker (4g in 800 mL water)

5MeTHF conversion factor:

1	ng/mL	=	2.179	nmol/L
100	µg/mL	=	217.9	µmol/L

To prepare 500 mL MBA stock solution (1 µmol/L):

217.90	µmol/L	x	**X**	uL	=	500	mL	x	1 µmol/L

x = 2294.6 µL

Blow a clean 500-mL volumetric flask with nitrogen gas (N_2) to get rid of air
Add **2294.6 µL** of 5MeTHf stock II (100 µg/mL) into the volumetric flask, add 0.5% ascorbic acid to the final volume of 500 mL.
The final concentration is 1 µmol/L (1000 nmol/L)

Aliqout 1000 vials of 400uL of MBA stock into cryovials that are blowed with N_2 before use
Store at -70°C freezer.

4) JA-4000-R&S-04-Prepare inoculation

a) Prepare growth medium (500 mL with folic acid)

- Add 23.5 g of Folic Acid Casei Medium into 500 mL of ultrapure water
- Add 100 µL of Tween-80
- Add 100 mg of chloramphenicol
- Heat to boiling, keep boiling for 2-3 minutes, then cool down to ambient temperature (+15°C to +30°C)
- Add 250 mg of ascorbic acid
- Add 750 µL of folic acid stock solution (100 ng/mL)
- Stir thoroughly and sterilize the medium by filtering through a 0.2 µm filter
- Aliquot 40 mL into sterile 50-mL tubes and keep frozen (-10˚C to -50°C).. The medium is good at least 1 year when stored frozen (-10°C to -50°C). For long term storage, store deep frozen (-50˚C to -90°C), should be stable at least 4 years.

b) Prepare *L. Rhamnosus* inoculation from frozen inoculation

- Step 1: Thaw 1 vial of *L. Rhamnosus* from deep frozen (-50°C to -90°C) and transfer ~ 1.5 mL into ~10 mL of growth medium, close the tube (not too tight) and incubate at 37±1°C for the first 24 hours. Include a blank tube that only contains medium without inoculation throughout the entire culture process to monitor contamination. Test OD at 590 nm from one of the culture tubes at different incubation time points by using sterile tips to transfer 200 µL of culture into a 96-well plate.

- Step 2: Transfer ~4 mL of the first 24-hour culture (OD ~ 0.3) into 3 new tubes containing ~10 mL of fresh medium and incubate at 37±1°C for the second 24 hours. It will produce ~ 45 mL culture with OD ~0.5.

- Step 3: Repeat Step 2 for the third 24-hour incubation if the ODs and/or culture volume are not enough.

- Step 4: Transfer ~2.5 mL of the second or third 24-hour culture (OD ~0.5) into multiple new 50-mL tubes (~15 tubes) containing 35 mL of medium and incubate at 37±1°C.

- Step 5: Monitor the log growth phase that is around ~ 20 hours incubation. When the OD reaches ≥ ~0.4 at ~20 hours incubation, mix the culture at 50/50 with 80% glycerol (sterilized by filtering through 0.2 um filter). Aliquot the mixture into 2-mL sterile cyrovials (~0.8 mL/per vial) and store deep frozen (-50°C to -90°C).

- Step 6: Test the new inoculation in a folate MBA run, prepare medium using the new inoculation. Calculate the volume that needs to be used in the 200 mL of medium based on the inoculation OD, e.g, 600 µL x OD 0.4 = 240 (usually use ~220-280). Prepare a standard plate and a sample plate including 3 levels of QC samples in duplicate and some samples. Evaluate the results by checking the blank OD and the highest calibration OD and the QC and samples results.

5) JA-4000-R&S-05-Reagents Tracking

Tracking chemicals and other materials used in the preparation of reagents, calibrators, and quality control materials

a) Reagent Preparation

- Frequently Prepared Reagents

  The following reagents specified in the APM are considered to be “frequently-prepared reagents”

  - Sodium ascorbate solution (0.5%)
  - Folic acid casei medium (folate free)

    The beaker is labeled with name of reagent. The beaker also has a label indicating to discard the reagent after each experiment. Chemicals used to prepare the reagent are documented on a reagent tracking sheets in the laboratory and in method specific network folder. The following information is included on the reagent tracking sheet:

    - Name of chemical, supplier and catalog number
    - Method and reagent(s) in which it is used
    - Lot number
    - CHaTS barcode (if available)
    - Date received, started and stopped use
    - Any important notes regarding these chemicals

    Information available on the chemicals used to prepare the “frequently-prepared reagents” identified above is tracked electronically in shared drive GML specific folder NBB_GML_GML_Tracking_Tracking - 4000

- Infrequently Prepared Reagents

  The following reagents specified in the APM are considered to be “infrequently-prepared reagents”:

  - Manganese sulfate stock solution (30 mg/mL)
  - Chloramphenicol stock solution (3 mg/mL)
  - Ascorbic acid stock solution (150 mg/mL)
  - Ascorbic acid solution (1%)
  - Glycerol (80%)
  - Folic acid casei medium for growing inoculation (with folic acid)

  Information available on chemicals used to prepare the “infrequently-prepared reagent” identified above is tracked electronically in shared drive GML specific folder NBB_GML\GML_Tracking\Tracking – 4000

b) Standards Preparation

- Stock Solutions

  The calibrator specified in the APM originates from single-analyte stock solutions. Information available on chemicals used to prepare stock solutions is tracked electronically in shared drive GML specific folder NBB_GML_GML_Tracking_Tracking – 4000 and NBB_GML_Folate MBA 4000_Standard_Standard preparation & Testing

- Working Standard Solutions

  Information available on the stock solution used to prepare the working solutions is tracked electronically in shared drive GML specific folder NBB_GML_GML_Tracking_Tracking – 4000

c) Quality Control Materials

Human serum and whole blood are used to prepare quality control materials. Available information on the human blood samples used is tracked electronically in shared drive GML specific folder NBB_GML_Folate MBA 4000_QC and NBB_LABS\QC pools

d) Other Materials

To facilitate tracking of solvents and other chemicals transferred from the original manufacturer containment into a secondary container (e.g., solvent bottle), include the expiration date provided by the manufacturer or the lot number on the secondary container.

C. Instruments:

1) JA-4000-I-01-Instrument Comparison and System Verification for Janus Liquid Handler and Plate Reader

- Instrument Comparison

  When a method is analyzed on multiple instruments, an initial instrument comparison must be conducted to establish analytical comparability and ruggedness. All calibrators, quality control materials, and multiple specimens that span the measurement range should be analyzed on each system. The same preparation should be analyzed on all systems on the same day. However, this may not be feasible. In this case, it is acceptable to prepare different aliquots of the samples and perform the analysis as close to the same time as possible. The results from each instrument should be plotted against the results from the original instrument. The parameters that are assessed are correlation (Pearson r >0.95), regression fit (r^2), and slope (m). The same data analysis and documentation procedure as described below under System Verification can be used.

- System Verification

  Following the initial instrument comparison, semi-annual system verification is performed to ensure that the systems are maintaining comparability.

  Requirement: According to DLS Policies and Procedures (section 12.2), if a DLS method is run on multiple instruments or at multiple sites, a set of at least five samples spanning the reportable range of the analytes must be run at least once every six months. The Pearson correlation coefficient of the compared results should be greater than 0.95, and if not, appropriate corrective action should be taken. In special situations, the Division Director may give written approval that the methods are sufficiently similar for the intended use of the data.

  Procedure: No separate sample preparation is needed. A set of samples spanning approximately the reportable range that were analyzed on the primary instrument, are re-analyzed shortly on the secondary instrument. The time delay should be within the processed sample stability parameters determined during method validation (e.g., sample kept in autosampler or refrigerated/frozen for a certain time).

  Data analysis:

  - Identify a subset of results (*n* ≥5) from the two analyses and describe any inclusion or exclusion criteria applied (e.g., only include samples with analyte results ≥3x the LOD and ≤ highest calibrator).
  - Determine the Pearson correlation coefficient. *Note:* Pearson correlation is a parametric test that requires normally distributed data. Most nutritional biomarkers show right-skewed analyte concentration distributions benefiting from a log-transformation to yield data that approximates a normal distribution. While Pearson analysis verifies correlation and not concordance, high concordance is expected for instrument comparisons because most critical variables are the same (measurement technique, sample preparation, operator, calibration, etc.) and only 1 variable changed (instrument).
  - Optional: Assess Lin's rho coefficient for concordance. Perform regression and Bland-Altman bias analysis. Assess whether a similar proportion of samples is <LOD on both instruments. *Note:* Most nutritional biomarkers show non-constant variance (constant CV with increasing concentration); thus, weighted Deming regression and relative (%) Bland-Altman analysis are generally preferred.

Documentation, review, and approval:

- Summarize the results in a spreadsheet that contains the raw data, the data analyses, and the summary information and request review by the supervisor. For an example, see: Instrument comparison - Template with data 4063 caffeine.xlsx
- General supervisor reviews the data and, if acceptable, approves the data. Convert summary information tab to a PDF and electronically sign the PDF in the designated field. Add the signed PDF to your electronic QA Manual. For an example, see: Instrument comparison - Example signed PDF 4063 caffeine.pdf

2) JA-4000-I-02-Janus liquid handler operation, function checks, maintenance, and performance evaluation

a) Operation, function checks, and maintenance

- Regularly check if there are any bubbles and bacteria growth in tubing
- Regularly check if screws connected to 8 pumps are tight. If not tight, tighten it by hand
- Regularly check 8 tips alignment. If not aligned, turn off the instrument and adjust the tips
- Regularly check if the racks of sample vials and glass tubes are in perfect positions for pipetting. If not, adjust the labware positions
- Wipe each tip using alcohol pad before each use
- Prime Janus with water using the default flush/wash program before starting an experiment
- Closely watch if there are any issues in Janus during a run, such as tip blockage, liquid dripping from tips, any error messages, or strange noises
- Open correct folate MBA instrument method file, operate the instrument following method protocol
- Strictly follow safety precaution rules,
    - Pause the program if need to move racks, plates or containers
    - Clean all tips using 1:200 diluted Lysol after use in each assay
    - Add concentrated bleach into liquid waste container to 1% final concentration, let it sit at least 30 minutes, and empty it into the drain

More maintenance information can be found in Janus User Guide Appendix B page 382.

b) Janus Performance Evaluation

- The Janus performance is directly assessed through:
    - Routine check of leaks, air bubbles in line, pump jam, needle blockage, bacteria growth in tubing, needle alignment, error message, etc.
    - Liquid sensor test quarterly or during PM, services, or trouble shootings
    - Function performance test during PM (once a year) and field services
    - Calibration verification at least twice a year using yellow dye or gravimetric balance
- Indirectly, the Janus performance is monitored through:
    - Routine monitoring of folate MA bench and blind QC results
    - Periodic comparison of folate MA subset sample repeat results
    - Calibration verification of folate MA using NIST SRM 1955 and NIBSC reference materials (at least twice a year)

  - Participation in UK NEQAS PT program for folate MA (twice a year)

- Comparison of Janus instruments for equivalent performance if two instruments are used:

  - At least twice a year, a set of at least five samples spanning the reportable range of the analyte of interest are run on both instruments. Pearson correlation coefficient of the compared results should be greater than 0.95, if not, appropriate remedial action should be taken.

3) JA-4000-I-03-Janus calibration verification using yellow dye

Test pipetting accuracy and precision of Janus automated liquid handler using yellow dye method. This is a fast and easy test method that can evaluate the exact pipetting steps used for the folate MBA procedure.

a) Equipment and supplies needed

- Accurately calibrated manual reference pipettes: repeater, 12-channel pipette, or single channel pipette
- Food yellow dye solutions: different concentrations for testing different volume range (filtered through 0.2µm filter)
- 96-well plate
- Microplate reader, 420 nm

b) Procedure

- Verify accurate calibration of manual reference pipettes gravimetrically for specific volumes, calculate the exactly pipetted water volumes and use the means as reference values to generate yellow dye standard curve

  - To test the step that Janus adds folate calibrator solutions into folate MBA plate, set the yellow dye test reference pipette to 25, 30, 40, 50, 60, 75, 80, and 100 µL.
  - To test the step that Janus adds 15 µL sample into glass tube, set the yellow dye test reference pipette to 10, 15, 30, and 40 µL.

- Use a 12-channel pipette to add 100 µL water into each well of a 96-well plate
- According to testing volume, choose appropriate concentration of yellow dye solution.
- Warm up the solution to ambient temperature (+15°C to +30°C).
- Use the reference pipettes to add appropriate volumes of yellow dye into indicated wells on the same plate to generate calibration curve (See Fig. 1a and 1b, yellow area)

  - To test volume range of ~1-7 µL, use yellow dye stock A (high concentration) to produce OD range ~0.6-3.5
  - To test volume range of ~10-30 µL, use yellow dye stock B (medium concentration) to produce OD range ~0.6-3.5
  - To test volume range of ~20-300 µL, use yellow dye stock C (low concentration) to produce OD range ~0.6-3.5

- Let Janus add specific volumes of the same yellow dye into indicated wells on the same plate (See Fig 1a and 1b).

- Mix the plate thoroughly (shaking on the plate reader) and read the OD in a microplate reader at 420 nm.

c) Data evaluation

- Gen5 calculates Janus pipetted volumes using the standard curve generated by the reference pipette.
- Use Excel to calculate the mean, SD, and CV of ODs and Janus pipetted volumes generated from Gen5.
- The difference of the mean volumes from Janus pipetting should be within pre-specified limits of the mean volumes from the reference pipette: within ±10% difference for 10-20 µL and within ±5% for 20-200 µL. The imprecision should be ≤5% for 15-200 µL.

Fig. 1a Plate layout to test the step that Janus pipettes 15 µL sample

Reference pipettes is calibrated and used to generated standard curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1 10.04	STD1 10.04	STD1 10.04	SPL1	SPL2	SPL3	SPL4	SPL5	SPL6	SPL7	SPL8	SPL9
B	STD1 10.04	STD1 10.04	STD1 10.04	SPL10	SPL11	SPL12	SPL13	SPL14	SPL15	SPL16	SPL17	SPL18
C	STD2 14.71	STD2 14.71	STD2 14.71	SPL19	SPL20	SPL21	SPL22	SPL23	SPL24	SPL25	SPL26	SPL27
D	STD2 14.71	STD2 14.71	STD2 14.71	SPL28	SPL29	SPL30	SPL31	SPL32	SPL33	SPL34	SPL35	SPL36
E	STD3 31.05	STD3 31.05	STD3 31.05	SPL37	SPL38	SPL39	SPL40	SPL41	SPL42	SPL43	SPL44	SPL45
F	STD3 31.05	STD3 31.05	STD3 31.05	SPL46	SPL47	SPL48	SPL49	SPL50	SPL51	SPL52	SPL53	SPL54
G	STD4 40.53	STD4 40.53	STD4 40.53	SPL55	SPL56	SPL57	SPL58	SPL59	SPL60	SPL61	SPL62	SPL63
H	STD4 40.53	STD4 40.53	STD4 40.53	SPL64	SPL65	SPL66	SPL67	SPL68	SPL69	SPL70	SPL71	SPL72

Fig. 1b Plate layout to test the step that Janus pipettes folate calibrator solutions

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1 25.4	STD1 25.4	STD1 25.4	STD1 25.4	SPL1	SPL9	SPL17	SPL25	SPL33	SPL41	SPL49	SPL57
B	STD2 30.77	STD2 30.77	STD2 30.77	STD2 30.77	SPL2	SPL10	SPL18	SPL26	SPL34	SPL42	SPL50	SPL58
C	STD3 40.29	STD3 40.29	STD3 40.29	STD3 40.29	SPL3	SPL11	SPL19	SPL27	SPL35	SPL43	SPL51	SPL59
D	STD4 50.39	STD4 50.39	STD4 50.39	STD4 50.39	SPL4	SPL12	SPL20	SPL28	SPL36	SPL44	SPL52	SPL60
E	STD5 59.99	STD5 59.99	STD5 59.99	STD5 59.99	SPL5	SPL13	SPL21	SPL29	SPL37	SPL45	SPL53	SPL61
F	STD6 75.57	STD6 75.57	STD6 75.57	STD6 75.57	SPL6	SPL14	SPL22	SPL30	SPL38	SPL46	SPL54	SPL62
G	STD7 80.2	STD7 80.2	STD7 80.2	STD7 80.2	SPL7	SPL15	SPL23	SPL31	SPL39	SPL47	SPL55	SPL63
H	STD8 100.62	STD8 100.62	STD8 100.62	STD8 100.62	SPL8	SPL16	SPL24	SPL32	SPL40	SPL48	SPL56	SPL64

4) JA-4000-I-04-Microplate reader operation, function checks, maintenance, and performance evaluation

a) Operation, function checks, and maintenance

- Turn on reader ~30 min before use
- Check reader function with each use, e.g., abnormal sound, shaking issue, plate holder jam
- Keep reader clean and free of dust
- Decontaminate when needed:
    - Turn off and unplug the instrument
    - Prepare 0.5% sodium hypochlorite (NaClO, or beach)
    - If use commercial bleach (10% NaClO), prepare 1:20 dilution by mixing 10 mL bleach mixed with 190 mL water. If use household bleach (5% NaClO), prepare 1:10 dilution by mixing 10 mL bleach mixed with 90 mL water.
    - Wipe down the carrier and all expected surfaces of the reader with the diluted bleach solution. Wear gloves and eye protection while doing cleaning. Allow to sit for ~20 minutes.
    - Wipe down with wet paper towel for a few times. Then wipe down with dry paper towel.

b) Performance evaluation

- Reader performance is directly assessed through routine function check: sound, shaking, plate holder jam.
- Indirectly, the reader performance is monitored through:
    - Reader system test monthly
    - Yellow dye liquid test twice a year
    - Calibration verification using Absorbance Plate at least twice a year
    - Participating in PT CAP-I program twice a year
    - Routine monitoring folate MA bench & blind QC results, folate calibration verification results, result reproducibility
- Comparison of reader instruments for equivalent performance if 2 readers are used:
    - At least twice a year, compare readers that are used for sample analysis by reading same plates. Pearson correlation coefficient of the compared results should be greater than 0.95, if not, appropriate remedial action should be taken.

5) JA-4000-I-05-Microplate reader calibration verification using Absorbance Test Plate

BioTek Absorbance Plate Test is to confirm the mechanical alignment; channel-to-channel variability; optical density accuracy, linearity, and repeatability; and wavelength accuracy of the PowerWave.

The Absorbance Test Plate needs to be sent to manufacture for annual calibration verification and receive certification. The certified plate is used to test the readers.

a) Setup Absorbance Test Plate in Gen5

- Open Gen5, click "System Menu_System_Diagnostics_Test plate_Add/modify plates…" to "Add…"plate
- Choose the plate type using the part number (P/N), enter the serial number of the test plate, enter the certification dates, and enter the date of next certification
- Use the Absorbance Test Plate Calibration Certificate supplied in the box with the test plate, enter the values of the Absorbance OD standards
- Select anywhere from 1 to 4 Peak Wavelength Accuracy tests that you want the system to preform every time you run the test. Enter the expected peak wavelength (using the values from the certificate provided) and enter the test range. Click "OK" and "CLOSE" the window.

b) Run the Absorbance Test Plate Calibration check in Gen5

- Open Gen5, click "System_Diagnostics_Test plate", and click "Run…"
- Enter your name under "User:" and make sure that "Perform Peak Wavelength Test" is checked.
- Hold the "Ctrl" key on the keyboard and select all of the wavelengths that you want to be tested in the calibration test
- Enter any comments under the "Comments" section and click "Start Test". Place the test plate in the BioTek plate reader and click "OK". The program will perform the peak wavelength tests and test the selected wavelength in duplicates.
- The screen will prompt you to rotate the plate 180°, click "OK" to continue the program.
- When completed, the "Test Plate Status" displays the results. Remove the test plate and place back in the casing.
- At the end of a Test Plate Run, "Test Plate Status" pops up, click "Save As" in \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_GML\GML_Instruments\Reader I-IV\Reader Check - Calibration Verification
- Supervisor will review and sign the results.
- To view diagnostics history, click" System Menu_System_Diagnostics_History…"

6) JA-4000-I-06-BioTek Reader yellow dye liquid test

This manufacture procedure tests repeatability and alignment, and reveals any problems with the optics of the system.

Required Materials:

- BioTek QC Check Solution No. 1 (PN 7120779, 25 ml; or PN 7120782, 125 ml)
- Deionized water
- 5-ml Class A volumetric pipette
- 100-ml volumetric flask

Preparation of Stock Solution:

- Pipette a 5-ml aliquot of BioTek QC Check Solution No. 1 into a 100-ml volumetric flask. (Note: to preserve volume, prepare a 1:20 dilution in a 25 mL volumetric flask by adding 1.25 mL of BioTek QC Check Solution No. 1.)
- Make up to 25 ml with DI water; cap and shake well.
- This should create a solution with an absorbance of about 2.000 OD when using 200 µl in a flat-bottom microwell. The OD value will be proportional to the volume in the well and the amount of QC Check Solution No. 1 used. You can use a larger or smaller well volume, or add more Check Solution or water to adjust the stock solution. Note that too small a well volume may result in increased pipetting-related errors.

Liquid Test Procedure

Note: A 96-well, flat-bottom microplate is required for this test (Corning Costar® #3590 is recommended). We use Thermal Fisher plate. Be sure to use a new microplate; any fingerprints or scratches may cause variations in the turnaround reading.

- Using freshly prepared stock solution (See Procedure A on page 66 or Procedure B on page 67), prepare a 1:2 dilution using deionized water (one part stock, one part deionized water; the resulting solution is a 1:2 dilution). The concentrated stock solution should have an optical density of approximately 2.000 OD or lower.
- Pipette 200 µL of the concentrated solution into the first column of wells in the microplate.
- Pipette 200 µL of the diluted solution into the second column of wells.

Important: After pipetting the diluted test solution into the microplate and *before* reading the plate, we strongly recommend shaking the plate at Variable speed for four minutes. This will allow any air bubbles in the solution to settle and the meniscus to stabilize. Alternatively, wait 20 minutes after pipetting the diluted test solution before reading the plate.

- Read the microplate five times at 405 nm using the normal/standard reading mode, single wavelength, no blanking (“Normal” plate position).
- Without delay, rotate the microplate 180° so that well A1 is now in the H12 position. Read the plate five more times (“Turnaround” plate position).

The ten sets of raw plate data can be exported to your own Excel spreadsheet using Gen5™, KC4™, or KCjunior™. The mathematical computations described on the following page may then be performed and the template kept for future data reduction.

Alternatively, you can type or paste the raw plate data into the supplied Liquid Test Worksheet. The worksheet performs data reduction and indicates PASS or FAIL for each well under test.

Channel-to-Channel Variation:

- Calculate the mean value for each physical well location in columns 1 and 2 for the five plates read in the Normal position, and then again for the five plates read in the Turnaround position. This will result in 32 mean values.
- Perform a mathematical comparison of the mean values for each microwell in its Normal and Turnaround positions (A1/H12, A2/H11, B1/G12, B2/G11, and so on). In order to pass this test, the differences in the compared mean values must be within the accuracy specification for the instrument.

  Example comparison calculation:

  If the mean value for well A1 in the Normal position is 1.902, where the specified accuracy is ± 1.0% ± 0.010 OD, then the expected range for the mean of the same well in its Turnaround (H12) position is 1.873 to 1.931 OD. 1.902 * 0.01 + 0.010 = 0.029; 1.902 - 0.029 = **1.873**; 1.902 + 0.029 = **1.931**

  Note: If any set of mean values is out of the expected range, review the other three sets of mean values for the same channel pair. For example, if the A1/H12 comparison fails (the wells are not within the expected range of each other), review the comparisons of A2/H11, H1/A12, and H2/A11. If two or more sets of mean values for a channel pair are out of the expected range, there is a problem with one of the eight read channels. If only one of the four mean values results in a failure, check the well for debris and the plate for scratches or fingerprints.

  Accuracy Specification:
  For comparison in this test, the following accuracy specifications are applied, using Normal/Standard mode and a 96-well microplate:
  ± 1.0% ± 0.010 OD from 0.000 to 2.000 OD
  ± 3.0% ± 0.010 OD from 2.000 OD to 3.000 OD

More detailed information can be found in manufacture manual: **BioTek Qualification Procedures_Appendix C, Page 58**

7) JA-4000-I-07-Seal plates using a heat plate sealer

- Pre-set the heat sealer to 130°C. When the light stops flashing, the temperature is stable and ready to use. Seal each plate when the temperature is stable.
- Apply manual sealing to the plates using a flat object, press the sealing membrane down to remove residual air.
- Place a piece of aluminum foil on top of the sealing membrane to protect the samples from overheating.
- Place the plate on the holder, and apply heat and pressure for 5 sec.
- Remove the foil membrane. Quickly press the membrane with a Kim-Wipe tissue and then immediately place an ice pack on top of the plate for a few seconds to cool down the membrane.

Apply additional manual sealing to prevent leakage and thus improve precision: using a flat object, apply pressure to the surface of the sealing membrane, then carefully seal all edges and corners.

D. Data review:

1) JA-4000-DR-01-Computerization and Data System Management

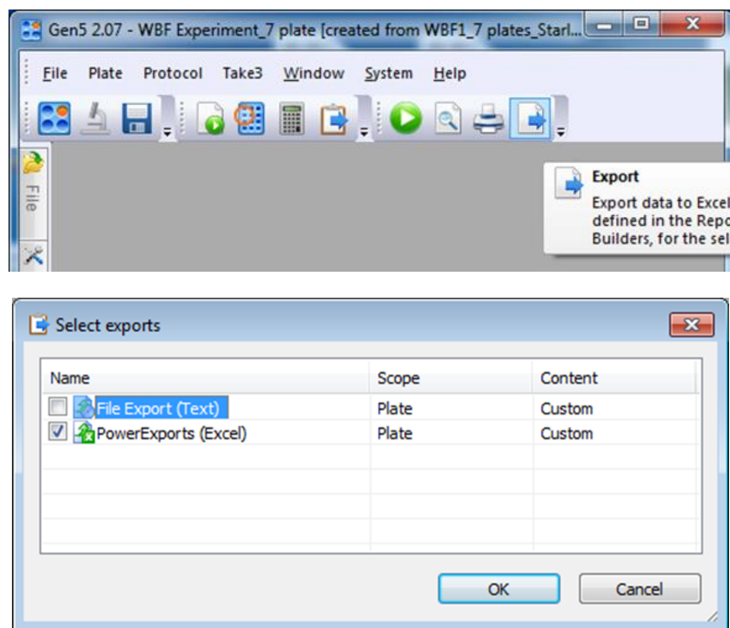
a) Sample Identification

During sample preparation and analysis, samples are identified by sample IDs. A sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by sample collectors.

b) Data Collection

The data is collected, analyzed, and stored using the GEN 5 Data Analysis software (Bio-Tek Instruments, Winooski, VT) which runs the PowerWave microplate readers. Three files are generated: (1) FOL MA Protocols containing serum and whole blood MBA protocols; (2) FOL MBA Experiments containing raw data for each assay such as sample IDs, standard curve and results (OD, final folate concentration); and (3) FOL MBA Power Export containing results for standard curve, QCs, and patient samples in Excel format. These files are stored on the network share drive in a method-specific folder (NBB_GML_Folate MBA 4000) and the third file is uploaded into STARLIMS.

To generate Power Export report, click on “Export” and select Power Exports (Excel), and save the report



- Add required experimental information of microorganism lot, calibrator lot, Janus, reader, Janus folate MBA method file name, medium lot and analyst in the Power Export report ONLY in the in assigned location: 8C – 8J and 9C-9J (see example below). The information will be imported into Starlims.
- Add any additional information in the area on the right side of the calibration curve. The information will be not automatically imported into Starlims.

- Add note for a sample on the column M and the first row of the 4 replicates (see example below)

WB Folate MA

Field Group

Reading Date/Time (Plate 1):	5/31/2018 7:43	L.casei batch	From Sean, OD 0.427 08/2014	Instrument	JANUS I - DG09106820	Reader	Reader I - PowerWave 200001		
Experiment File Name:	052918 WBF MX N3209-3210_R61.xpt	Calibrator batch	LCMS 5MeTHF 12/2015 (stock II),02/2018	FOL WB Janus I method	WBF 2-step std+1-step sample 04/01/2015	Medium Lot #	5362942	Analyst	Mary Xu

Note: Today used new L.casei Sean OD: 0.427 Aug-14

Curve [590 raw]

.200 .000 .800 .600 .400 .200 .000

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.00

Curve [590 raw] Fitting Results

Curve Name	Curve Formula	A	B	C	D	R2	Fit F Prob
Curve [590 raw]	Y=D*X^3+C*X^2+B*X+A	0.0947	2.19	-1.87	0.725	0.999	?????

590

Well ID	Name	Plate	Well	590	Conc-Raw	Formula [Conc X Dilution]	Conc X Dilution	Count	Mean	Std Dev	CV (%)	590 - Mean	CV (%)	Conc/Dil
BLK		1	A1	0.094	<0.000	x*1	?????	0	?????	?????	?????	0.09	3.335	
		1	B1	0.087	<0.000	x*1	?????							
		1	C1	0.092	<0.000	x*1	?????							
		1	D1	0.09	<0.000	x*1	?????							
		1	E1	0.09	<0.000	x*1	?????							
		1	F1	0.086	<0.000	x*1	?????							
		1	G1	0.087	<0.000	x*1	?????							

Sheet1

Ready

Well ID	Name	Plate	Well	590	Conc-Raw	Formula [Conc X Dilution]	Conc X Dilution	Count	Mean	Std Dev	CV (%)	M
SPL1	954550003	2	C1	0.428	0.151	X*3080	464.034	4	442.725	19.347	4.37	bright red color, code 64
		2	D1	0.423	0.147	X*3080	451.588					
		2	C2	0.577	0.283	X*1540	435.857					
		2	D2	0.566	0.272	X*1540	419.419					
SPL2	479818003	2	G1	0.398	0.127	X*3080	390.376	4	352.742	27.707	7.855	
		2	H1	0.37	0.105	X*3080	323.745					
		2	G2	0.515	0.225	X*1540	346.342					
		2	H2	0.518	0.228	X*1540	350.506					

c) Data Review

After the data from each run is carefully reviewed and appropriate notes are made, a Power Export result file (containing standard curve, patient, and QC data) is generated and imported into STARLIMS for statistical evaluation and QC check as described below. The final result files containing patient data as well as QC data are also stored on the network.

d) Data Back-up

- Power Export raw data files and sequence files from instrument computers are saved in method-specific network folder (NBB_GML_Folate MBA 4000_Pre-Macro Files) and automatedly backed up.

- Other instrument-related files from instrument computers (e.g. instrument method, experiment files) are saved in method-specific network folder (NBB_GML_Folate MBA 4000_GEN5) and automatedly backed up.

e) STARLIMS Data Review

Level I – Analyst

- Double click the STARLIMS icon on desktop
- Under 'Run-based Tasks', select 'Pending Runs Assigned to My Labs'
- Choose 'Show Pending Tests' and select '4000B or 4000S'
- Click on 'Add' and select the Instrument
- Run# and Equipment ID will be populated
- [0] Run Instrument Macro – select the result file to run macro for STARLIMS import
- [1] Upload Instrument File – import the post-macro result file to STARLIMS
- [2] Mark Null Results – click this button to properly transfer null WFOL or SFOL results
- [3] Evaluate Sample QC – check the sample QC flags according to the defined criteria
- [4] Evaluate Run QC – evaluate bench QC via the DLS SAS Multi-Rule System QC program to determine QC pass/fail
- [5] Set Run QC Statuses – set analytes pass/fail based on SAS out-of-control assessment
- [6] Attach SAS QC file – upload both the SAS input file (.csv) and output file (.pdf)
- Enter run information in Run Comment and User Field 1-10
- Click on 'Manage Attachments' and upload Level I Checklist for the run
- Click 'Finish Results' located under the test workflow steps and notify Project Lead

Level II –General Supervisor (Team Lead)

- Double click the STARLIMS icon on desktop
- Under 'Run-based Tasks', select 'Run Approval'
- Choose 'Show Pending Tests' and select '4000B or 4000S'
- Review analyst run sheet, Checklist Level I
- Review and confirm Run QC evaluation
- Assess blind QC results - click on 'Blind QC Results Only' tab, 'Assess Blind QC', 'OK'
- Print blind QC report - click on 'BQC Reports', 'All data displayed in the datagrid', 'A paper-based report from template', 'NBB Blind QC Report', 'Proceed to the SSRS Report', Save PDF
- Enter bench QC (SAS) and blind QC evaluation status in the Run Comments column
- Set results final - in 'All Results (S)' tab, click on 'Set Final' Wizard, select 'Process all samples displayed in the datagrid' and 'Run the Set Final Wizard' and click 'Proceed'
- Choose set final criteria - check 'Required Sample QC Passed' and 'Required Run QC Passed'; check 'Pass' and 'Warn' for 'Allowable Results Statuses for Set Final'; choose date range to cover runs that include the previous analysis of these samples; click 'Proceed'
- Resolve samples with retest results and set final
- Submit sample IDs and repeat instructions to the analyst to schedule the repeats
- Click on 'Manage Attachments' and upload Level II Checklist for the run
- In Run Approval tab, and click Release Run and notify QA Officer

Level III – QA Officer

- Refer to the Checklist Level III located in NBB_LABS_Databases_STARLIMS_Data review checklists

General Supervisor (Lab Chief)

- Conduct random “spot checks” to verify proper handling of lab results
- Discuss with Team Lead or QA Officer course of action on difficult questions

2) JA-4000-DR-02-STARLIMS Data Review Process and Criteria

In STARLIMS, sample QC evaluation can be accessed via ***Pending Runs Assigned to Me***, ***Pending Runs Assigned to My Labs*** or ***Run Approval*** windows. Generally, the calibration curve, bench QC and sample QC evaluation step is performed by the Analyst during the Level I run review and confirmed by the General Supervisor (Team Lead) during the Level II run review.

To perform sample QC evaluation, click on **[3] Evaluate Sample QC** in STARLIMS. This will flag each sample/analyte.

a) Check calibration curve and blank

1) Look for any calibrator of 2-11 that has been flagged for >15% different from the target value. This does not apply to calibrator 1, because at the low end of the curve calibrators with ±20% difference from target are still acceptable. If any of the other calibrators are >15% different to target, or multiple calibrators are flagged, then the run fails and is set Sample QC Rejected.

2) Excluding results from up to 2 wells from the 8 replicates per calibrator level is permitted due to leakage, instrument error, or random error. It is not permitted to exclude an entire calibration point.

3) Check polynomial 3 regression equation and R^2

   - Verify that the A, B, C, and D parameters of the regression equation are consistent with previous runs.
   - Check the CVs of each calibration point. The CVs are usually ≤ 15% at the low end of the curve and ≤ 10% at the high end of the curve.
   - Check R2
     [$R^2 > 0.98$, Status code: ***Pass*** → Action: none]

     [$R^2 > 0.95$ - ≤0.98, Status code: **Check** → Action: requires Branch Chief’s approval]

     [$R^2 \leq 0.95$, Status code: **Fail** → Action: code 26 and repeat run]

b) Check blank OD

- The mean blank OD is usually at ~0.1 - 0.2 when using *Himedia Folic Acid Casei medium*. Sometimes it is slightly elevated to ~0.2 - 0.25: ***Warn*** → Action: check QC results, if QC results pass and are close to limit means, accept the run. If mean blank QC result ≥ 0.25: **Fail** → Action: repeat the run, use code 26 for all samples.

c) Evaluate bench QC and blind QC

1) Run SAS program to evaluate bench QC. If pass, set Run QC **Pass;** if fail, set Run QC **Fail**, enter code 61 for all samples, repeat the entire run.

2) Assess blind QCs based on **JA-4000-DR-04-Blind QC Review Rules**. If **fail,** do 10% subset repeat to confirm; if any problem is found, enter code 921 for all samples, repeat the run.

d) Review sample QC for serum or WB unknown samples

1) **Pass** – sample result can be reported if all following criteria are met

   a. Raw folate concentration is ≥ 0.05 nmol/L and ≤ 1 nmol/L
   b. Final folate result has N = 4 and CV ≤ 15% or N = 3 and CV ≤ 10% (code 114)
   c. WB folate ≥ 44 nmol/L (LOD) or serum folate ≥ 2 nmol/L (LOD)
   d. WB folate ≥ 44 nmol/L (LOD) and < 127 nmol/L, or serum folate ≥ 2 nmol/L (LOD) and < 7 nmol/L, is confirmed

2) ***Check***, **Warn**, **Incomplete** and **Fail** – Flag on QC criteria needing review

   a. Result is null, use code 26, ***Check***, need to repeat
   b. WB folate < 44 nmol/L (LOD) or serum folate < 2 nmol/L (LOD), if confirmed, report “<LOD “as a final result, use code 37
   c. WB folate ≥ 44 nmol/L (LOD) and ≤ 127 nmol/L or serum folate ≥ 2 nmol/L (LOD) and ≤ 7 nmol/L

      - If the first analysis result has N = 3 and CV ≤ 10% or N = 4 and CV ≤ 15%, **Incomplete**, use code 33, repeat with lower dilution to confirm.
      - If the repeat analysis result with the lower dilution has N = 3 and CV ≤ 10% or N = 4 and CV ≤ 15%, **Warn**, use code 33 and 115, and if the difference of the lower result / the higher result is within ± 25%, report the 2nd result with code 33. If the difference is > ±25%, repeat the sample again.
      - If N < 3, or N = 3 and CV > 10% or N = 4 and CV > 15%, **Fail**, code 26, repeat

   d. WB folate > 127 nmol/L or serum folate > 7 nmol/L

      - If N = 3 and CV ≤ 10% or N = 4 and CV ≤ 15%, report
      - If N < 3, or N = 3 and CV > 10% or N = 4 and CV > 15%, **Fail**, code 26, repeat

   e. If the raw folate concentration is > 1 nmol/L, repeat with higher dilution, use code 97
   f. If the raw folate concentration is < 0.05 nmol/L, repeat with lower dilution, use code 115
   g. No reportable final result
   h. After confirmed no reportable, use “Modify SQC assessment” tool to set Result as “?????” and Final Result as “NN”, manually enter comment code

      - No reportable result due to inhibition, manually enter code 116
      - No reportable result due to invalid samples

         - Only ascorbic acid solution in sample vial and no WB added (no specific comment code assigned for this case), enter code 98.
         - Invalid hemolysate with wrong WB lysate color due to incorrect lysate solution used; or wrong WB lysate volume in sample vial (> or <1.1 mL for NH sample), manually enter code 156.

  - Ascorbic acid solution is not added into WB sample, only whole blood is present in sample vial, manually enter code 64.
  - The volume in WB lysate vial looks right, but the color is bright red, it likely has wrong solution used instead of ascorbic acid, manually enter code 35.

Because bench QC samples have prespecified acceptability criteria, the sample QC criteria are limited to unknown samples and not applied to bench QC results. Bench QC results with fewer replicates or higher CV are rare and are handled at the discretion of the supervisor.

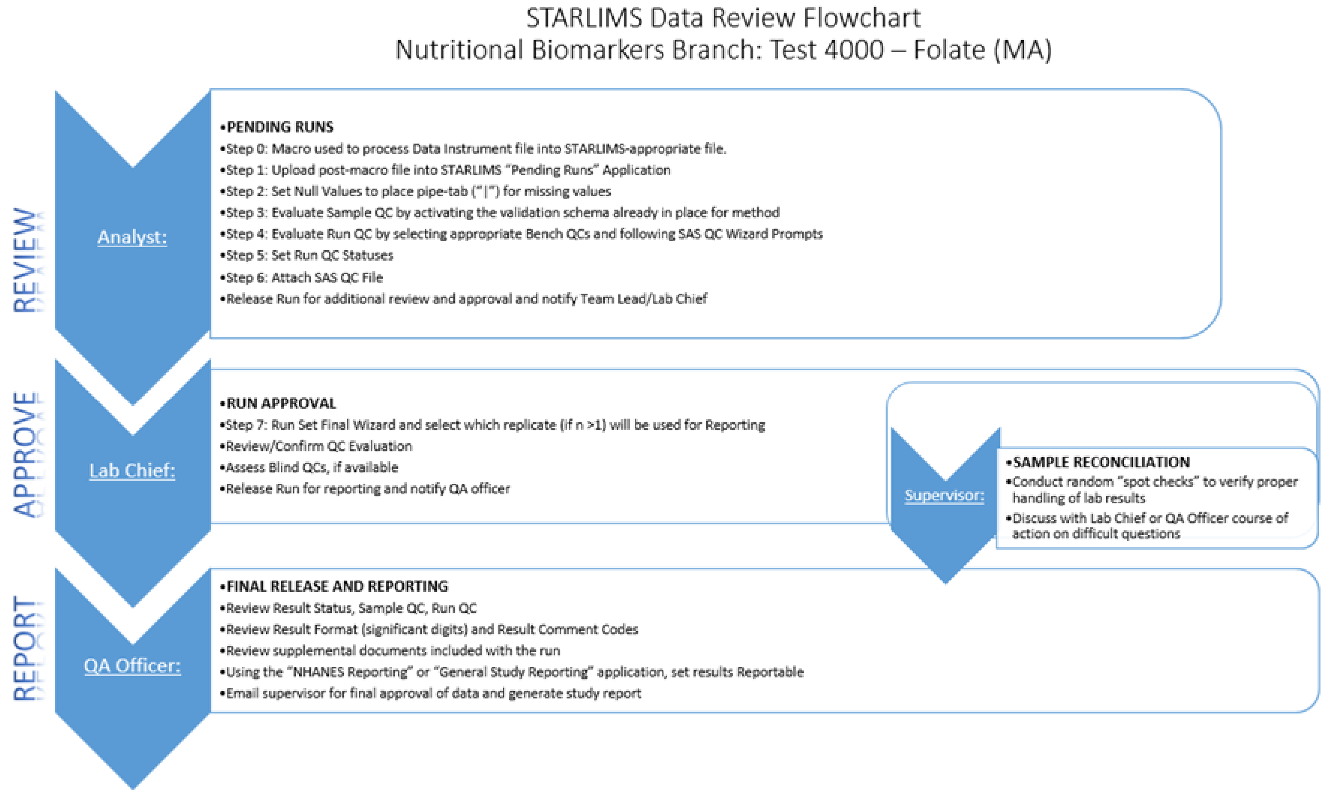
e) Check sample reproducibility

Click on “Set Final Wizard”, the multiple results of a sample are shown if there are any, review all results, make sure to set final on the result that meets bench QC and sample QC criteria.

The difference of the lower valid result / the higher valid result should be within ±25%.

3) JA-4000-DR-03-STARLIMS Data Review Flow Chart

The following instructions reflect general steps for data review, reporting, and sample reconciliation in STARLIMS. The users may deviate from this procedure as necessary.



4) JA-4000-DR-04-Blind QC Review Rules

Evaluate Blind QC results in 4000

- If maximum one blind QC result is outside 2s limits (but within 3s limits), blind QC will be accepted.
- If more than one blind QC result is outside 2s limits or one or more blind QC results are outside 3s limits, the run will be held. The failed blind QCs and a 10% random subset of patient samples need to be repeated in a subsequent run.
    - If previous patient sample results are confirmed (typically ≤25% difference between results by dividing the lower result by the higher result) and new blind QC results are within 2s limits, original patient sample results and the new blind QC results are set final. Report the original run.
    - If previous patient sample results are not confirmed (i.e., potential frame shift or racking error), add code 921 to the entire run and do not set final. The run needs to be repeated to obtain valid final results.
    - If previous patient sample results are confirmed, but new blind QC results are still out of control, original patient sample results are set final, but blind QC results in subsequent runs are monitored for potential problems or trends with these pools.

5) JA-4000-DR-05-QC Failure Corrective Actions

a) Bench QC (SAS) and Blind QC pass/fail noted in STARLIMS in the “Run Comments” column

- List failed Bench QC
- List failed Blind QC

b) Investigating QC failures and corrective action routine for SAS QC failed runs:

- Verify if the calibration curve is abnormal in ODs of each point of calibrator, curve shape, and blanks.
- If yes, the curve preparation has a problem, code the entire run with code 61 and make a note in Run Comment. Repeat the run.
- Verify analyst remarks stating a problem in result of the bench QC in question:

  If there is a problem such as no sample pickup, dark wells, leaking, or other obvious issue, mask the invalid wells and make a note, check QC through SAS. If bench QC fails, code the entire run with code 61 and make a note in Run Comment. Repeat the run.
- Verify analyst remarks stating a problem in result of the blind QC in question.

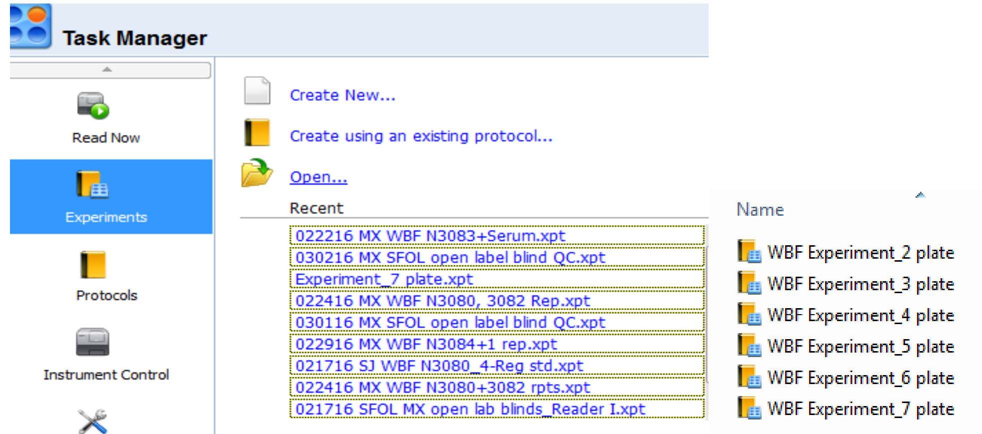
  If there is a problem such as no sample pickup, dark wells, leaking, other obvious issue or failed SQC criteria, set SQC fail and add code 26. Review blind QC results following blind QC rules.
- Track OOC and problematic runs in Data Review Status file located in method specific folder NBB_GML_Folate MBA_Database - Starlims - MA 4000_ Data Review Status. Investigate the possible cause, provide a solution and make a decision to repeat the samples.
- Track QC results in QC tracking file located in method specific folder NBB_GML_Folate MBA 4000_QC_QC tracking. Investigate trending.

c) Enter QC corrective action write-up in STARLIMS in the “Run Comments” column

- QC failure corrective action: applied corrective action routine, “outcome”

6) JA-4000-DR-06-Set Up Folate MBA Experiment in Gen5

1) Open Gen5, click on “Experiment” and “Open”, open the folate MBA experiment template saved in the method specific folder



2) Select correct plate number for your experiment

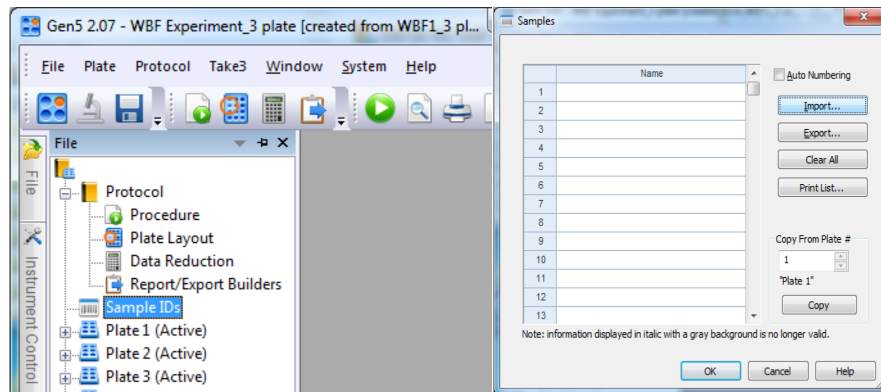
Open the experiment, save it as a new experiment with a name containing date, analyst, matrix, sample group, Janus and reader

Example: **2022-06-28 SP WB N3360-63_J I_R I**

3) Scan the sample IDs analyzed in the experiment into a Text file (notepad). The scanned sample list should be in the exact same sequence as they are measured in the plate. Temporarily save the list on the Desktop and name it by the assay date.

File Edit Format
2345787
2458076
4567890
5432678
7654321

4) Import the sample ID list into the new experiment by clicking on “Sample ID” to import the IDs from the Notepad file saved on Desktop. Click “save”.



5) Check sample dilution factors by clicking on “Protocol_Data Reduction_Transformation.” Make changes if irregular dilutions (not as default dilution) are used for all or certain samples. Save the new experiment.

Transformation

Data In: Conc-Raw

Select multiple data sets...

New Data Set Name: Conc X Dilution

Show

Data Format: <Decimal,3>

Color Effect: <None>

Formula

Use single formula for all wells

Current Formula: X*3080

Open formula editor

Apply to all wells

Mode

Edit Formula

Difference Between Rows

Difference Between Columns

Direction: A-B / 1-2 B-A / 2-1

Plate 1 | Plate 2 | Plate 3 | Plate 4 | Plate 5 | Plate 6 | Plate 7

	1	2	3	4	5	6	7	8	9	10	11	12
A	lowQC2 X*3080	lowQC2 X*1540	SPL23 X*3080	SPL23 X*1540	SPL27 X*3080	SPL27 X*1540	SPL31 X*3080	SPL31 X*1540	SPL35 X*3080	SPL35 X*1540	SPL39 X*3080	SPL39 X*1540
B	lowQC2 X*3080	lowQC2 X*1540	SPL23 X*3080	SPL23 X*1540	SPL27 X*3080	SPL27 X*1540	SPL31 X*3080	SPL31 X*1540	SPL35 X*3080	SPL35 X*1540	SPL39 X*3080	SPL39 X*1540
C	medQC2 X*3080	medQC2 X*1540	SPL24 X*3080	SPL24 X*1540	SPL28 X*3080	SPL28 X*1540	SPL32 X*3080	SPL32 X*1540	SPL36 X*3080	SPL36 X*1540	SPL40 X*3080	SPL40 X*1540
D	medQC2 X*3080	medQC2 X*1540	SPL24 X*3080	SPL24 X*1540	SPL28 X*3080	SPL28 X*1540	SPL32 X*3080	SPL32 X*1540	SPL36 X*3080	SPL36 X*1540	SPL40 X*3080	SPL40 X*1540
E	hiQC2 X*3080	hiQC2 X*1540	SPL25 X*3080	SPL25 X*1540	SPL29 X*3080	SPL29 X*1540	SPL33 X*3080	SPL33 X*1540	SPL37 X*3080	SPL37 X*1540	SPL41 X*3080	SPL41 X*1540
F	hiQC2 X*3080	hiQC2 X*1540	SPL25 X*3080	SPL25 X*1540	SPL29 X*3080	SPL29 X*1540	SPL33 X*3080	SPL33 X*1540	SPL37 X*3080	SPL37 X*1540	SPL41 X*3080	SPL41 X*1540
G	SPL22 X*3080	SPL22 X*1540	SPL26 X*3080	SPL26 X*1540	SPL30 X*3080	SPL30 X*1540	SPL34 X*3080	SPL34 X*1540	SPL38 X*3080	SPL38 X*1540	SPL42 X*3080	SPL42 X*1540
H	SPL22 X*3080	SPL22 X*1540	SPL26 X*3080	SPL26 X*1540	SPL30 X*3080	SPL30 X*1540	SPL34 X*3080	SPL34 X*1540	SPL38 X*3080	SPL38 X*1540	SPL42 X*3080	SPL42 X*1540

Undo | OK | Cancel | Help

Appendix C: Information on Absorption Maxima, Absorption Coefficient, and Formulas to Calculate the Folate Concentration

Compound	**Diluent for primary stock solution I**	**Absorption maximum (nm)**	**Molar extinction coefficient (L/mol*cm)**	**Reference**	**Molecular weight (g/mol)**
5-MethylTHF	20 mM phosphate buffer with 0.1% cysteine (pH 7.2)	290	31,700	**[1]**	459.46

Additional information can be found in reference **[2].**

Conversion factors from conventional (ng/mL) to SI units (nmol/L) for 5-MethylTHF is 2.176

Formulas to calculate the concentration of folate stock solutions based on molar absorptivity:

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

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

ε max = molar extinction coefficient and 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**

References:

(1) Gupta VS, Huennekens M. Arch. Biochem. Biophys. 1967;120:712.

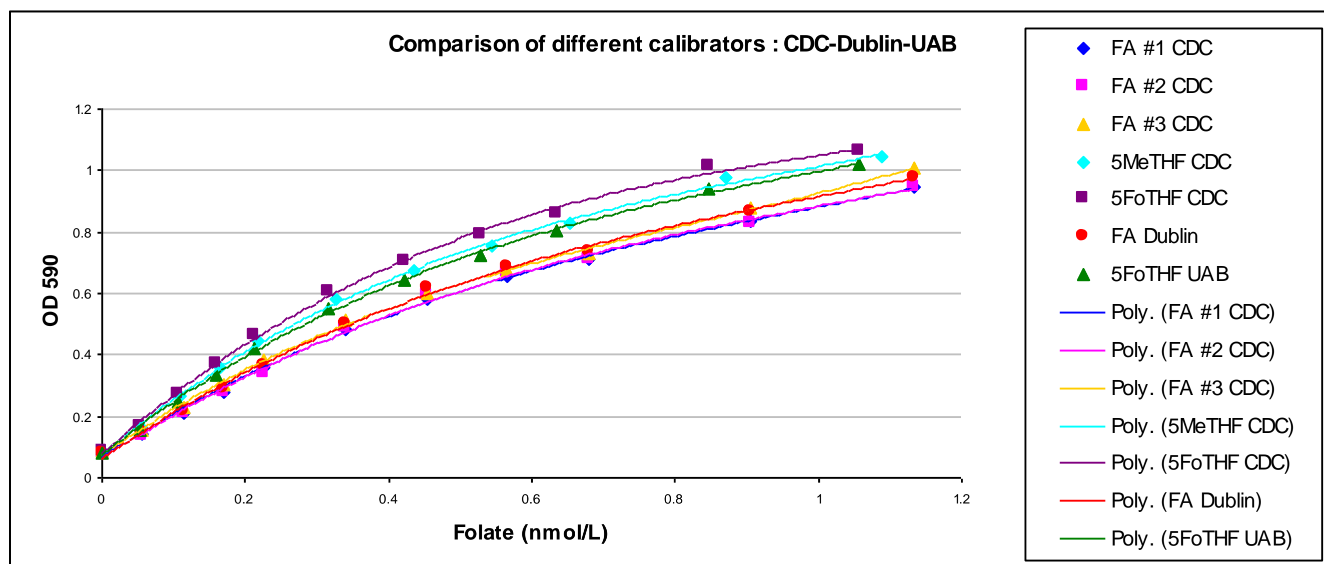
(2) Blakley R.L. The biochemistry of folic acid and related pteridines. In: Neuberger A, Tatum EL, eds. Frontiers of biology. Amsterdam: North Holland Publishing Company, 1969:91–5.

Appendix D: Information on Response of Microorganisms to Different Calibrators and on Comparison of Microbiologic Assay to other Assays

Response of *L. Rhamnosus* to different folate calibrators

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

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



Comparison of microbiologic assay to other assays

We have performed in-house comparisons of the microbiologic assay, the LC-MS/MS assay, and the BioRad assay. Microbiologic assay is still considered an accurate "reference point" for total folate. For serum samples, there is excellent correlation between the three assays. The microbiologic assay produces results that are within ±10% of the LC-MS/MS results **[2, 3]**. 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 **[4]**. We have shown that calibration of the microbiologic assay with 5-methylTHF produces results that are ~25% lower than calibration with folic acid **[1]**.

References:

(1) 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.

(2) 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.

(3) Fazili Z, Whitehead Jr. RD, Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. Anal Bioanal Chem. 2013;405:4549–60.

(4) 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.

Appendix E: 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.

A. Turbidity reading of mixed microplates

(1) 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±1°C for ~42 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.

(2) Purpose: To vary the time window within which mixed plates are read.

(3) 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 3 QC levels, each in duplicate

(4) 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.

B. Delayed turbidity reading of microplates

(1) 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 ambient temperature (+15°C to +30°C) or refrigerated (+2°C to +8°C) prior to reading.

(2) Proposal: To assess effects of delayed reading plates. Plates were first read as specified in the method and then re-read after some delay.

(3) Findings:

	Method specifies	High level a	High level b
Reading time point	Directly after ~45 h incubation	After storing refrigerated at +2˚C to +8°C for 4 days	After storing ambient temperature (+15°C to +30°C) 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%

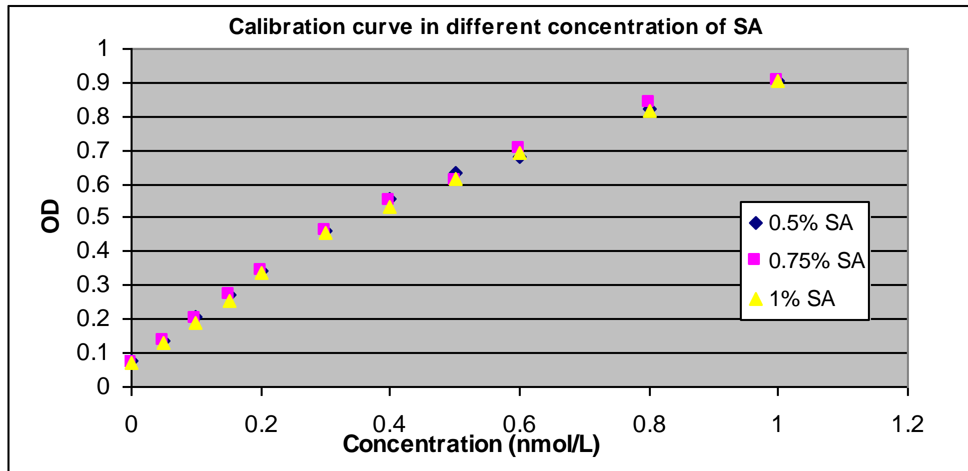
(4) Conclusion: Delaying the plate reading does not appear to affect serum folate concentrations, as long as the plate is stored overnight at ambient temperature (+15°C to + 30°C) or up to 4 days refrigerated (+2°C to +8°C).

C. Sodium ascorbate concentration

(1) 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.

(2) Proposal: To assess the potential protective effects of higher SA concentration.

(3) 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%

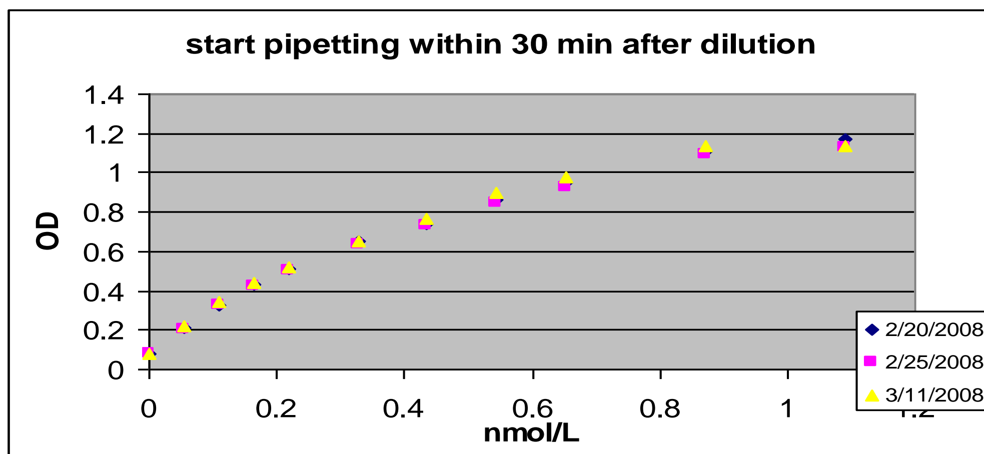
(4) Conclusion: Increasing the sodium ascorbate concentration from 0.5% to up to 1% does not appear to provide a protective effect to serum and WB folate concentrations, or to the 5-methyltetrahydrofolate standard in the calibration curve.

D. Stability of diluted calibrator

(1) 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.

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

(3) 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.



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

E. Incubation time for microorganism growth

(1) Principle: The microorganism requires ~41-43 h of incubation at 37±1°C for reproducible growth.

(2) 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.

(3) 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%)

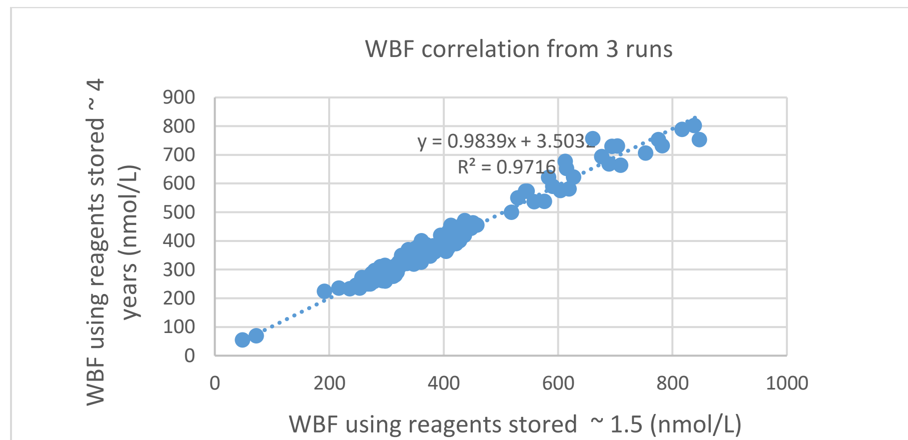
(4) Conclusion: Incubating plates for a few extra hours beyond the regular time appears to be generally ok but should be avoided if possible. Plates should not be incubated for only 24 h due to inconsistent results and high variability from sample to sample (as reflected in the relatively large SD).

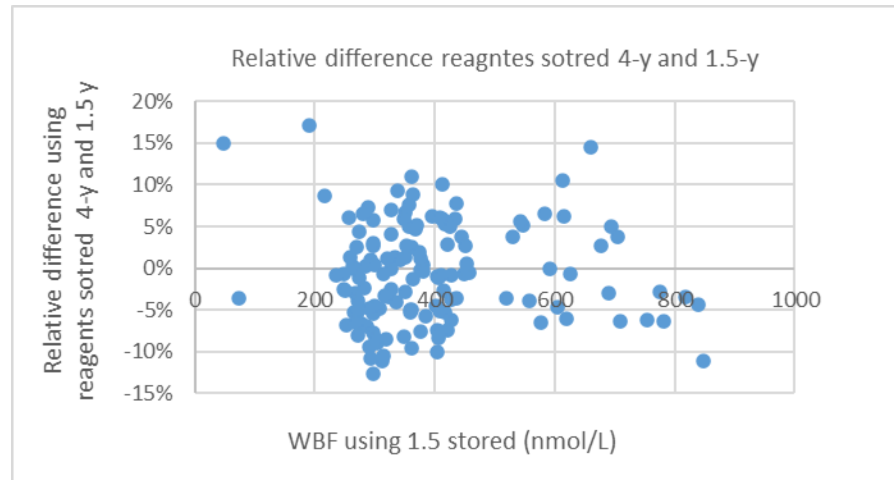
F. Stability of three reagents stored deep frozen (-50°C to -90°C)

(1) Principle: The extended stability of frozen stock solutions of ascorbic acid, chloramphenicol, and manganese sulfate stored deep frozen (-50°C to -90°C) needs to be determined.

(2) Proposal: Compare stock solutions kept deep frozen (-50°C to -90°C) for ~1.5 years vs. ~4 years

(3) Findings: The comparison data (from 3 runs with 140 WB samples) showed high correlation r=0.986 and a low mean difference of -0.7% (SD 5.9%).





(4) Conclusion: The three reagent stock solutions are stable up to 4 years when stored deep frozen (-50°C to -90°C). We changed the three reagent stocks storage date from 2 years to 4 years in Aug 2021.

G. Stability of calibrator stored deep frozen (-50°C to -90°C)

(1) Principle: The extended stability of 5-MeTHF calibrator stock solution (1 umol/L) stored deep frozen (-50°C to -90°C) needs to be determined.

(2) Proposal: Perform 4 runs using QC samples to compare 5-MeTHF calibrator stored deep frozen (-50°C to -90°C) for 4 years vs. the regular Std lot (~ 2-y storage deep frozen -50°C to -90°C) and QC limits

(3) Findings:

Mean relative difference of WBF and SFOL QC results between Std Lot 2017-08 (~4-year storage) and Regular Std Lot 2019-07 (~2-year storage) and QC Limits

	Date	N	Difference vs. Regular Std	Difference vs. QC Limits
Run 1	8/3/2021	20	-4.5%	-12.3%
Run 2	8/9/2021	20	5.3%	-3.4%
Run 3	8/11/2021	20	2.0%	-0.4%
Run 4	8/17/2021	20	7.9%	3.3%
Mean difference			**2.7%**	**-3.2%**
SD			**5.3%**	**6.6%**

(4) Conclusion: The comparison between 4-year and ~2-years storage time and QC limits showed acceptable difference (2.7 and -3.2%). We changed the calibrator storage date from 1.5 year to 4 years in Aug 2021.

Appendix F: Folate MBA Assay Procedure Steps

Step 1	1	Arrange frozen QC, blind QC and unknown samples in regular 12x4 racks; thaw in ambient temperature (+15°C to +30°C) water bath. Also thaw the 3 frozen reagent stocks
	2	Prime Janus several times using "Beginning of run" program, check water waste bottle
	3	Turn on heat sealer
Step 2	1	Prepare medium, add Tween-80, heat to boil, keep boiling for ~2-3 min, move the beaker off the heater using thermal gloves
	2	Cool down the medium at ambient temperature (+15°C to +30°C) for ~30-45 min to ~37°C
Step 3	1	Prepare sodium ascorbate (SA) solution while medium is cooling down
Step 4	1	Label glass tubes, arrange them in 12x4 racks
	2	Dispense SA into glass tubes using Digiflex
	3	Place glass tubes on Janus, cover with plastic wrap
Step 5	1	Mix sample vials in hood, remove bubbles, place samples in Janus sample rack, place rack on Janus
	2	As soon as finished mixing samples, immediately start sample dilution on Janus (from vials to glass tubes)
	3	Keep eyes on the water waste bottle, empty it if needed, add bleach to reach ~ 1% in final concentration with water waste
	4	After dilution is done, cover tubes with plastic wrap; close sample vials and temporarily keep the vials at refrigerated while entering IDs into Gen5
Step 6		If medium temperature is cooled to ~37°C (not hot to touch), add thawed 3 reagent stocks into the medium, stir ~5-10 min
If needed, it is a good time to take a quick lunch break		
Step 7	1	Add bacteria into medium ~15-30 min before pipetting medium into plates, continuously gently stir; cover the beaker with foil
Step 8	1	Label plates with #, date & analyst; place plates in correct position on Janus
	2	Janus adds medium and SA into standard and sample plates
Step 9	1	A few minutes before adding diluted samples into plates, thoroughly mix glass tubes on multitube mixer, place tubes on Janus
	2	Janus adds diluted samples into sample plates
Step 10	1	Thaw frozen standard stock in hood for ~15 min in a separate room
Step 10	1	Dilute standard, place through with work solution I & II in correct position on Janus

	2	When sample transferring is done, continue with Janus transferring diluted std into plate; move completed sample plates out of Janus.
	3	Seal all plates
Step 11	1	Start plates incubation at an appropriate time; record the time; check incubator temperature and log in the logsheet
Step 12	1	Scan sample IDs into Gen5, create experiment file; it is usually required to create the experiment on the same day as setting the assay up.
Step 13	1	Move samples back to original sample boxes and store deep frozen
	2	Clean Janus with Lysol 1:200 dilution
	3	Empty waste bottles with 1% bleach
Step 14	1	Clean all used glassware