Laboratory Procedure Manual

Analyte: **Folate Forms**

Matrix: **Blood**

Method: **Liquid Chromatography Tandem Mass Spectrometry**

Method No: 4015.06

Revised: September 2024

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 following table:

File Name	Variable Name	SAS Label
FFMR_L	LBXRF7SI	Total folate (nmol/L)
	LBXRF1SI	5-Methyl-tetrahydrofolate (nmol/L)
	LBDRF2SI	Folic acid (nmol/L)
	LBXRF3SI	5-Formyl-tetrahydrofolate (nmol/L)
	LBXRF4SI	Tetrahydrofolate (nmol/L)
	LBXRF5SI	5,10-Methenyl-tetrahydrofolate (nmol/L)
	LBXRF6SI	Mefox oxidation product (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 **[1]**. Prolonged folate deficiency leads to megaloblastic anemia. Low folate status has been causally linked to an increased risk in women of reproductive age to have an offspring with neural tube defects. Low folate status also increases plasma homocysteine levels, a potential risk factor for chronic diseases such as cardiovascular disease or cognitive function. Potential roles of folate and other B vitamins in modulating the risk for diseases (e.g., heart disease, cancer, and cognitive impairment) are under investigation.

While serum folate is an indicator of recent intake, red blood cell (RBC) folate is an indicator of long-term status. RBCs contain mainly 5-methyltetrahydrofolate (5-methylTHF) in the polyglutamate form to ensure cellular retention. In people with a polymorphism in the 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme, a portion of the methyl folate is replaced by formyl folates. The measurement of folate forms present in RBCs may elucidate the role of folate vitamers relative to various health outcomes, while the calculated total folate (tFOL, sum of the individual folate forms), provides information on the folate status of the individual. Because concentrations of the three minor folate forms THF, 5-formylTHF, and 5,10-methenylTHF represent the non-methyl folate portion of the total folate and can be a result of folate interconversions at slightly acidic pH during sample preparation **[2]**, it is recommended to express the sum of these three forms as non-methyl folate to simplify data interpretation **[3]**.

B. Test Principle

RBC folate status can be determined directly by measuring folate forms in washed RBCs **[4]** or indirectly by measuring folate forms in a whole blood (WB) lysate **[5,6]**. The direct measurement requires the addition of exogenous γ-glutamyl hydrolase (exo-GGH) to deconjugate folate polyglutamates to monoglutamates **[4]**. It also requires the measurement of hemoglobin (Hb) in the RBC lysate to correct for residual moisture (~20%) in the washed RBCs. By using the mean corpuscular hemoglobin content (MCHC), RBC folate can be calculated. The indirect measurement can either utilize the endogenous plasma-based γ-glutamyl hydrolase (no addition of enzyme; 4 h incubation at 37°C) or preferably, for faster deconjugation, use the addition of exo-GGH (30 min incubation at ambient temperature [+15°C to +30°C]). To calculate RBC folate, it requires the measurement of serum folate to correct for the serum folate contribution to WB folate and hematocrit (Hct) to normalize to the proportion of packed red cells.

Five folate forms, 5-methylTHF, pteroylglutamic acid (PGA), tetrahydrofolate (THF), 5-formyl-tetrahydrofolic acid (5-formylTHF), 5,10-methenyltetrahydrofolic acid (5,10-methenylTHF), and one oxidation product of 5-methylTHF called MeFox (pyrazino-s-triazine derivative of 4-α-hydroxy-5-methylTHF) are measured by isotope-dilution high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The current method is a modification of previously published methods **[5,6]** to add the measurement of an oxidation product **[7]** and to scale down the amount of specimen needed and increase the sample throughput **[8].** The method distinguishes 5-formylTHF from 10-formylTHF on the basis of mass transitions, however, during LC-MS/MS in the acidic mobile phase, 10-formylTHF converts within minutes to 5,10-methenylTHF and trace amounts of 5-formylTHF, THF, and 10-formyl-folic acid. Thus, this method is not capable of quantifying 10-formylTHF; formylated folates are quantified as 5-formylTHF and 5,10-methenylTHF, which are stable under these conditions **[2].**

The assay is performed by combining specimen (150 µL of WB lysate or RBC lysate) with an internal standard mixture containing exo-GGH enzyme (5 µg per mL of WB lysate or RBC lysate) and incubation at ambient temperature (+15°C to +30°C) for 30 min to deconjugate folate polyglutamates to monoglutamates prior to folate extraction. Ammonium formate buffer (1%) is added to the samples and extraction and clean-up is performed by automated 96-probe solid phase extraction (SPE) using 96-well phenyl SPE plates which takes ~1 h for a 96-well plate. Folate forms are separated within 4 min using isocratic mobile phase conditions and measured by LC-MS/MS (7 min to next injection). Quantitation is based on peak area ratios interpolated against a five-point aqueous linear calibration curve using $1/x^2$ weighting. The following analytes are quantified **(Table I)**:

Table I: Analytes and abbreviations

Compound	Abbreviation	
	Scientific literature	Database analyte code[1]
5-Methyltetrahydrofolic acid	5-methylTHF	MET
5-Formyltetrahydrofolic acid	5-formylTHF	FOT
Tetrahydrofolic acid	THF	THF
5,10-Methenyltetrahydrofolic acid	5,10-methenylTHF	MYT
Pteroylglutamic acid (folic acid)	PGA	PGA
Pyrazino-s-triazine derivative of 4-α-hydroxy-5-methyltetrahydrofolate	MeFox	MFO
Total folate (sum of folate forms)	tFOL	RBF7

[1]MET and MFO are the major analytes; FOT, THF, MYT, and PGA are the minor analytes

2. Safety Precautions

Consider all blood specimens as potentially positive for infectious agents including HIV, hepatitis B and hepatitis C. We recommend the hepatitis B vaccination series for all analysts working with whole blood and /or serum. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place all disposable plastic, glassware, and paper (pipette tips, autosampler vials, gloves etc.) that contact serum/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 after use. Also, wipe down all contaminated work surface with 1:10 diluted bleach solution (which corresponds to ~0.5-1% sodium hypochlorite) or other appropriate disinfectant when work is finished.

Handle acids (e.g., formic, and acetic acid) and bases (e.g., ammonium hydroxide; produces strong fumes; handle only in chemical fume hood) used in sample and reagent preparation with extreme care; they are caustic and toxic. Handle organic solvents (e.g., methanol, acetonitrile) only in a well-ventilated area or, as required, under a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Safety data sheets (SDSs) for all chemicals are readily available in the SDS section as hard copies in the laboratory. SDSs for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at Chemical Safety | Safety Practices | OSSAM (cdc.gov).

Additional information on hazard identification, risk evaluation, and risk mitigation for this method can be found in the method risk assessment form. Refer to the **DLS Online Safety Manual** for general information regarding protection from health hazards associated with the laboratory environment. The DLS Online Safety Manual is updated annually and can be found at https://intranet.cdc.gov/nceh-atsdr/dls/safety_manual/.

Observe universal precautions (i.e., PPE) during operation of automated liquid handlers (e.g., Hamilton, Zephyr G3 SPE); keep instrument doors always locked when it is running; wipe down contaminated decks (10% bleach solution or other appropriate disinfectant) after work is finished. Either turn off the instruments or keep in standby mode.

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 and respective batch file from the tandem mass spectrometer are collected using the instrument software and stored on the instrument workstation. The data file and batch file are transferred to the CDC network where the data file is processed into a results file that is also saved on the CDC network. Results are typically generated by auto-integration but may require in some cases manual integration. The results file (including analyte and internal standard names, peak areas, retention times, sample dilution factor, data file name, acquisition time, etc.) is imported into a STARLIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. For details, refer to **Appendix B_D: JA-4015-DR-01-Computerization and Data System Management**.

(C) The batch and the raw data file from the instrument workstation are typically backed up to the CDC network after a run is completed. This is the responsibility of the analyst under the guidance of the project lead. Files stored on the CDC network are automatically backed up nightly by ITSO support staff.

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

(A) Collecting a fasting specimen is not required to interpret RBC folate.

(B) RBC folate analysis is typically performed on frozen specimens, either WB lysate or RBC lysate. It is not recommended to freeze intact whole blood and generate the lysate later because thawing whole blood is particularly sensitive to folate degradation **[4]**.

(C) A 600-µL lysate specimen is required to allow for repeat analysis and for automated sample pipetting; a volume of 150 µL is required for analysis.

(D) WB is collected using lavender-top Vacutainers containing EDTA as an anticoagulant.

- WB lysate: 100 µL EDTA WB is added to 1.0 mL of 1 g/dL ascorbic acid, corresponding to a 1/11 dilution; the WB lysate is frozen promptly typically around -70°C (-50°C to -90°C) to keep folates in the reduced state. A Hct measurement is made at the time of blood collection to allow RBC folate calculation.

- RBC lysate: packed RBCs are triple washed with cold physiologic saline (1:1 ratio), then diluted 1/2 with saline (1+1); 100 µL diluted RBCs are dispensed into a plastic screw-capped vial and 1.0 mL of 1 g/dL ascorbic acid is added, corresponding to a 1/11 dilution; the RBC lysate is frozen promptly typically around -70°C (-50°C to -90°C) to keep folates in the reduced state. A separate hemoglobin measurement is made on the lysate at the time of the folate measurement to allow RBC folate calculation.

(E) Specimens collected in the field should be kept cold (+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, these should be kept 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 these are transferred to the testing laboratory for longer storage at deep frozen condition (-50°C to -90°C). Folates are stable for only a few weeks if the specimen is frozen (-15°C to -30°C). For long-term storage, specimens should be stored deep frozen (-50°C to -90°C). Up to three short (2 h) freeze-thaw cycles cause only minor folate degradation.

(F) Specimens should generally arrive frozen. Specimens received at ambient temperature (+15°C to +30°C) are rejected. Refrigerated intact WB samples may be used provided they are brought promptly from the site where the blood was collected. Samples should be processed for WB lysates or RBC lysates as soon as possible.

(G) Specimen handling conditions are outlined in the DLS Policies and Procedures Manual. The protocol discusses in general collection and transport of specimens and the special equipment required. If there is more than one test of interest in the specimen and it needs to be divided, the appropriate amount of blood specimen should be transferred into a sterile Nalgene cryovial labeled with the participant's ID to avoid cross-contamination.

(H) The known rejection criteria that would necessitate rejecting a specimen for blood folate analysis is when specimen volume received is < or > than expected (1.0 mL); specimen is viscous (not appropriately diluted); specimen shows precipitate, and the vial received is either damaged or leaking. However, these specimens, if processed for analysis, are assigned an appropriate description and a comment code in the database during data review. The results for these specimens are set "no reportable".

(I) A series of standard comment codes are available in the STARLIMS database to identify any issues related to sample quality (e.g., specimen not enough for initial analysis; [code 21; set no reportable]; not enough specimen for repeat analysis [code 22; set no reportable]; lab error, spills, contamination etc. [code 24; set no reportable]; or instrument error/failure [code 23; repeat analysis]). These and other 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.

(J) For information on freezer and refrigerator management refer to **SOP NBB-OC-EQUIP.01. Freezer Management** (use newest version).

(K) For information on sample receipt, tracking, and disposition refer to **SOP NBB-OC-SAMPLE.01. Sample Management** (use newest version).

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 as indicated in **Appendix B_B: JA-4015-R&S-02-Reagent 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 (for traceability) as well as the storage condition (e.g., temperature; ambient +15°C to +30°C; refrigerated +2°C to +8°C; frozen -10°C to -50°C; deep-frozen -50°C to -90°C) on the secondary container. If

the manufacturer does not specify an expiration date, the expiration date should be established and documented based on scientific merit. An expiration date can be indefinite if the material is stored under proper storage conditions and its suitability for intended use can be verified prior to use through inspection of physical properties (e.g., color, viscosity, granularity, presence of particulate matter). Alternatively, a recertification date can be established based on scientific merit. The recertification of materials should be based on their verified and documented performance with the analytical method.

A. Reagent Preparation

Prepare all reagents with filtered (0.45 µm or smaller; cellulose Nitrate-filters) deionized water with a resistivity of at least 18 MΩ·cm. Use Class A volumetric glassware where a volumetric flask is specified. 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. Log sheets maintained in the laboratory are used to track the information for chemicals used to prepare the reagent.

1) 1% Ammonium formate buffer, pH 3.2

Into a 1-L reagent bottle, add 980 mL deionized water (measured by graduated glass cylinder) and 10 mL concentrated formic acid (measured by graduated glass pipette). This mixture is titrated with ammonium hydroxide (30%) to adjust the pH to 3.2. Transfer the buffer into a 1-L graduated glass cylinder and make up to final volume with deionized water. Transfer back into the 1-L reagent bottle and degas under vacuum for 3–5 min. This buffer can be stored at ambient temperature (+15°C to +30°C) for ~1 month.

Note: 10x concentrated ammonium formate buffer can be prepared as above (use 100 mL of concentrated formic acid instead of 10 mL measured by graduated glass cylinder). This buffer can be stored at ambient temperature (+15°C to +30°C) for 6 months. At the time of use, dilute 100 mL of the 10x buffer to 1 L using deionized water and degas under vacuum.

a) Conditioning solvent for 96-well SPE plates

   1% ammonium formate buffer (pH 3.2) described above is used as is to condition the sorbent of the 95-well SPE plate (1.3 mL/well). Typically, 200 mL is used daily. Other solvents used to condition the sorbent are 0.5 mL/well each of acetonitrile and methanol.

b) **Solvent #1:** 1% ammonium formate buffer, pH 3.2, with 0.5% ascorbic acid

   Add 0.5% ascorbic acid (0.5 g/100 mL) to this buffer at the time of use (prepared for single use).

c) Buffer for folate calibrator Mix A: 1% ammonium formate buffer, pH 3.2, with 0.1% ascorbic acid.

   Add ascorbic acid (0.1 g/100 mL) to this buffer at the time of use (prepared for single use).

d) Solvent for ISTD Mix B: 0.1% ascorbic acid in deionized water.

   Add ascorbic acid (0.1 g/100 mL) to water (prepared daily for single use).

e) Wash buffer (**Solvent #2**): 0.05% ammonium formate, pH 3.4.

   Dilute 50 mL of 1% ammonium formate buffer, pH 3.2 to 1 L with deionized water in a graduated glass cylinder. Transfer into a 1-L reagent bottle, check pH, and degas under vacuum for 3–5 min. This buffer can be stored at ambient temperature (+15°C to +30°C) for 2

weeks. Add ascorbic acid powder to a final concentration of 0.1% (0.1 g/100 mL) prior to use (typically 200 mL prepared for single use).

2) Sample elution buffer (**Solvent #3**): 49% deionized water, 40% methanol, 10% acetonitrile, 1% acetic acid, 0.5% ascorbic acid.

   Using graduated glass cylinders (500 mL and 100 mL), add 490 mL of deionized water, 400 mL of methanol, and 100 mL of acetonitrile to a 1-L reagent bottle and degas under vacuum for 3–5 min. At the time of use add 1% (final concentration) acetic acid (1 mL/100 mL) and 0.5% (final concentration) ascorbic acid powder (0.5 g/100 mL). Typically, 100 mL prepared for single use.

3) HPLC mobile phase: 49.5% deionized water, 40% methanol, 10% acetonitrile, 0.5% acetic acid

   Using graduated glass cylinders (500 mL and 100 mL), add 495 mL of deionized water, 400 mL of methanol, and 100 mL of acetonitrile to a 1-L reagent bottle and degas under vacuum for 3–5 min. At the time of use, add acetic acid to a final concentration of 0.5%. Typically, 300 mL prepared for single use.

4) HPLC needle wash: 50% methanol and 50% water

   Using a 500-mL graduated glass cylinder, add 250 mL methanol and 250 mL deionized water to a 1-L reagent bottle. Solution is used by the HPLC after each injection. Prepare this solution as needed.

5) HPLC Rinse and Shutdown solvent: 90% methanol and 10% water

   Using a 500-mL graduated glass cylinder, add 450 mL methanol and 50 mL deionized water to a 1-L reagent bottle. Solution is used by the HPLC at the end of an analytical run. Prepare this solution as needed.

6) L-Ascorbic acid solution (1% or 1 g/dL, pH 2.7)

   To a 50-mL falcon tube, add 0.5 g ascorbic acid and 40 mL deionized water and mix well to dissolve. Add deionized water to the 50-mL mark. The solution should be made fresh before use.

   The same solution is also used as a lysate buffer. To prepare lysates, add 100 µL of either intact WB of saline-diluted washed RBCs into 1.0 mL of this solution (1/11 dilution), vortex mix and store at the appropriate temperature.

   If the ascorbic acid is used to dilute folate stock solutions, degas each tube under a stream of nitrogen for a few minutes and filter using 0.45 µm (or smaller) 10-mL sterile syringe filter (mixed cellulose esters) just prior to use.

7) Phosphate buffer (0.1 M)

   Dissolve 1.4 g potassium phosphate monobasic and 2.2 g dibasic in 95 mL of deionized water (measured by graduated glass cylinder), measure the pH and adjust to 7.2 with KOH. Make up to final volume (100 mL) with deionized water. Filter and degas under a stream of nitrogen for a few minutes. To obtain a 20 mM concentration, dilute 20 mL buffer to 100 mL with degassed deionized water (using graduated glass cylinder and pipette). Prepared fresh at the time of use.

8) PPG dilution solvent for mass spectrometer calibration

Dissolve 15.4 mg of ammonium acetate completely in 49.9 mL of water first. Mix 49.9 mL of methanol with 0.1 mL of formic acid and 0.1 mL of acetonitrile. Then mix the above two solutions together to make the final PPG dilution solvent. Use a 1/50 dilution (400 µL of PPG standard 2000 + 19.6 mL of PPG dilution solvent) for positive ion calibration of the SCIEX tandem mass spectrometer. This solution is stable when refrigerated (+2°C to +8°C) for 6 months. For negative ion calibration, PPG standard 3000 and diluent is supplied in the kit by the manufacturer (SCIEX).

Note: *Glassware used for reagent preparations are washed with warm soapy water and rinsed thoroughly with deionized water before use. HPLC mobile phase reagent bottle is rinsed once a week thoroughly with deionized water; washed once a month with warm soapy water followed by rinsing thoroughly with deionized water. HPLC reagent bottles for needle wash, rinse, and shutdown solutions are washed thoroughly with warm soapy water once a month and bottles are rinsed thoroughly with deionized water before use.*

B. Standards Preparation

1) Primary and intermediate individual stock solutions

The concentrations of primary stock solutions (for each individual folate) are calculated using molar absorptivity. Information on absorption maxima, absorption coefficients, and formulas to calculate the concentration are provided in **Appendix C**. **Table II** summarizes diluent information for primary and intermediate stock solutions. Information on the preparation of individual folate stock solutions is maintained on the shared network drive.

Note: *Glassware used for standards preparation are washed with warm soapy water; rinsed thoroughly with deionized water and dried with N2 gas before use.*

Table II. Diluents used for primary and intermediate stock solutions

Compound	**Diluent for primary stock solution I**	**Diluent for intermediate stock solution II (100 µg/mL)**	**Diluent for intermediate stock solution III (20 µmol/L)**
5-MethylTHF	20 mM phosphate buffer (pH 7.2) [D&F]	1% ascorbic acid [D&F]	0.5% ascorbic acid [D&F]
5-FormylTHF	20 mM phosphate buffer (pH 7.2) [D&F]	1% ascorbic acid [D&F]	0.5% ascorbic acid [D&F]
THF	20 mM phosphate buffer (pH 7.2) [D&F]	1% ascorbic acid [D&F]	1% ascorbic acid [D&F]

Compound	**Diluent for primary stock solution I**	**Diluent for intermediate stock solution II (20 µmol/L)**
5,10-MethenylTHF	1 M HCl [D]	0.5 M HCl with 1.0% ascorbic acid [D&F]
PGA	20 mM phosphate buffer (pH 7.2) D&F]	Deionized water [D&F]
MeFox	0.1 N NaOH [D&F]	Deionized water [D&F]

[D] degassed with nitrogen; [F] filtered using 0.45 µm (or smaller) sterile filters (mixed cellulose esters membrane)

a) **Preparation 5-MethylTHF, 5-FormylTHF, and THF stocks**:

5-MethylTHF, 5-formylTHF and THF reduced folate forms are treated the same way. The stock solutions for both unlabeled and $^{13}C_5$-labeled compounds (used as internal standards) are prepared in the same way as described below.

1. Prepare a **primary stock solution I (≤200 µg/mL)** in a volumetric flask by dissolving an accurately known mass (±0.2 mg) of the pure solid compound in degassed 20 mM phosphate buffer (pH 7.2, containing 0.1% cysteine), targeting a final concentration of ≤200 µg/mL (e.g., ≤5 mg in 25 mL). Vortex briefly to help dissolve the contents and make up to final volume.

2. Remove a small aliquot (~1 mL) of the primary stock solution I to a microcentrifuge vial to determine its concentration by UV spectrophotometry. From this aliquot, prepare two dilutions (e.g., 1/10 and 1/20), each in duplicate, measure the UV absorbance at the peak maximum (5-methylTHF 290 nm and 245 nm; 5-formylTHF 285 nm; THF 298 nm) using scan analysis against phosphate buffer as a blank, and calculate the concentration of primary stock solution I (**Appendix C**). For 5-methylTHF, 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.

3. To the remaining primary stock solution I, add ascorbic acid powder to a final concentration of 1%. Vortex to help dissolve the ascorbic acid. Aliquot the primary stock solution I with 1% ascorbic acid into labeled cryovials (typically 1 mL/vial for single use) and store deep frozen (-50°C to -90°C). The primary stock solution I is stable for at least 2 years.

4. Prepare an **intermediate stock solution II (100 µg/mL)** by diluting the primary stock solution I in a 25-mL volumetric flask using the diluent specified in the Table II. Aliquots of the intermediate stock solution II are stored deep frozen (-50°C to -90°C) in labeled cryovials (typically 1.2 mL/vial for single use) and used approximately every three months to generate a fresh intermediate stock solution III (20 µmol/L). The intermediate stock solution II is stable at least 2 years.

5. Prepare an **intermediate stock solution III (20 µmol/L)** by diluting a portion of the intermediate stock solution II (100 µg/mL) in the diluent specified in **Table II** (typically 10 mL volume). Aliquots of the intermediate stock solution III (typically 0.2 mL/vial for single use) are stored deep frozen (-50°C to -90°C) in labeled microcentrifuge vials and used to generate daily working solutions. This stock III is stable at least 3 months and is verified before use.

b) **Preparation for 5,10-methenylTHF stocks**:

Although 5,10-methenylTHF is also a reduced folate, it is treated differently because it is only stable at acidic pH. At neutral pH it is in equilibrium with 5-formylTHF and at alkaline pH it converts to 10-formylTHF. The $^{13}C_5$-labeled compound is used as internal standard and is prepared in the same way as described below.

1. Prepare a **primary stock solution I (~100 µg/mL)** in a volumetric flask by dissolving an accurately known mass (±0.1 mg) of the pure solid compound in 1 M HCl, targeting a final concentration of ~100 µg/mL (e.g., 5 mg in 50 mL). Vortex briefly and keep the flask for ~10 min in a beaker with warm water (~70°C) to help dissolve the contents. Vortex a few times in between. After contents are completely dissolved, keep the volumetric flask at

ambient temperature (+15C° to +30°C) for ~30 min to let it cool down. Make up to final volume.

2. Remove a small aliquot (~1 mL) of the primary stock solution I to a microcentrifuge vial to determine its concentration by UV spectrophotometry. From this aliquot, prepare two dilutions (e.g., 1/10 and 1/20), each in duplicate, measure the UV absorbance at the peak maximum using scan analysis against 1 M HCl as a blank, and calculate the concentration of primary stock solution I (**Appendix C**).

3. To the remaining primary stock solution I, add ascorbic acid powder to a final concentration of 1%. Vortex to help dissolve the ascorbic acid. Aliquot the primary stock solution I with 1% ascorbic acid into labeled cryovials (typically 1 mL/vial for single use) and store deep frozen (-50°C to -90°C). The primary stock solution I is stable for at least 2 years and used approximately every three months to generate a fresh intermediate stock solution II (20 µmol/L).

4. Prepare an **intermediate stock solution II (20 µmol/L)** by diluting the primary stock solution I in a 10-mL volumetric flask using the diluent specified in the **Table II**. Aliquots of the intermediate stock solution II are stored deep frozen (-50°C to -90°C) in labeled microcentrifuge vials (typically 0.2 mL/vial for single use) and used to generate daily working solutions. This stock II is stable at least 3 months and is verified before use.

c) **Preparation for PGA stocks:**

The solubility of PGA decreases as the pH decreases from alkaline to acidic **[9]**. To maintain optimum solubility, keep higher concentration stock solutions (µmol/L) at neutral (or alkaline) pH and ensure buffering when working at slightly acidic pH at much lower PGA concentrations (nmol/L). The $^{13}C_5$-labeled compound is used as internal standard and is prepared in the same way as described below.

1. Prepare a **primary stock solution I (~50 µg/mL)** in a volumetric flask by dissolving an accurately known mass (±0.1 mg) of the pure solid compound in degassed 20 mM phosphate buffer (pH 7.2), targeting a final concentration of ~50 µg/mL (e.g., 5 mg in 100 mL). Vortex briefly to help dissolve the contents. Keep the volumetric flask in a luke-warm water bath (~30 °C) for ~20 min to ensure complete solubility. Keep the volumetric flask at ambient temperature (+15°C to +30°C) for ~20 min to let it cool down. Make up to final volume.

2. Remove a small aliquot (~1 mL) of the primary stock solution I to a microcentrifuge vial to determine its concentration by UV spectrophotometry. From this aliquot, prepare two dilutions (e.g., 1/10 and 1/20), 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 (**Appendix C**).

3. Aliquot the remainder of the primary stock solution I into labeled cryovials (typically 1 mL/vial for single use), and store deep frozen (-50°C to -90°C). The primary stock solution I is stable for at least 2 years and used approximately every three months to generate a fresh intermediate stock solution II (20 µmol/L).

4. Prepare an **intermediate stock solution II (20 µmol/L)** by diluting the primary stock solution I in a 10-mL volumetric flask using the diluent specified in the **Table II**. Aliquots of the intermediate stock solution II are stored deep frozen (-50°C to -90°C) in labeled

microcentrifuge vials (typically 0.2 mL/vial for single use) and used to generate daily working solutions. This stock II is stable at least 3 months and is verified before use.

d) **Preparation for MeFox stocks:**

MeFox is an oxidation product of 5-methylTHF. The $^{13}C_5$-labeled compound is used as internal standard and is prepared in the same way as described below.

1. Prepare a **primary stock solution I (~100 µg/mL)** in a volumetric flask by dissolving an accurately known mass (±0.1 mg) of the pure solid compound in degassed 0.1 N NaOH (pH ~12.4), targeting a final concentration of ~100 µg/mL (e.g., 5 mg in 50 mL). Vortex briefly to help dissolve the contents and make up to final volume.

2. Remove a small aliquot (~1 mL) of the primary stock solution I to a microcentrifuge vial to determine its concentration by UV spectrophotometry. From this aliquot, prepare two dilutions (e.g., 1/10 and 1/20), each in duplicate, measure the UV absorbance at the peak maximum using scan analysis against 0.1 N NaOH as a blank, and calculate the primary stock solution concentration (**Appendix C**).

3. Aliquot the remainder of the primary stock solution I into labeled cryovials (typically 1 mL/vial for single use) and store deep frozen (-50°C to -90°C). The primary stock solution I is stable for at least 2 years and used approximately every three months to generate a fresh intermediate stock solution II (20 µmol/L).

4. Prepare an **intermediate stock solution II (20 µmol/L)** by diluting the primary stock solution I in a 10-mL volumetric flask using the diluent specified in the **Table II**. Aliquots of the intermediate stock solution II are stored deep frozen (-50°C to -90°C) in labeled microcentrifuge vials (typically 0.2 mL/vial for single use) and used to generate daily working solutions. This stock II is stable at least 3 months and is verified before use.

> ***Note**: Fresh individual primary stock solutions stock I for all folate forms and their respective $^{13}C_5$-labeled compounds are prepared approximately every 2 years. Individual intermediate stock solution II (100 µg/mL) for 5-methylTHF, 5-formylTHF and THF are prepared at the same time with primary stock solution preparation. Individual intermediate stock solutions II or III (20 µmol/L) are prepared approximately every 2 months. Analyte and internal standard stock solutions are always prepared together. In-house long-term storage stability data showed that 5-methylTHF and $^{13}C_5$-5-methylTHF stock solutions (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) **[7]**. Buffers and diluents are degassed with nitrogen & filtered using 0.45 µm (or smaller) sterile filters (mixed cellulose esters membrane) before use.*

2) Mixed calibrator solutions

At the beginning of each run, prepare a mixed calibrator (Mix A) and a mixed internal standard solution (Mix B) using intermediate (20 µmol/L) stock solutions. (Stock II: 5,10-methylene, PGA, MeFox and Stock III: 5-methylTHF, 5-formylTHF, THF).

a) Calibrator mix (Mix A)

Contains a mixture of each standard prepared in 1% ammonium formate buffer (pH 3.2), with 0.1% ascorbic acid, as shown in **Table III**. The total volume of Mix A is 1.0 mL.

Table III: Information for calibrator mix A

Mix A	5-MethylTHF	PGA	5-FormylTHF	MeFox	THF	5,10-MethenylTHF
Intermediate stock solution II 20 µmol/L (µL)	--	50	--	50	--	50
Intermediate stock solution III 20 µmol/L (µL)	100	--	50	--	50	--
1% Ammonium formate buffer with 0.1% ascorbic acid (µL)	650					
Concentration in Mix A (µmol/L)	2.0	1.0	1.0	1.0	1.0	1.0
Note: Vortex Mix A prior to use to ensure complete homogeneity						

b) Internal standard mix (Mix B)

The internal standard Mix B (total volume of 16 mL) contains a mixture of each internal standard prepared in water with 0.1% ascorbic acid, as shown in **Table IV**.

Table IV: Information for internal standard Mix B

Mix B	$^{13}C_5$-5-MethylTHF	$^{13}C_5$-PGA	$^{13}C_5$-5-FormylTHF	$^{13}C_5$-MeFox	$^{13}C_5$-THF	$^{13}C_5$-5,10-MethenylTHF
Intermediate stock solution II 20 µmol/L (µL)	--	20	--	20	--	20
Intermediate stock solution III 20 µmol/L (µL)	80	--	20	--	20	--
Deionized water with 0.1% ascorbic acid (mL)	15.82					
Concentration in Mix B (nmol/L)	100	25	25	25	25	25
Note: Vortex Mix B prior to use to ensure complete homogeneity						

Vortex Mix B (16 mL) for complete homogeneity and aliquot 7.8 mL of Mix B in a new tube for mixing with 200 µL of exo-GGH enzyme.

The concentration of commercially available exo-GGH enzyme is 500 µg/mL. For 1 mL of RBC lysate, 5 µg of exo-GGH are required for complete folate polyglutamate deconjugation (0.75 µg enzyme/150 µL RBC lysate) **[4]**. To optimize enzyme use, and avoid sample dilution, the enzyme is directly added from the original vial to the internal standard mix vial (200 µL enzyme solution containing 100 µg enzyme in 7.8 mL Mix B). Vortex this mix well before dispensing into the sample wells. The internal standard Mix B (total volume of 8.0 mL) contains a mixture of the exo-GGH enzyme, and each internal standard prepared in 0.1% ascorbic acid, as shown in **Table IV**.

3) Calibration standards

Prepare mixed calibrators S1 to S5 for the calibration curve in 1.5-mL microcentrifuge vials: add 50 µL mix A to 950 µL ammonium formate buffer (Solvent #1) to prepare the highest calibrator S5 (100 nmol/L 5-methylTHF, and 50 nmol/L each of PGA, MeFox, THF, 5-formylTHF, and 5,10-methenylTHF). Prepare calibrators S4 to S1 from calibrator S5 by using the amounts specified in **Table V**.

Table V: Information for mixed calibrators S1 to S5

Calibrator level	Calibrator mix	Solvent #1	Concentration (nmol/L): 5-methylTHF/PGA/5-formylTHF/MeFox/THF/5,10-methenylTHF
S5	50 µL Mix A	950 µL	100/50/50/50/50
S4	200 µL S5	800 µL	20/10/10/10/10
S3	40 µL S5	960 µL	4/2/2/2/2
S2	20 µL S5	980 µL	2/1/1/1/1
S1	10 µL S5	990 µL	1/0.5/0.5/0.5/0.5

C. Preparation of Quality Control Materials

Quality control materials for this assay are prepared in-house from blood products acquired from blood banks or from other volunteer blood donors. Approximate QC target values for RBC tFOL are ~350 (low), ~600 (medium), and ~1000 nmol/L (high), respectively. The low QC aims to be close to the deficiency cutoff value of 317 nmol/L. The high QC aims to be in the top third of the population distribution (75th percentile is ~1400 nmol/L). Because the United States has mandatory food fortification with folic acid, it is difficult to find donors with low RBC folate levels and the prevalence of clinical folate deficiency is <1% in the US population **[10]**.

In addition to tFOL, concentrations of individual folate forms are considered. If specimens don't contain the approximate target values for the individual folate forms as shown in **Appendix D** manipulation through spiking with standard compounds or dilution with physiologic sodium chloride solution or albumin solution (4%) is considered. It is advisable to including a few blood donors with MTHFR T/T genotype to obtain blood that has endogenous levels of THF and 5,10-methenylTHF. Not all folate forms have to be low in the "low" pool, medium in the "medium" pool, and high in the "high" pool, just as long as there is sufficient distinction between the pools.

To generate WB QC pools, fresh EDTA WB (~40 mL) is collected from blood donors. The Vacutainers are placed on a rocker for 5-10 min at ambient temperature (+15°C to +30°C); WB is then diluted with 1% ascorbic acid to achieve a 1:11 dilution. WB lysate (usually 700 µL) QC pools are aliquoted into 2.0-mL Nalgene cryovials vials for single use and stored deep frozen (-50°C to -90°C). The QC pools are stable for at least 3 years.

To generate RBC QC pools, fresh EDTA WB (~40 mL) is collected from blood donors. The Vacutainers are placed on a rocker for 5-10 min at ambient temperature (+15°C to +30°C); WB is then centrifuged (1100 g) at 4°C for 10 min and the plasma layer is removed; the RBC pellet is washed 3x with cold saline. The packed RBC pellet is diluted with cold saline (1/2; 1 part RBC + 1 part saline) and mixed well. The diluted RBCs are further diluted (1/11) with 1% ascorbic acid (10 g/L) to produce RBC lysate. The RBC lysate (~700 µL) QC pools are aliquoted into 2.0-mL Nalgene cryovials for single use and vials are stored deep frozen (-50°C to -90°C) to maintain stability. The QC pools are stable for at least 5 years and can be recertified in increments of 2 years (see **Appendix A_C: Stability**).

Characterization limits are established by analyzing duplicates of each pool for at least 20 consecutive runs.

Information on the preparation of quality control pools is maintained on the shared network drive. For more detailed information on the preparation of QC materials, homogeneity testing, and characterization refer to **SOP "NBB-OC-LABOP.01 QC Materials"** (use newest version).

D. Other Materials

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

case of standards, internal standards, chemicals, and reagents, the chemical and/or isotopic purity of the substitute must meet or exceed that of the listed product. In the case of the HPLC column and guard cartridge, equivalent performance must be demonstrated experimentally in accordance with DLS Policies and Procedures.

(1) Consumables for automated sample dilution on Hamilton liquid handler

a) 96-well 2 mL sample/collection plate (Whatman)

b) 96-well collection plate seals (Agilent Technologies)

c) 1 mL and 0.3 mL plastic pipette tips (Hamilton)

d) 300 mL plastic reusable reagent and water troughs (Hamilton)

(2) Consumables for automated solid phase extraction (SPE) on 96-probe Zephyr G3 SPE instrument (Revvity)

a) 96-well Bond Elute SPE plate (50 mg phenyl sorbent, Agilent Technologies)

b) Captiva 96-well filter plates (0.45 µm PVDF embedded well, Agilent Technologies) for efficient automated filtration with vacuum manifold (IST VacMaster-VCU)

(3) General consumables

a) C-8(2) analytical HPLC column, 150 x 3.2 mm, 5 µm (Phenomenex)

b) 0.5 µm stainless frits A-102X (Upchurch Scientific)

c) PEEK tubing 0.005 and 0.007 ID (Upchurch Scientific)

d) HPLC Solvent glass inlet filters, purge frits, gold seal and outlet caps (Agilent Technologies)

e) Eppendorf pipette tips (0.5-10 µL, 10-100 µL, 100-1000 µL, and 10 mL)

f) Eppendorf Combitip advanced pipette tips (0.5 mL and 1 mL) for repeater pipette

g) Gilson Pipetman positive displacement pipette tips (50 µL, 100 µL, and 1000 µL)

h) Nunc 30 mm, 1-mL 96-well HPLC plate for 96-well autosampler (Fisher Scientific)

i) Fisher Brand silicone plate seal for 1 mL 96-well HPLC plate (Fisher Scientific)

j) HPLC solvent filter degasser, model FG-256 (Lazar Research Laboratories)

k) 0.22 µm cellulose nitrate nonpyrogenic sterile water filter system 500 mL capacity (Corning)

l) 0.45 µm (or smaller) mixed cellulose esters (MCE) membrane syringe filter (Millipore)

m) 2.0 mL polypropylene cryovials (Nalgene)

n) 1.0 mL disposable syringes (Hamilton)

o) Various glass beakers, volumetric flasks (class A), graduated glass cylinders, and bottles

(4) Folate Standards

a) PGA (Pteroylglutamic acid, free acid or Na2-salt), 5-methylTHF ([6S]-5CH3-H4PteGlu, Ca-or Na2-salt), 5-formylTHF ([6S]-5CHO-H4PteGlu, Ca-or Na2-salt), MeFox ([6S](pyrazino-s-triazine derivative), THF ([6S]-H4PteGlu, free acid, Ca-or-Na2-salt) and 5,10-methenylTHF ([6S]-5,10-CH=H4PteGlu-Cl x HCl salt) (Merck & Cie., https://www.emdgroup.com)

b) $^{13}C_5$-PGA, $^{13}C_5$-5-methylTHF, $^{13}C_5$-5-formylTHF, $^{13}C_5$-MeFox, $^{13}C_5$-THF and $^{13}C_5$-5,10-methenylTHF (Merck & Cie., https://www.emdgroup.com)

(5) Chemicals and Solvents

a) Exo-GGH enzyme (500 µg/mL, Novus Biologicals)

b) Ammonium hydroxide (28–30% as NH3, ACS reagent, J.T. Baker Brand by Avantor)

c) L-Cysteine (Sigma Aldrich)

d) Potassium phosphate dibasic and monobasic salts (Fisher Scientific)

e) Formic acid (>95% reagent grade, Sigma Aldrich)

f) Acetic acid (99% reagent grade, Fisher Scientific)

g) L-ascorbic acid (vitamin C min 99% purity, Spectrum Chemicals, Fisher Scientific)

h) Hydrochloric acid (36.5-38%, JT Baker)

i) Water (18 MΩ/cm, HPLC grade, Aqua Solutions)

j) Methanol and acetonitrile (LC-MS grade, Honeywell/Burdick & Jackson Laboratories)

k) Nitrogen ultrapure (>99.99 % purity, Air Products)

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with an equivalent product from a different manufacturer provided if it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., HPLC components, tandem quadrupole mass spectrometer) equivalent performance must be demonstrated experimentally in accordance with DLS Policies and Procedures if a product substitution is made. For details, refer to **Appendix B_C: JA-4015-I-01-Instrument Comparison and System Verification**.

To provide adequate throughput for this method as well as backup instrumentation during times of repair and maintenance, we utilize multiple HPLC-MS/MS systems of the SCIEX type. Equivalent performance must 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.

(1) Agilent 1260 Infinity HPLC systems (Agilent Technologies) equipped with control pilot, degasser, binary pump, autosampler with thermostat, and column compartment.

(2) SCIEX 6500 QTrap triple quadrupole mass spectrometer with Turboionspray (TIS) as ion source in ESI mode equipped with Analyst software (SCIEX)

(3) Peak Scientific Infinity 31N model nitrogen generator (Peak Scientific Instruments Ltd)

(4) Zephyr G3 SPE automated 96-probe liquid handler (Revvity, previously Perkin Elmer); can also be used for sample transfer for filtration on Captiva 96 well-plate filters

(5) Microlab Starlet automated liquid handler (Hamilton)

(6) Eppendorf repeater pipette (volume range from 1uL to 10 mL, Eppendorf)

(7) Eppendorf single channel pipettes (10 µL, 100 µL, 200 µL, 1000 µL, and 10 mL; Eppendorf)

(8) Gilson MICROMAN positive air displacement pipettes (50 µL, 100 µL, and 1000 µL; Gilson)

(9) Digiflex CX (ICN Biomedicals, Inc. Diagnostics Division)

(10) Vortex Genie 2 mixer (VWR)

(11) Magnetic stirrer (Baxter Scientific Products)

(12) pH meter (Corning Pinnacle 530 or Accumet XL150 -pH/MV; Fisher Scientific)

(13) Analytical Balance Model AG104 (Mettler Instrument Corp.)

(14) Cary 3500 Bio UV-visible spectrophotometer (Agilent Technologies)

(15) Centrifuge Model J6-MI (Beckman Coulter)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

Because WB lysates are 1:11 diluted WB, and RBC lysates are ~1:22 diluted RBCs, we do not expect to see a matrix effect, which is also supported by complete spiking recoveries (100% ± 10%) for most analytes in WB lysates using an aqueous calibration curve **[5]**. In-house studies showed that aqueous calibration provides equivalent results to calibration in RBC lysates and slopes for the two calibration curves (RBC lysate vs. water) were less than ±5% different for all folate forms. Aqueous calibrators (at the beginning of each run) are carried through the entire sample processing procedure. Calculation of folate concentrations in QC and unknown patient samples is based on results obtained from a daily 5-point calibration curve (S1 to S5). A blank sample (S0, containing internal standard mix) and a double blank (containing reagents only) are also included in each run. Area ratios of analyte to internal standard from single analysis of each calibrator are calculated and a linear regression equation ($1/x^2$ weighting) is generated. At the end of each run, the calibration curve is re-injected to assess potential calibrator drift. The measured concentrations of the calibrators at the beginning of each run should generally agree within 15% of their set values, although >15% agreement will be observed at concentrations approaching the LOD.

This method is linear from 0-220 nmol/L for each folate form **[2,8]**. The calibration range is from 1–100 nmol/L for 5-methylTHF and from 0.5–50 nmol/L for all other folate forms. The removal of calibration point(s) is not suggested for this assay. This assay uses a freshly prepared mixed calibration curve of all six analytes. In our experience excluding a calibrator point(s) for a particular analyte(s) didn't affect the accuracy of major analyte results, but the impact was noticeable on minor analyte results. We recommend repeating the sample analysis for the analyte(s) where a calibrator(s) point error is noticed. Samples with concentrations that exceed the calibration range are diluted with 0.1% ascorbic acid and re-analyzed.

Since calibrators are included in every run, there is no additional calibration verification required. However, for good laboratory practice, calibration verification is conducted at least twice a year using the only available WB international reference material (NIBSC 95/528; lyophilized, one level) (no RBC-based reference material is available) to monitor the assay performance. For details, see **Appendix B_A**: **JA-4015-G-01-Calibration and Calibration Verification**. The folate concentration in the NIBSC material has been determined by consensus value assignment but is mainly representative for the microbiologic assay. We analyze this SRM for assay performance, analyst training and/or competency evaluations.

For information on the proficiency testing (PT) process refer to **SOP NBB-OC-LABOP.02.PT Management** (use newest version). Details about our PT activities can be found in the proficiency testing form. While no proficiency testing (PT) program is available for RBC samples, we participate in the external PT program from the College of American Pathologists (CAP) Ligand Survey for WB folate twice a year. General information on the handling, analysis, review, and reporting of proficiency testing materials is saved on network.

In a matrix comparison study using blood samples from 60 donors we found excellent correlation (r ≥0.98) and good agreement (within ±5% for 5-methylTHF, PGA, and non-methyl folate; within ~±15% for MeFox) for RBC folate calculated from 3 matrices: conventionally prepared WB lysates (4 h at 37°C to allow the endogenous GGH to deconjugate polyglutamates; serum folate and Hct correction), WB lysates incubated with exo-GGH for 30 min at ambient temperature (+15°C to +30°C); serum folate and Hct correction), and RBC lysates incubated with exo-GGH for 30 min at ambient temperature (+15°C to +30°C); Hb and MCHC correction) **[11]** (**Appendix E**). This reinforces the validity of using the external CAP PT exercises for WB folate to track assay performance.

In a method comparison study, we compared HPLC-MS/MS results for RBC lysates treated with exo-GGH (30 min at ambient temperature (+15°C to +30°C)) to results obtained with the microbiologic assay, still considered an accurate “reference point” for total folate (tFOL). We obtained excellent correlation (r = 0.95) and good agreement (within ~±10%) **[11]** (**Appendix F**). A method comparison study using traditional WB lysates has been described previously **[12].**

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 G**.

B. Instrument Calibration

Equipment listed below requires calibration and/or calibration verification. Other small equipment used in this method that is not listed below does not require calibration and/or calibration verification. For more information on equipment management throughout its lifecycle refer to **SOP NBB-OC-EQUIP.02. Equipment Management** (use newest version).

1) Tandem mass spectrometer

The calibration of the mass spectrometer is scheduled on an annual or semi-annual basis as part of a preventative maintenance program and is performed by an authorized service engineer.

The tuning and mass calibration of the first (Q1) and third (Q3) quadrupoles of the mass spectrometer is performed using a solution of polypropylene glycol (PPG) by infusion and running the instrument in either Manual Tuning mode or using Automatic Mass Calibration. Resolution for Q1 is always set at “Unit” while the resolution for Q3 is sample matrix dependent and can be set either “Unit or High”. The method for the analysis of serum folate forms operates in positive ion mode with “Unit” resolution for both Q1 & Q3 quadrupoles.

The PPG calibration solution kit contains a PPG standard 2000 vial for positive ion mode calibration and a PPG standard 3000 vial for negative ion mode calibration. Preparation of the PPG standard 2000: SCIEX 6500 uses a 1:50 dilution (for calibration of positive ion mode add 0.4 mL of PPG standard to 19.6 mL of PPG dilution solvent). PPG 3000 is used directly without any dilution for calibration of negative ion mode.

After preventative maintenance (PM) or repair service is completed, the engineer performs tuning and mass calibration of the instrument; saves PPG calibration profiles and data in respective PM folder on the instrument computer. The analyst (and/or team lead) reviews the PPG data and peak responses (intensities and peak widths); verifies the instrument mass calibration table with the calibration data saved (pdf file) in PM folder; compares the data and PPG responses with previously conducted services (PM or repairs) and updates. PPG peak widths and peak responses should either exceed or compare well with previous performance otherwise new instrument service (e.g., deep cleaning, repairs, tuning and re-calibration) and updated mass calibration is requested.

2) HPLC system

This system does not require calibration. Preventative maintenance is performed annually by an authorized service engineer and parts replaced as needed. With use, the analyst performs routine maintenance as described in section 8G.

3) Hamilton Microlab Starlet liquid sample handler

Twice a year a Hamilton service engineer performs preventative maintenance including volume verification at 10 µL and 1000 µL. User checks instrument performance through gravimetric volume verification of the various steps of the method using the Volume Field Verification kit from Hamilton. For details refer to **Appendix B_C: JA-4015-I-02- Hamilton Microlab STARlet Liquid Handler Maintenance and Calibration Verification using the Volume Field Verification (VFV) Kit.**

4) Zephyr G3 SPE instrument

A 96-probe automated SPE instrument is used for sample extraction and cleanup in a non-volume-critical sample and/or solvent transfer mode and thus does not require calibration of volume verification. Once per year an authorized service engineer performs preventative maintenance and replacement of parts if needed.

5) Pipettes (air displacement and positive displacement)

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

6) Cary 3500 UV-Vis spectrophotometer

The vendor performs annual preventative maintenance which may include a system calibration. System calibrations are only required when the instrument returns a ‘calibration required’ message when powering up or when system self-tests fail. Otherwise, system calibration should not be performed. NBB staff performs calibration verification using certified filters at least once a year, ideally at times off-set from the annual vendor calibration service. Recertification of the certified filters is performed externally every other year. Additional performance testing is done three times per year by participation in the CAP instrumentation survey.

7) Balances

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

8) pH meter

Calibration verification is performed by the analyst prior to use as needed using commercial calibration solutions.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A typical run consists of the following sequence of samples: reagent blank (double blank), blank (contains internal standard mix), 5 calibrators, first set of QC, 83 patient samples, and second set of QC, for a total of 96 samples (96-well plate format). Three levels of blood QC are analyzed in duplicate in each run as bench QC materials. In preparation for SPE, samples are typically pipetted by a liquid sample handler, but they can also be pipetted manually. SPE is carried out using an automated 96-probe instrument.

A. Preliminaries

(1) Thaw frozen WB lysate or RBC lysate specimens (QC and unknown patient samples), folate intermediate stock solutions (calibrator and internal standard); it takes about 40 min for the samples to reach ambient temperature (+15°C to +30°C).

(2) Prepare buffers and mobile phase (can be prepared ahead of time).

(3) Add 0.5% acetic acid to the pre-made mobile phase prior to use.

(4) Prepare fresh sample solvent #1 (with 0.5% ascorbic acid), sample solvent # 2 (with 0.1% ascorbic acid) and sample solvent #3 (with 0.5% ascorbic acid and 1% acetic acid).

(5) Prepare fresh 1% ammonium formate buffer (pH 3.2, with 0.1% ascorbic acid) for calibrator Mix A and prepare fresh 0.1% ascorbic acid for internal standard Mix B.

(6) Mark the 96 well-plate rows for the number of samples to be analyzed.

(7) Prepare calibrator Mix A and internal standard Mix B as described in section 6.B.(2).

(8) Prepare calibration standards S1 to S5 as described in section 6.B.(3). Vortex thoroughly for adequate mixing.

(9) Vortex all thawed specimens thoroughly prior to pipetting and visually check for any unusual sample volume, specimen color, or debris/precipitate.

B. Automated Sample Pipetting using a Liquid Handler to Prepare for SPE (routine runs)

Hamilton Microlab Starlet is used for automated pipetting of calibrators and of WB lysates or RBC lysates from cryovials into a 2-mL 96-well plate; after addition of IS mix containing exo-GGH, samples are incubated for ~30 min at ambient temperature (+15°C to +30°C). Next, the Hamilton is used to add water (blanks, and calibrators only) and buffer (blanks, calibrators, and specimens) to the 96-well plate. Finally, the plate is subjected to automated SPE sample extraction and clean-up.

With use, maintenance and function checks are performed prior to sample pipetting. For details, refer to **Appendix B_C: JA-4015-I-02- Hamilton Microlab STARlet Liquid Handler Maintenance and Calibration Verification using the Volume Field Verification (VFV) Kit**.

(1) Check and restock tip racks.

(2) Fill in two reagent troughs with solvent #1 and deionized water respectively and load on to the reagent carrier.

(3) Put calibrators, internal standard mixture, QC samples, and unknown patient samples onto respective sample carriers.

(4) Put a 2-mL 96-well collection plate (sample destination plate) in place.

(5) Using Microlab Star Run Method Software the pipetting program is executed and dispenses according to the scheme in **Table VI**.

Table VI: Pipetting Scheme I

Well #	Sample Type	Internal Standard Mix with exo-GGH	Calibrator Mix	Sample Solvent #1	QC or Patient Specimen	Final Volume
1	Reagent blank (Double blank)	--	--	400 µL	--	400 µL
2	Calibrator S0 (Blank)	60 µL	--	150 µL	--	210 µL
3	Calibrator S1	60 µL	150 µL		--	210 µL
4	Calibrator S2	60 µL	150 µL		--	210 µL
5	Calibrator S3	60 µL	150 µL		--	210 µL
6	Calibrator S4	60 µL	150 µL		--	210 µL
7	Calibrator S5	60 µL	150 µL		--	210 µL
8	Low QC – Set 1	60 µL	--		150 µL	210 µL
9	Medium QC – Set 1	60 µL	--		150 µL	210 µL
10	High QC – Set 1	60 µL	--		150 µL	210 µL
11-92	Patient Samples	60 µL	--		150 µL	210 µL
93	Low QC – Set 2	60 µL	--		150 µL	210 µL
94	Medium QC – Set 2	60 µL	--		150 µL	210 µL
95	High QC – Set 2	60 µL	--		150 µL	210 µL

(6) After calibrators, IS mix, QC and patient samples are dispensed, the sample plate is removed, covered with the 96-well plastic seal, and let the plate is let sit at ambient temperature (+15°C to +30°C) for 30 min to allow endogenous folate to equilibrate with IS mix, and also to allow the exo-GGH to deconjugate folate polyglutamates.

(7) After the 30-min incubation at ambient temperature (+15°C to +30°C), a second pipetting program is executed that dispenses according to the scheme shown in **Table VII** for a final volume of 550 µL. This prepares the samples for SPE clean-up on the 96-probe instrument (Zephyr G3 SPE).

Table VII: Pipetting Scheme II

Well #	Sample Type	Deionized Water	Sample Solvent #1	Final Volume (Tables VI + VII)
1	Reagent blank (Double blank)	150 µL	--	550 µL
2	Calibrator S0 (Blank)	150 µL	190 µL	550 µL
3	Calibrator S1	150 µL	190 µL	550 µL
4	Calibrator S2	150 µL	190 µL	550 µL
5	Calibrator S3	150 µL	190 µL	550 µL
6	Calibrator S4	150 µL	190 µL	550 µL
7	Calibrator S5	150 µL	190 µL	550 µL
8	Low QC – Set 1	--	340 µL	550 µL
9	Medium QC – Set 1	--	340 µL	550 µL
10	High QC – Set 1	--	340 µL	550 µL
11-92	Patient Samples	--	340 µL	550 µL
93	Low QC – Set 2	--	340 µL	550 µL
94	Medium QC – Set 2	--	340 µL	550 µL
95	High QC – Set 2	--	340 µL	550 µL

Notes: *Use positive displacement pipettes wherever possible for the preparation of the calibration standards and Internal standards. Use positive displacement pipettes or an automated positive displacement pipettor for transferring the preliminary assay volume of standards, QC, and patient samples into the sample plates.*

Folate forms are light sensitive. Perform all sample preparation under yellow subdued light.

C. Manual Sample Pipetting to Prepare for SPE (occasional run)

(1) Mark 96-well plate with date, study ID and mark the number of wells that need to be used to prepare samples for SPE.

(2) Follow the pipetting scheme shown in **Table VI** to construct a 5-point calibration curve, prepare a reagent blank that contains only reagents and a blank that contains reagents and only the internal standard mix (S0), and to prepare QC and patient samples.

(3) Seal the 96-well plate with silicone plate seal and mix the contents gently on plate shaker (typically 1 min.).

(4) Incubate at ambient temperature (+15°C to +30°C) for 30 min.

(5) Follow the pipetting scheme shown in **Table VII** to prepare samples for SPE.

D. Automated Solid Phase Extraction Method

(1) A 96-probe instrument (Zephyr G3 SPE) is used for automated SPE. All SPE steps, such as SPE plate conditioning, sample loading, SPE plate washing, and sample elution are performed automatically. The instrument processes one 96-well plate extraction in about an hour which includes blanks, calibrators, 2 sets of QC, and 82 unknown patient specimens.

(2) Open the “Maestro Software” on the desktop and select the Folate SPE method.

(3) The SPE sample plate, collection plate, conditioning solvents, wash, and elution buffers are placed on their respective deck positions on the SPE instrument.

(4) Start the Folate SPE method within the software.

(5) The SPE method performs the following steps:

a) **Condition:** SPE plate is conditioned with acetonitrile and methanol (0.5 mL each) in 2 steps each, followed by conditioning with 1.3 mL solvent #1 in 3 steps (0.5 mL x2 and 0.3 mL x1)

b) **Load:** 500 µL sample is loaded in 4 steps (125 µL x4)

c) **Wash:** SPE plate is washed in 3 steps with 1.3 mL of solvent #2 (0.5 mL x2 and 0.3 mL x1)

d) **Elution:** Sample elution is carried out in 2 steps (0.3 mL and 0.2 mL) with solvent #3

(6) The chemical waste (~2L/week) from this procedure consists of water, methanol, acetonitrile, ammonium formate, ascorbic acid, and blood hemolysate (~4.0%) and is discarded appropriately.

E. Automated Sample Filtration for HPLC-MS/MS Analysis

(1) After SPE is completed, samples are filtered using Captiva filter plates (96-well, 0.45 µm PVDF).

(2) Zephyr G3 SPE liquid handler is used to transfer 300 µL of the extracted sample into a fresh Captiva filter plate.

(3) Captiva filter plate is placed on top of a fresh HPLC collection plate (30 mm Nunc plate).

(4) The samples in the Captiva filter plate are filtered into the HPLC collection plate using a vacuum manifold (IST) at 5 psi pressure within <5 min.

(5) The HPLC collection plates are sealed with the pre-slit seals and arranged on the HPLC autosampler for analysis.

F. Cleanup and Disposal of Reagents

Unused or expired chemicals and reagents must be disposed of as indicated in the CDC Chemical Hygiene Plan. Once empty, glassware and reusable plasticware should be thoroughly rinsed first with tap water, followed by deionized water, and allowed to dry on a drying rack or other appropriate surface following use. For general information on safe handling of other disposable labware, see Section 2.

G. HPLC-MS/MS Instrument Preparation

(1) The Agilent HPLC system coupled to the SCIEX MS/MS system is used to quantitate folate vitamers in extracted WB lysates or RBC lysates. Typical MS/MS method parameters for each folate vitamer and the corresponding internal standards are listed in **Appendix H**.

(2) Prior to each run, HPLC instrument function checks are performed:

a) Switch all HPLC components to “ON” mode.

b) Purge each solvent line with the respective solvent bottle.

c) Prime the HPLC column with a series of solvents as described in the next steps.

(3) Methanol and water (90:10 v/v) are used for line purging for ~5 min at a flow rate of 5 mL/min. The column is primed with this solvent for ~20 min at a flow rate of 500 µL/min.

(4) The lines are purged with mobile phase for 5 min at a flow rate of 5 mL/min and column is primed ≥15 min at a flow rate of 250 µL/min. Pressure is recorded. HPLC system is ready for analysis; samples are analyzed (20 µL injection volume) at a flow rate of 250 µL/min for 7 min that includes column equilibration time.

(5) Typically, once per week (or as needed) acetonitrile and water (65:35 v/v) is used for line purging (~5 min) at a flow rate of 5mL/min. The column is primed with this solvent ~20 min at a flow rate of 500 µL/min.

(6) Typically, to increase sample throughput on column and/or improve peak resolution, the column is reversed once it has run in one direction; primed as described below prior to its use for routine sample analysis.

(7) Brand new column is primed with acetonitrile and water (65:35) at 800 µL/min for 60 min; with methanol and water at 800 µL/min for 30 min; equilibrated with mobile phase at 250 µL/min for 60 min prior to sample run. Typically, a new column is also primed with matrix by injecting a set of processed serum samples (15-20 samples) prior to its use for routine sample analysis.

(8) Prior to sample analysis, MS/MS instrument maintenance and function checks are performed:

a) Put the instrument in standby mode and remove the curtain plate from source. Clean curtain plate first with water, then methanol, and wipe dried with lint-free Kimwipe.

b) Wipe orifice plate with methanol damped lint-free Kimwipe.

c) Check ion spray needle for any blockage and clean if necessary.

d) Check instrument vacuum (1.0 to 2.0 e^{-5}Torr) and gas generator for curtain (~60 psi), source (90-110 psi), and exhaust (~70 *psi*) status.

e) Load the appropriate instrument method and create an acquisition batch containing the sample sequence.

(9) Prior to sample analysis, the HPLC-MS/MS instrument is equilibrated:

a) At least 2 sample solvent injections are performed prior to sample analysis to verify that the system is performing properly.

b) Either a blank (S0, mixture of internal standards) or the low QC sample from the previous run can be re-injected prior to the analysis of the current run to check the instrument performance.

(10) The sample plate is loaded on the autosampler, and the sample acquisition batch created in the earlier step is submitted for analysis.

(11) The HPLC rinse method is loaded at the end of the sample batch. It runs isocratic (90:10 v/v of methanol and water) for 20 min in Q1 scan mode at the end of the batch to clean the HPLC column and MS/MS system. The data is recorded in an acquisition rinse batch file so that it can be reviewed later. If necessary, multiple batches can be submitted for analysis, each separated by the HPLC rinse method.

(12) The HPLC shutdown method is loaded after the HPLC rinse method. It runs isocratic (90:10 v/v of methanol and water) for 5 min in MRM mode after the rinse method. Finally, the instrument

goes to standby mode until the next run and the sample plate in the autosampler is maintained at ≤10°C during standby mode.

(13) The chemical waste (~2L/week) from this procedure consists of water, methanol, acetonitrile, acetic acid and is discarded appropriately.

H. Processing and Reporting a Run

(1) The SCIEX Analyst software is used to review and process a run. For details see **Appendix B_D: JA-4015-DR-02-Processing and Reporting a Run**.

(2) A STARLIMS database is used for additional levels of data review by the analyst, project lead, QA officer, and supervisor and for data reporting.

(3) Import the results file into the STARLIMS database for further data review, see **Appendix B_D: JA-4015-DR-01-Computerization and Data System Management**.

(4) Calculate the results using Excel (exception, for R&D runs or troubleshooting):

a) The final integrated results can either be directly imported to the STARLIMS database (typical) or alternatively can be processed by importing into an Excel template sheet for final calculations and interpretation.

b) Transfer the peak areas for the analyte and the internal standard for each sample into the appropriate fields in the Excel sheet.

c) The calibration curve with slope, intercept, and R^2 is automatically generated (linear, not forced through zero, no weighting) based on area ratios.

d) The concentrations of QC and unknowns are automatically calculated using the slope and intercept information.

e) The file is saved and maintained for documentation.

f) For studies where data is not imported into the database, we directly transfer the results from the "**Analyst Result**" table into the Excel sheet for final summary and results interpretation since our results are from a weighted calibration curve ($1/x^2$ weighted).

I. Exporting a Run

The procedure to export a run to a LIMS database is described in section 3 and in **Appendix B_D: JA-4015-DR-01-Computerization and Data System Management**.

J. Calculations

For WB lysate samples, we obtain results for each folate form as nmol/L lysate. These results have to be multiplied by 11 to obtain individual folate form results as nmol/L WB. Then, the individual folate form results are summed up to obtain a WB tFOL result using the automated "Calculated Analyte Test 4901" in STARLIMS (see **SOP NBB-WSML-DATA.4901** [use newest version]).

If a folate form result is less than LOD, a fill value of LOD divided by square root of the 2 [LOD/SQRT (2)] is used for summation. If one of the folate forms is missing, the WB tFOL result is also missing. The WB tFOL result is then converted to RBC folate result by using the Hct (expressed as a fraction) and the serum tFOL result:

RBC folate = (((WB lysate tFOL x 11) – (Serum tFOL x (1-Hct)))/Hct

For RBC lysate samples, results for each folate form are also obtained as nmol/L lysate. Because Hb (expressed as g/L) is measured in the same lysate by the sodium lauryl sulfate (SLS) method **[13]**, it corrects for the residual moisture content in the packed RBCs. The Hb result can be used in conjunction with the MCHC (expressed as g/L) to calculate RBC folate. This can be done separately for each individual folate form using the following formula in the automated "Calculated Analyte Test 4908" in STARLIMS (see **SOP NBB-WSML-DATA.4908** [use newest version]).

RBC folate form = (RBC-lysate folate form/Hb)*MCHC

To obtain a RBC tFOL result, the individual folate form results are added up (excluding MeFox; see special method notes) using the automated "Calculated Analyte Test 4909" in STARLIMS (see **SOP NBB-WSML-DATA.4909** [use newest version]). If a folate form result is less than the LOD, a fill value of LOD divided by the square root of the 2 [LOD/SQRT (2)] is used for summation. If one of the folate form results is missing, the RBC tFOL result is also missing. Thus, the use of washed RBCs does not require the measurement of serum folate or Hct, it only requires the measurement of Hb in the same lysate.

The calculated RBC tFOL results are reviewed and released by general supervisor or higher. For more information on data reporting, refer to **SOP NBB-OC-DATA.01. Data Reporting** (use newest version).

RBC folate results are needed to interpret folate status as clinical cutoffs are defined based on RBC folate levels. If the Hct value is not available for a patient or QC sample, assume a Hct of 40% or alternatively the RBC folate result is not reportable (study dependent). If the serum folate value is not available, assume a serum folate value of 18 nmol/L or alternatively the RBC folate result is not reportable (study dependent). When the serum folate value is low compared to the RBC folate value (i.e., non-fortified population), the formula can be simplified by ignoring the serum folate term.

K. System Maintenance

Major equipment used in this method is listed in section 7B with its calibration and calibration verification requirements. Preventative maintenance is performed by authorized service engineers on all major equipment (MS/MS, HPLC, Zephyr G3 SPE, Hamilton, and Cary UV spectrophotometer) at least once a year. For more information on equipment management throughout its lifecycle refer to **SOP NBB-OC-EQUIP.02. Equipment Management** (use newest version).

1) HPLC system

Preventative maintenance is performed annually by an authorized service engineer. The analyst performs routine system maintenance before and after each run that consists mainly of the different prime, purge, and wash cycles described in Section 8 of this document. With use, column connections are checked for leaks and are wiped with a water-moistened tissue if any residues have built up. Solvent bottles are filled as needed and cleaned typically once a month (or as needed). Filters in the solvent bottles are replaced as needed (typically every 6-8 months). The pre-column filters are typically replaced after ~500 injections (or as needed).

2) Mass spectrometer

The preventative maintenance (PM), tuning and mass calibration of the mass spectrometer is scheduled on an annual or semi-annual basis and is performed by an authorized service engineer. After PM or repair service is completed, the analyst conducts at least one test run (including folate forms calibration curve, and 2 sets of QC) to verify method performance. If the method performs

within acceptable criteria for all analytes, the service call is closed, otherwise service is continued until acceptable performance is achieved.

Rough pump oil (1L) in the mass spectrometer is replaced during PM as part of service and waste oil is discarded appropriately.

With use, the analyst performs the routine maintenance of mass spectrometer (described in section 8F) which includes: cleaning of the curtain plate first with water, then wiped with lint free Kimwipes dabbed in methanol; wiping the orifice plate with methanol dabbed lint-free Kimwipes.

Note: In preparation for an occasional power shutdown or repairs, the HPLC-MS/MS instruments are turned off following the steps below:

a) Complete or stop any ongoing scans and put all instruments on standby

b) Close all software, shut-down the computer, and unplug the computer power cable

c) Close the air supply valve and vent the MS/MS (press vent button for ~3 sec)

d) Wait for ~15 minutes for the turbo pump to spin down

e) Power off all HPLC modules and MS/MS system

3) Hamilton Microlab Starlet liquid handler

Preventative maintenance is performed on an annual basis by an authorized service engineer. With use maintenance of the system is executed through the instrument operated software that checks for deck cleaning, tip waste, and 8 channel tightness. For details, refer to **Appendix B_C: JA-4013-I-02-Hamilton Microlab STARlet Liquid Handler Maintenance and Calibration Verification using the Volume Field Verification (VFV)**.

4) Zephyr G3 SPE Instrument

The Preventative maintenance is performed on an annual basis by an authorized service engineer. With use the analyst performs routine maintenance and function checks which includes: checking vacuum pump pressure (maintained at 15±5 psi during SPE); rinsing solvent troughs with deionized water after each run (troughs replaced as needed); checking appropriate deck position for SPE supplies (tip boxes, solvent troughs, SPE plate and collection plate); checking air compressor gauge limits; weekly instrument cleanup (dry wipe) and silicone lubrication of the 96-probe SPE head.

5) Cary 3500 UV-Vis spectrophotometer

The Preventative maintenance which may include system calibration is performed annually by a vendor authorized service engineer.

L. Special Method Notes

Since the majority of folate in blood occurs in the polyglutamate form which needs to be deconjugated to monoglutamates for measurement by LC-MS/MS, the WB or RBC samples need to be lysed with 1% ascorbic acid, pH 2.7 (1:11 dilution) and incubated for 30 min at ambient temperature (+15°C to +30°C) in the presence of commercially available exo-gamma glutamyl hydrolase (exo-GGH) prior to SPE and analysis **[4,11]**. Use of exo-GGH enzyme shortens the incubation time which is useful

to recover the relatively unstable THF in blood samples, often found in samples from persons with the MTHFR T/T genotype.

Blood total folate may or may not include MeFox, depending on the study and investigator request. It is still of scientific debate whether MeFox is only generated *in vitro* or may already be present *in vivo* **[-14]**. Including MeFox into the total folate may slightly overestimate folate status, while excluding it may slightly underestimate status, however the difference between the two approaches is rather small (~5%) **[14]**.

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

This method is linear from the LOD (see section 7) to 100 nmol/L for 5-methylTHF, and 50 nmol/L for 5-formylTHF, PGA, MeFox, THF, and 5,10-methenylTHF. Samples with 5-methylTHF lysate instrument results <11 nmol/L (corresponding to an RBC folate of <317 nmol/L at an approximate dilution of 1:30 to 1:35) are re-analyzed for confirmation before results are released. Samples with folate concentrations exceeding the highest calibrator are re-analyzed after appropriate dilution with 0.1% ascorbic acid. There is no known maximum acceptable dilution. When possible, avoid small volume pipetting and minimize use of serial dilutions when generating diluted samples. Note: the theoretical sample dilution is 1:22, however washed RBCs contain only ~70-75% of RBCs and ~25-30% of saline, which increases the dilution factor by ~1.5-fold to ~1:33.

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 at least 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. After a run is completed, used blind QC are removed from the run, marked with a black dot on the cap to indicate that the vial has been thawed, and returned to the blind QC box. This helps to identify which vials have been used. If a run needs to be repeated, the same blind QC can be inserted as in the initial run.

The use of blind QC is optional but encouraged. Blind QC are used in this method as a supplementary tool to assist in monitoring accuracy, precision, and aid in detecting errors; these are not used as part of the primary control procedures to determine if a run is out of control.

B. Bench Quality Controls

Bench QC specimens are prepared from a minimum of two pools that represent low and high levels of 6 different folate forms. This assay typically uses 3 blood pools (WB lysate or RBC lysates), which represent low, medium, and high levels of 5-methylTHF, 5-formylTHF, PGA, MeFox, THF and 5,10-methenylTHF. These QC samples are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The QC results are checked after each run using of a multi-rule quality control program **[15]** based 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 S_i limit or
2. 2 2S Rule—Two or more of the three run results are outside the same 2 S_i 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 S_i

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

b) 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**

c) 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 analytical 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. For more detailed information on non-conforming event (NCE) and corrective and preventive action (CAPA) management refer to **SOP NBB-OC-ADMIN.01. NCE and CAPA Management** (use newest version).

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 set of criteria used to evaluate the quality of individual test result 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 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.

The following parameters are subject to sample QC evaluation in this method. For details on how the sample QC criteria are used refer to **Appendix B_D: JA-4015-DR-03-STARLIMS Data Review and Criteria.**

(1) Calibration curve R^2 should be:

   a) ≥0.98 for MET, MFO, FOT and MYT (pass; code 0)

   b) ≥0.95 for PGA and THF (pass; code 0)

(2) Calibrator difference to the target and calibration drift (drift between front and back calibrator injections) should be:

   a) Calibrator S1 within 30% for all analytes (pass; code 0)

   b) Calibrators S2-S5 within 15% for MET, PGA and MFO (pass; code 0)

   c) Calibrator S2 within 20% for FOT, THF and MYT (pass; code 0)

   d) Calibrators S3-S5 within 15% for all analytes (pass; code 0)

(3) Relative retention time (retention time quantitation ion/retention time ISTD) <minimum or >maximum threshold should be:

a) ≥0.98 to 1.02 min for MET and PGA (pass; code 0)

b) ≥0.985 to 1.02 min for MFO (pass; code 0)

c) ≥0.985 to 1.018 min for FOT (pass; code 0)

d) ≥0.963 to 1.06 min for THF (pass; code 0)

e) ≥0.982 to 1.02 min for MYT (pass; code 0)

(4) ISTD peak area minimum threshold:

a) For major analytes: ≥10000 for MET; and ≥9000 for MFO; (pass; code 0)

b) For minor analytes: ≥7000 for PGA; ≥9000 for FOT; ≥6000 for THF; and ≥8000 for MYT; (pass; code 0)

c) If ISTD peak area is less than minimum threshold (check; enter code 23), then verify instrument result, troubleshoot, and address appropriately (perform instrument maintenance, repair, etc.). Finally, perform a test run to check instrument response and repeat sample analysis.

(5) Patient sample result:

a) >High calibrator (fail; code 26); dilute/repeat (pass; code 97)

d) Null or no peak for MET (fail; code 26); repeat/confirm (warn; code 37)

e) <LOD or <LOQ for MET (check; code 37); repeat/confirm (warn; code 37)

f) MET result ≥LOD and <11 nmol/L (incomplete; code 33); repeat/confirm (warn; code 33)

g) Null or no peak for MFO (check; code 37); repeat/confirm (warn; code 37)

h) <LOD for MFO (warn; code 37); repeat/confirm (warn; code 37)

i) Delta difference between repeat results ≤15%; otherwise, repeat/confirm

j) Signal/noise ratio <3 & raw result >LOD (warn; code 0); check dilution factor

k) Specimen volume received less than expected for the analysis (code 21); set no reportable

(6) Manual integration (check; no code assigned); verify instrument peak integration is correct; enter code ‘0’ if result is correct.

(7) Check and update results for appropriate comment codes, e.g., not enough specimen for first or repeat analysis (code 21 or code 22; set no reportable); lab error, spills, contamination etc. (code 24; set no reportable); or instrument error/failure (code 23; repeat analysis; set no reportable).

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-4015-DR-04-Out-of-Control Corrective Action**. The following steps are provided 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 is indicated below.

(A) Look for sample preparation errors, i.e., if the analyst forgot to add internal standard, specimen, right volume of buffer etc.

(B) Check the calibrations of the pipettes.

(C) Check to make sure that the Instrument hardware is functioning properly. Check the autosampler for proper sample injections. Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator. Make sure the mass spec calibrations are ok. Run PPG’s in Q1 and Q3 scan mode to verify the instrument calibration.

(D) Run folate standards in Q1 scan mode to see if molecular ion is detected.

(E) 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.

(F) Do not report analytical results for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

(A) The most common causes of error are intermittently inaccurate micro-pipettors or pipetting errors.

(B) Calibrators, internal standards, and specimens should be vortex-mixed thoroughly before pipetting.

(C) Handling calibrators and internal standards in stepwise sequential manner will minimize the chances of cross-contamination.

(D) Working bench should be cleaned and small bench top waste bags should be emptied daily into the metal waste bins. The blue pads should be replaced weekly to keep the work area clean and free of contamination.

(E) Change of gloves after the preparation of stock and working standards and internal standards are recommended to avoid any contamination.

(F) All solvents should be degassed before use.

(G) Buffers should be made fresh as needed and pH checked when prepared.

(H) Ascorbic acid powder should be added to sample solvents #1 (0.5% w/v), #2 (0.1% w/v), and #3 (0.5% w/v) only prior to use.

(I) Acetic acid should be added to sample solvent #3 (1% v/v) and to the mobile phase (0.5% v/v) only prior to use.

(J) WB lysate or RBC lysates should be prepared carefully and accurately. Blood samples should be thoroughly mixed before aliquoting. Inappropriate mixing of packed RBC samples before aliquoting for lysate preparations (1/11 dilution with 1% ascorbic acid) may give incorrect values.

(K) Samples (including calibrators) should be prepared in yellow subdued light.

(L) Multiple freeze/thaw cycles of specimens for extended time at ambient temperature (+15°C to +30°C) will cause degradation of folates and should be avoided.

(M) HPLC system (lines and column) should be purged and primed properly.

(N) Interference testing was performed on this method as part of its method validation and is documented in **Appendix A**. This method has also undergone a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. At least 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 G**. Please refer to the *DLS Policies and Procedures Manual* for further information on ruggedness testing.

13. Reference Ranges (Normal Values)

RBC folate values are indicative of body stores. RBC folate levels <317 nmol/L are interpreted as risk of megaloblastic anemia **[16]**, while levels <748 nmol/L in women of reproductive age are interpreted as increased risk of neural-tube birth defects in the offspring **[17]**.

Clinical reference ranges reported for RBC folate are 317-1422 nmol/L with the CPB radioassay **[18]**.

The newest post-fortification RBC folate reference ranges for the U.S. population generated with the microbiologic assay for NHANES 2011-2016 are shown below **[10]**. Pfeiffer et al. also reported microbiologic assay-equivalent reference ranges for pre-fortification (NHANES 1988-1994) and early post-fortification (NHANES 1999-2004) periods **[19]**.

RBC folate: 505–2,510 nmol/L (2.5^{th} –97.5^{th} percentile in persons ≥1 year; *n* = 24,150)

466–2,270 nmol/L (2.5^{th} –97.5^{th} percentile in women 12–49 years; *n* = 5,583)

New reference ranges for individual folate forms based on HPLC-MS/MS will be determined using data from NHANES 2019-2020.

14. Critical Call Results (“Panic Values”)

Any NHANES samples with RBC folate levels <317 nmol/L (140 ng/mL) are considered to require follow-up. Since survey data are transmitted approximately weekly to the NCHS contractor, abnormal reports are automatically forwarded to the NCHS survey physician for follow-up. For smaller, non-NHANES studies, abnormal values are identified to the study principal investigator. Emails sent concerning abnormal results are maintained by the supervisor for the duration of the study. Most of these studies are epidemiological in nature.

15. Specimen Storage and Handling during Testing

Specimens should be brought and 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

If only RBC tFOL is of interest, the microbiologic assay could be performed instead of the HPLC-MS/MS method under some circumstances.

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 an 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 either transmitted electronically on a regular basis (approximately weekly for rapid turnaround analytes) or, as is the case for this assay, once study cycle is completed. 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 some smaller studies, electronic copies of a data report are sent and upon request hard copies can be sent as well.

For more information on data reporting refer to **SOP NBB-OC-DATA.01. Data Reporting** (use newest version).

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.

A STARLIMS database is used to keep records and track specimens for NHANES 1999+. If blood folate analyses are used for smaller, non-NHANES studies, records may be kept in Excel files on the 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 WB lysate or RBC lysate from these analyses for non-NHANES studies are retained for at least 1 year after results have been reported and may be then returned or discarded at the request of the principal investigator. Very little residual material will be available after NHANES analyses are completed, however residual WB lysate or RBC lysate is retained for 1 year after results have been publicly released; at that point, samples 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 a database and the specimens stored deep frozen (-50°C to -90°C). The specimen ID is read of the vial by a barcode reader used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the result file is 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 the STARLIMS database.

For more information on sample tracking and disposition refer to **SOP NBB-OC-SAMPLE.01. Sample Management** (use newest version).

19. Method Performance Documentation

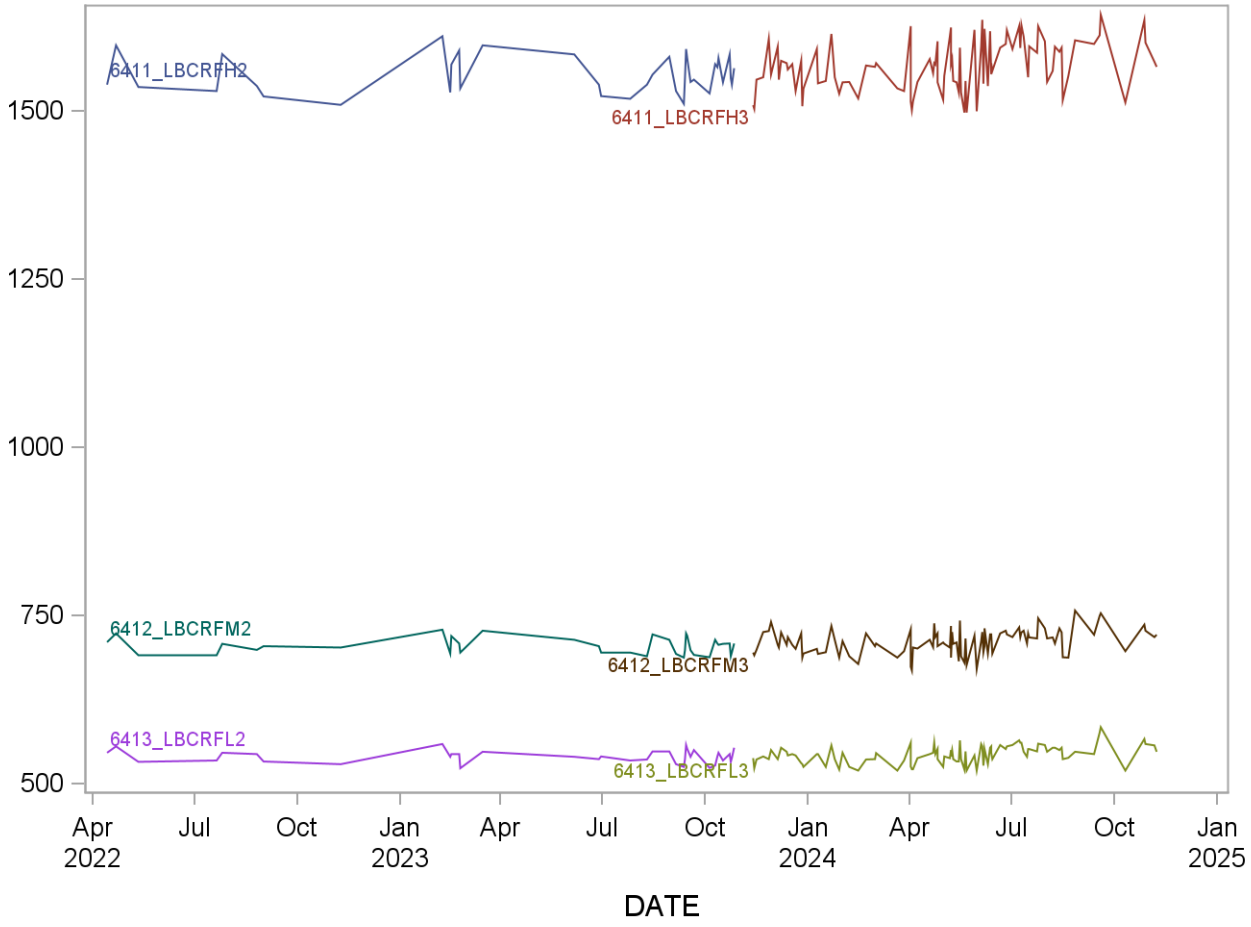
Method performance documentation for this method including accuracy, precision, sensitivity, specificity, and stability is provided in **Appendix A**. The approval of this procedure by the Branch Chief and CLIA Director denote that the method performance is fit for the intended use of the method.

20. Summary Statistics and QC Graph

Please see following pages.

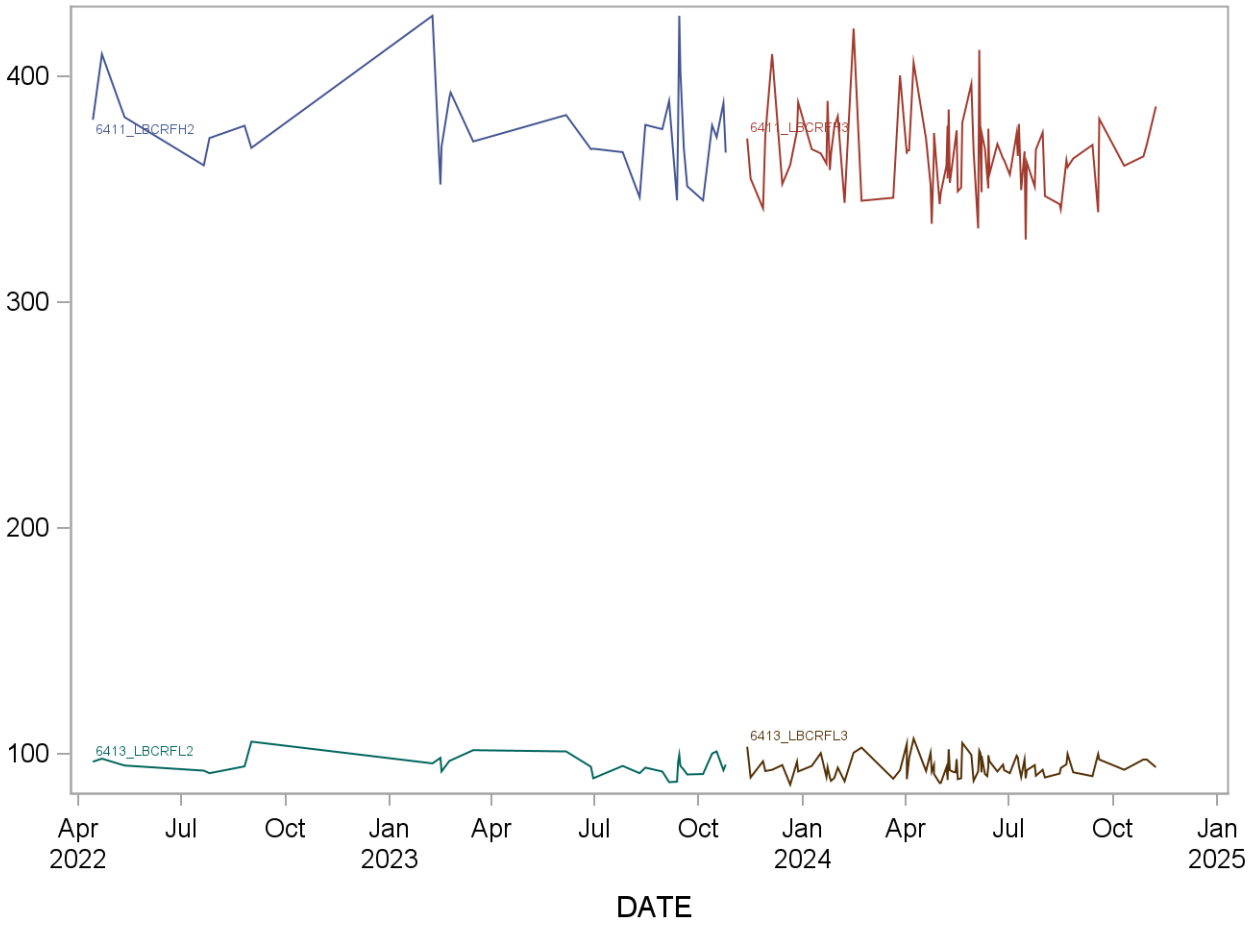
August 2021-August 2023 Summary Statistics and QC Chart
LBXRF1 (5-Methyl-tetrahydrofolate, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCRFH2	37	14APR22	27OCT23	1554.11	27.58	1.8
6413_LBCRFL2	37	14APR22	27OCT23	540.31	9.40	1.7
6412_LBCRFM2	37	14APR22	27OCT23	704.77	12.11	1.7
6411_LBCRFH3	97	13NOV23	08NOV24	1568.20	37.86	2.4
6413_LBCRFL3	97	13NOV23	08NOV24	543.40	13.48	2.5
6412_LBCRFM3	97	13NOV23	08NOV24	711.62	18.86	2.7



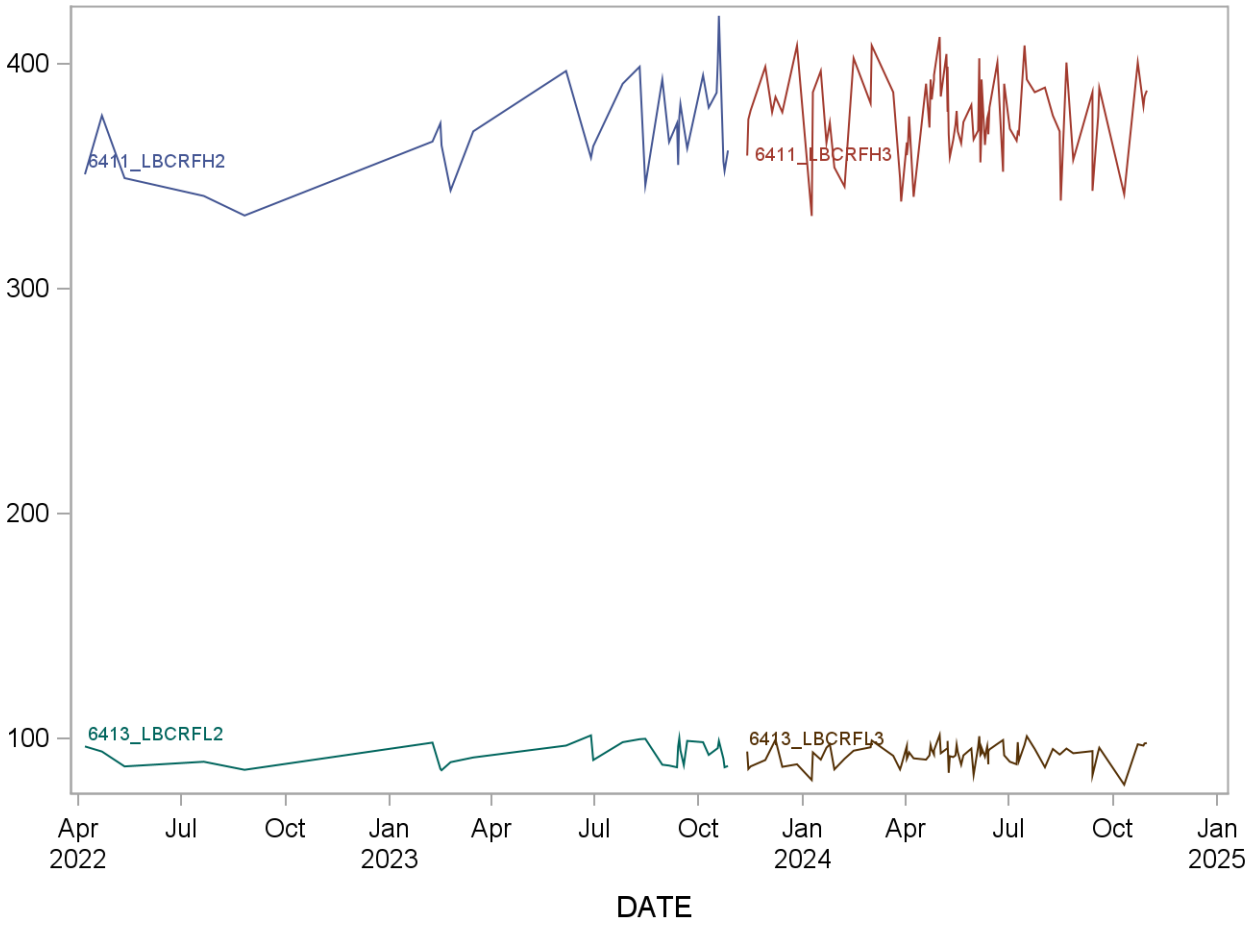
August 2021-August 2023 Summary Statistics and QC Chart
LBXRF2 (Folic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCRFH2	32	14APR22	25OCT23	376.7209	20.2363	5.4
6413_LBCRFL2	32	14APR22	25OCT23	94.8395	4.1756	4.4
6411_LBCRFH3	83	13NOV23	08NOV24	365.6413	17.8211	4.9
6413_LBCRFL3	83	13NOV23	08NOV24	93.9940	4.7146	5.0



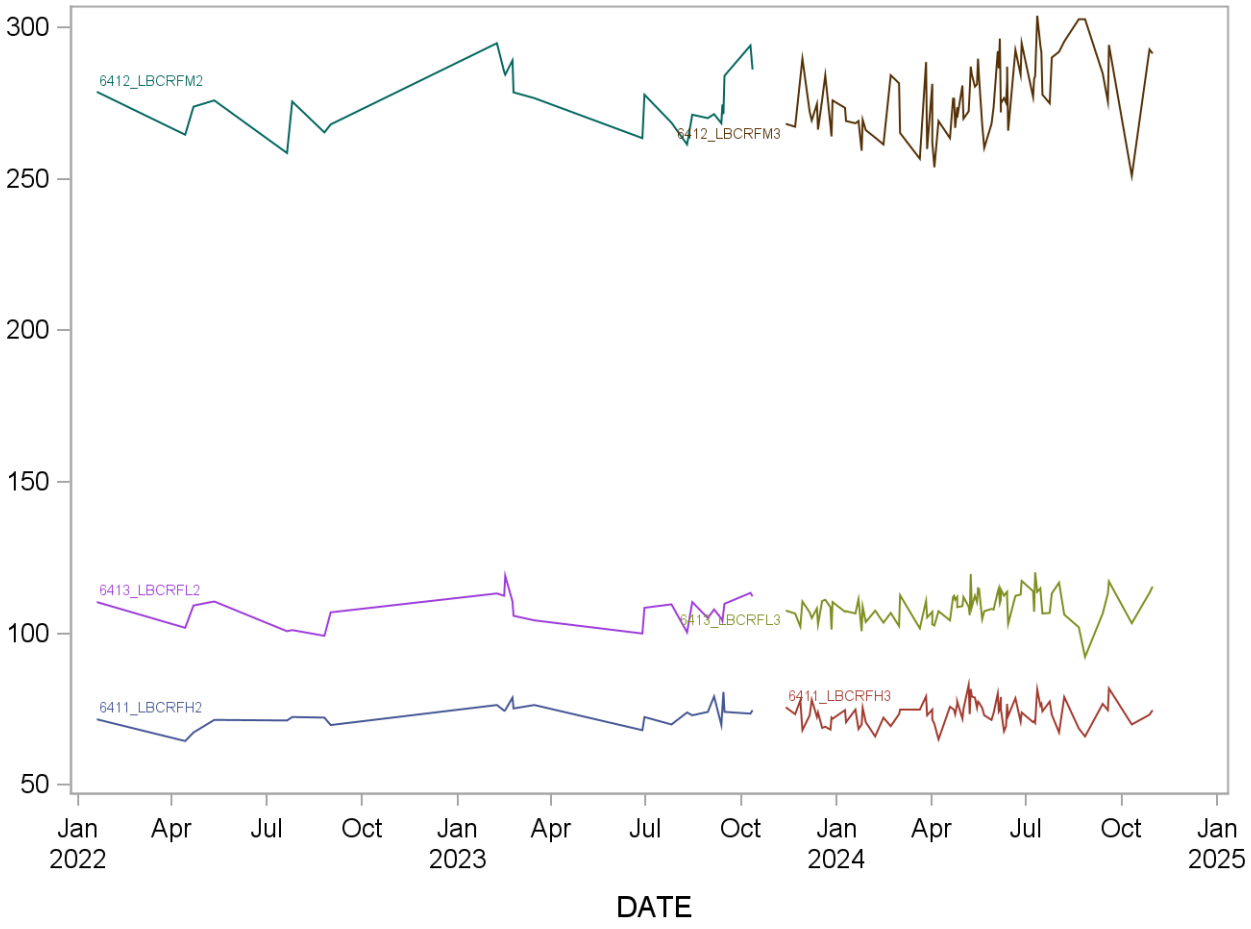
August 2021-August 2023 Summary Statistics and QC Chart
LBXRF3 (5-Formyl-tetrahydrofolic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCRFH2	32	07APR22	27OCT23	370.3758	20.2951	5.5
6413_LBCRFL2	32	07APR22	27OCT23	93.2106	5.0580	5.4
6411_LBCRFH3	82	13NOV23	31OCT24	376.6376	18.2745	4.9
6413_LBCRFL3	82	13NOV23	31OCT24	93.2052	4.5059	4.8



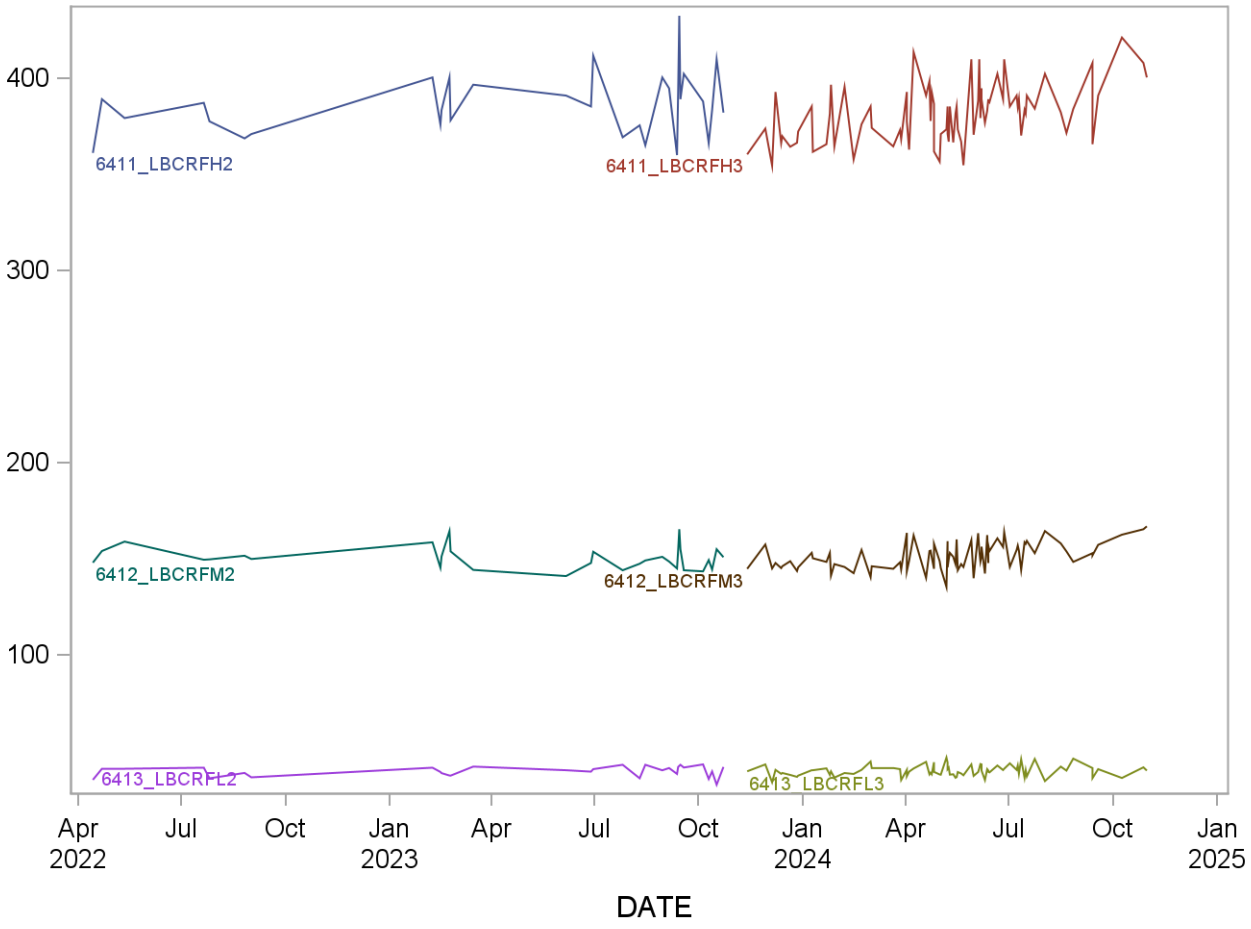
August 2021-August 2023 Summary Statistics and QC Chart
LBXRF4 (Tetrahydrofolic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCRFH2	27	19JAN22	12OCT23	73.0998	3.6403	5.0
6413_LBCRFL2	27	19JAN22	12OCT23	107.3035	4.9152	4.6
6412_LBCRFM2	27	19JAN22	12OCT23	275.3019	9.5807	3.5
6411_LBCRFH3	84	13NOV23	31OCT24	73.8085	3.8725	5.2
6413_LBCRFL3	84	13NOV23	31OCT24	109.1057	4.8325	4.4
6412_LBCRFM3	84	13NOV23	31OCT24	277.5114	11.8373	4.3



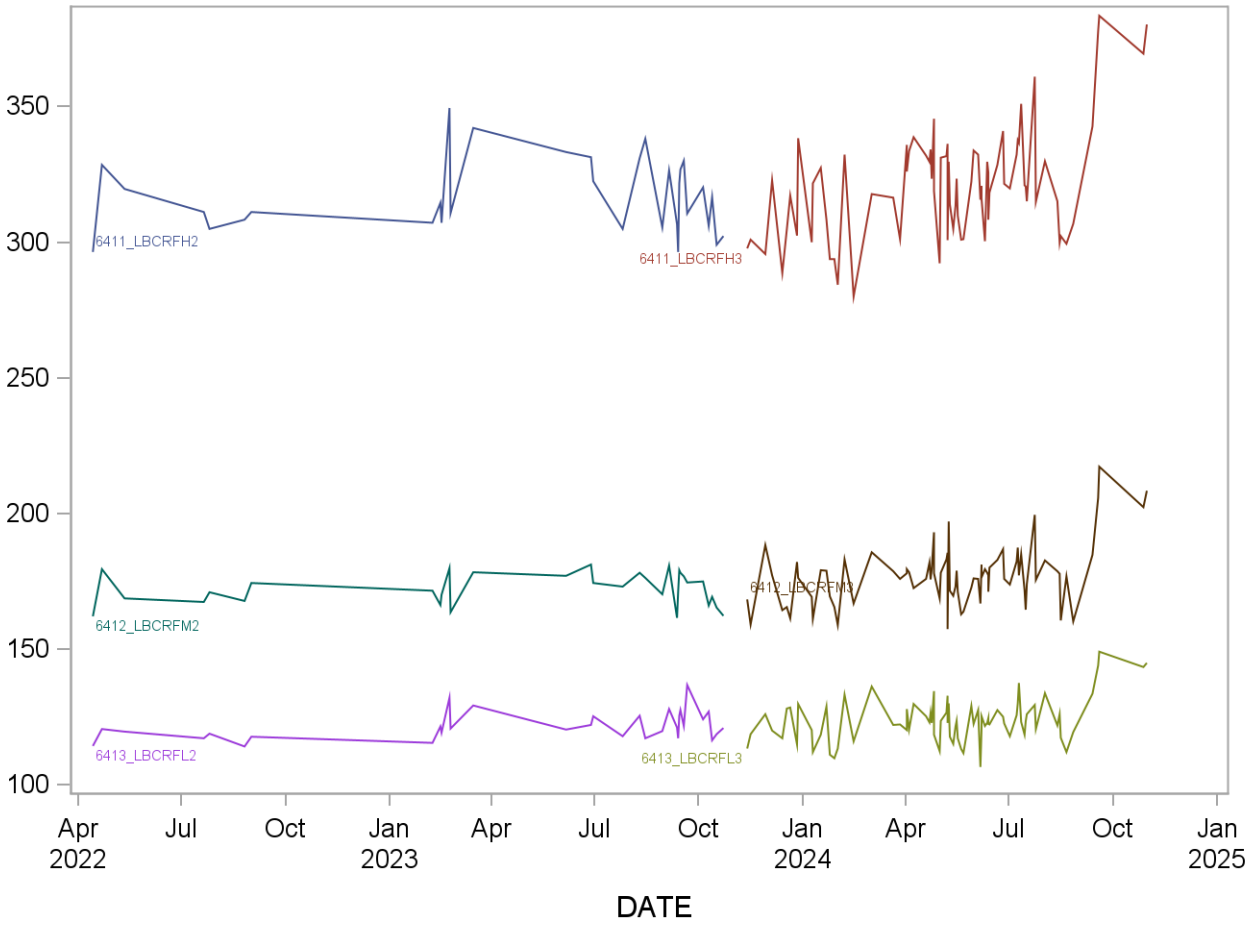
August 2021-August 2023 Summary Statistics and QC Chart
LBXRF5 (5,10-Methenyl-tethrofolic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCRFH2	31	14APR22	23OCT23	386.0948	16.2514	4.2
6413_LBCRFL2	31	14APR22	23OCT23	39.3506	2.7415	7.0
6412_LBCRFM2	31	14APR22	23OCT23	150.5082	5.8647	3.9
6411_LBCRFH3	80	13NOV23	31OCT24	381.6192	15.0253	3.9
6413_LBCRFL3	80	13NOV23	31OCT24	39.5525	2.8220	7.1
6412_LBCRFM3	80	13NOV23	31OCT24	151.3989	7.1046	4.7



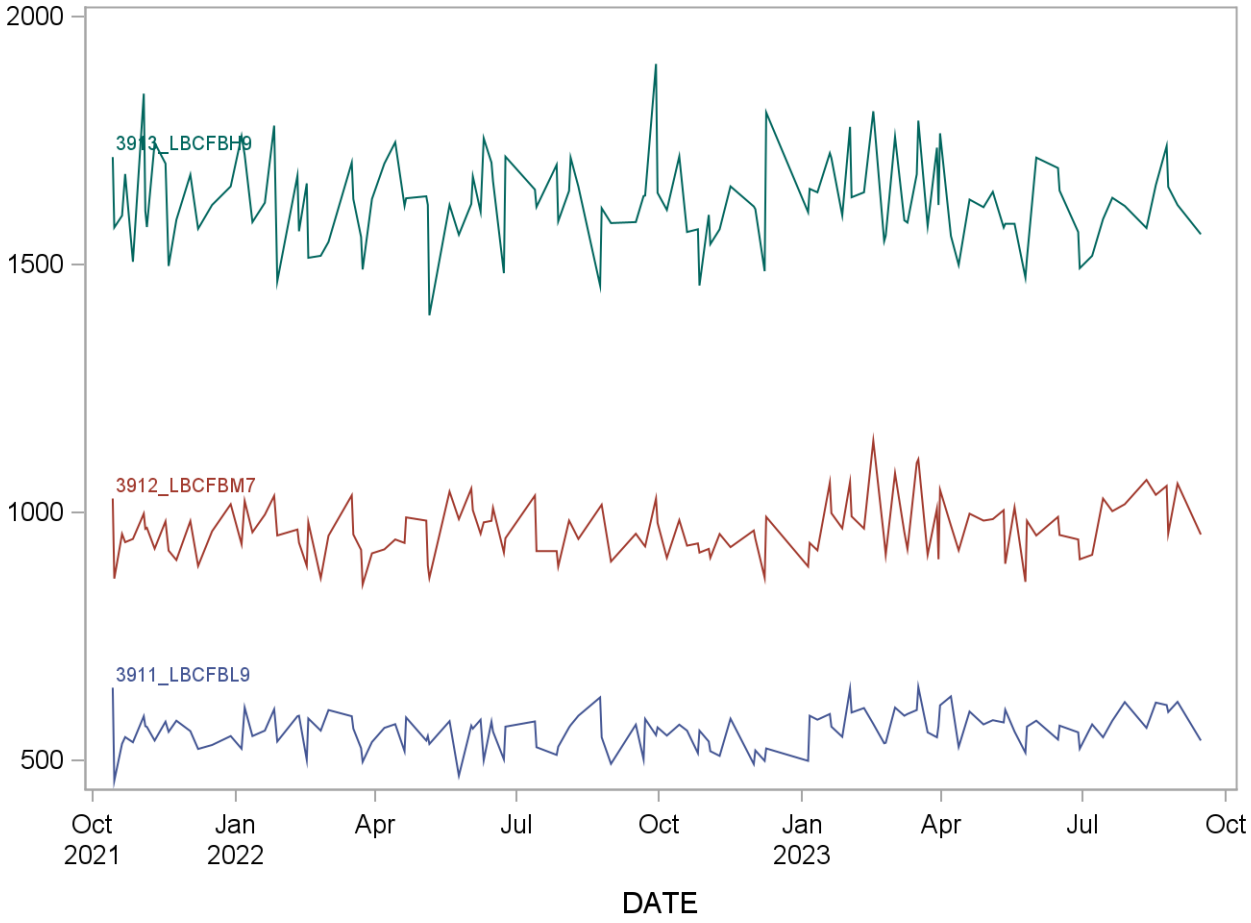
August 2021-August 2023 Summary Statistics and QC Chart
LBXRF6 (Mefox oxidation product, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCRFH2	32	14APR22	23OCT23	316.855	13.702	4.3
6413_LBCRFL2	32	14APR22	23OCT23	121.627	5.230	4.3
6412_LBCRFM2	32	14APR22	23OCT23	172.178	5.930	3.4
6411_LBCRFH3	79	13NOV23	31OCT24	321.273	20.811	6.5
6413_LBCRFL3	79	13NOV23	31OCT24	123.698	8.203	6.6
6412_LBCRFM3	79	13NOV23	31OCT24	177.122	11.265	6.4



August 2021-August 2023 Summary Statistics and QC Chart
LBXRBF (Folate, RBC(nmol/L RBC))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
3913_LBCFBH9	124	14OCT21	15SEP23	1627.5	87.7	5.4
3911_LBCFBL9	124	14OCT21	15SEP23	560.7	36.8	6.6
3912_LBCFBM7	123	14OCT21	15SEP23	965.6	54.9	5.7



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Acknowledgements

We gratefully acknowledge the contributions of Zia Fazili-Qari, Ph.D., Neelima Paladugula, M.S., and Christine M. Pfeiffer, Ph.D. who assisted in developing the methodology and preparing the manuscript for this chapter.

Appendix A: Method Performance Documentation

A. Accuracy

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be **85-115%** except at **3*LOD** where can be **80-120%**

Method name:	Folate Forms by LC-MS/MS
Method #:	4015
Matrix:	RBC
Units:	nmol/L

Analyte: 5-MethylTHF

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)	Spike concentration	Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)		
Sample	1	0	25.20	24.2	24.95		0	29.0	27.9	28.88		98	8.7
	2		25.20	24.6				29.6	29.1				
	3		25.00	25.5				29.2	28.5				
Sample + Spike 1	1	5	30.50	30.0	30.33	108	5	33.5	33.3	32.98	82		
	2		31.00	29.8				33.8	31.9				
	3		30.10	30.6				32.6	32.8				
Sample + Spike 2	1	10	35.50	35.5	35.15	102	10	40.0	37.5	38.67	98		
	2		35.40	34.6				39.5	38.3				
	3		34.80	35.1				38.3	38.4				
Sample + Spike 3	1	20	46.80	44.6	45.27	102	20	48.4	47.0	48.13	96		
	2		45.10	44.2				50.3	47.0				
	3		46.10	44.8				47.8	48.3				

Analyte: Folic acid

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)	Spike concentration	Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)		
Sample	1	0	0.10	0.10	0.09		0	0.11	0.11	0.11		106	2.3
	2		0.11	0.10				0.12	0.13				
	3		0.07	0.09				0.10	0.10				
Sample + Spike 1	1	5	5.73	5.18	5.4	107	5	5.61	5.46	5.4	106		
	2		5.02	5.34				5.56	5.14				
	3		5.95	5.37				5.23	5.38				
Sample + Spike 2	1	10	10.6	10.5	11.0	109	10	10.6	10.5	11.0	109		
	2		11.4	10.9				10.8	11.9				
	3		12.2	10.2				11.4	11.0				
Sample + Spike 3	1	20	22.6	19.7	20.8	103	20	20.8	21.5	21.0	104		
	2		20.4	20.9				19.2	21.1				
	3		20.9	20.2				21.5	21.7				

Analyte: MeFox

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Replicate	Spike concentration	Measured concentration Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)		
Sample	1	0	6.38	5.85	6.1		0	5.59	5.59	5.63		116	3.1
	2		5.83	6.22				5.73	5.76				
	3		6.36	6.11				5.62	5.48				
Sample + Spike 1	1	5	12.20	12.00	12.2	121	5	11.10	11.00	11.3	113		
	2		13.30	11.90				11.50	11.10				
	3		12.00	11.70				11.80	11.20				
Sample + Spike 2	1	10	17.90	16.80	17.7	116	10	18.60	16.70	17.2	115		
	2		18.20	17.60				17.10	17.30				
	3		18.10	17.50				16.90	16.40				
Sample + Spike 3	1	20	30.3	28.5	29.2	116	20	27.6	27.5	28.1	112		
	2		29.4	29.1				29.5	27.3				
	3		29.9	28.2				28.8	27.8				

Analyte: 5-FormylTHF

		Sample 1					Sample 2					Mean recovery (%)	SD (%)
	Replicate	Spike concentration	Measured concentration Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1 (05/23/19)	Day 2 (05/31/19)	Mean	Recovery (%)		
Sample	1	0	0.04	0.09	0.06		0	0.03	0.07	0.06		101	1.9
	2		0.06	0.08				0.10	0.06				
	3		0.03	0.09				0.04	0.09				
Sample + Spike 1	1	5	5.22	4.87	5.06	100	5	4.99	5	5.02	99		
	2		5.01	5.08				4.74	4.89				
	3		5.53	4.67				5.19	5.28				
Sample + Spike 2	1	10	10.2	9.96	10.13	101	10	10.2	9.89	10.14	101		
	2		10.5	10.1				10.4	10.2				
	3		10.2	9.81				10.4	9.75				
Sample + Spike 3	1	20	21.4	20.4	21.0	105	20	21.5	19.6	20.3	101		
	2		21.9	20.1				20.3	20.6				
	3		20.4	21.6				20.5	19.3				

Analyte: 5, 10 -MethenylTHF

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1 (05/23/19)	Sample 1 Measured concentration Day 2 (05/31/19)	Sample 1 Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1 (05/23/19)	Sample 2 Measured concentration Day 2 (05/31/19)	Sample 2 Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.19	0.20	0.20		0	0.43	0.34	0.39		113	3.0
	2		0.20	0.21				0.44	0.37				
	3		0.20	0.21				0.38	0.36				
Sample + Spike 1	1	5	6.30	6.17	6.00	116	5	6.10	5.86	5.80	108		
	2		5.46	5.77				5.69	5.94				
	3		6.41	5.89				5.12	6.09				
Sample + Spike 2	1	10	12.30	11.20	11.82	116	10	11.60	11.00	11.48	111		
	2		10.90	11.60				10.80	11.60				
	3		12.90	12.00				12.40	11.50				
Sample + Spike 3	1	20	23.2	23.6	22.8	113	20	24.0	23.3	22.9	112		
	2		22.2	23.2				21.4	22.2				
	3		21.0	23.3				23.3	22.9				

Analyte: Tetrahydrofolate

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1 (05/23/19)	Sample 1 Measured concentration Day 2 (05/31/19)	Sample 1 Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1 (05/23/19)	Sample 2 Measured concentration Day 2 (05/31/19)	Sample 2 Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.39	0.63	0.50		0	0.85	0.99	0.97		92	1.7
	2		0.44	0.60				0.94	1.11				
	3		0.43	0.54				0.87	1.07				
Sample + Spike 1	1	5	5.08	5.22	5.15	93	5	5.12	5.63	5.42	89		
	2		5.07	5.11				5.16	5.56				
	3		5.22	5.19				5.49	5.53				
Sample + Spike 2	1	10	9.91	10.10	9.83	93	10	9.40	10.10	10.10	91		
	2		9.85	9.64				9.87	10.30				
	3		9.91	9.55				10.50	10.40				
Sample + Spike 3	1	20	19.40	18.70	19.07	93	20	20.80	20.20	19.57	93		
	2		18.50	18.90				19.30	18.80				
	3		19.30	19.60				19.30	19.00				

B. Precision

Precision - fill in yellow shaded cells

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

Instruments	Hazel
Method name	Folate Forms by LC-MS/MS
Method #:	4015
Matrix:	RBC
Units:	nmol/L
Run dates:	02/19/2019 to 03/20/2019

Analyte: 5-MethylTHF

Quality material 1 (LR-18530_LC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	14.6	14.4	14.5	0.013	0.013	421
2	14.8	14.7	14.8	0.001	0.001	436
3	14.9	14.9	14.9	0.001	0.001	445
4	14.3	14.9	14.6	0.078	0.078	427
5	14.6	14.9	14.7	0.028	0.028	435
6	14.7	15.2	14.9	0.062	0.062	446
7	14.7	14.8	14.7	0.004	0.004	434
8	14.2	14.5	14.4	0.014	0.014	412
9	15.0	14.7	14.9	0.018	0.018	441
10	15.0	15.1	15.1	0.007	0.007	454
Grand sum	294.9	Grand mean	14.75			
		Std Dev	0.21			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.4510	0.0451	0.2124	1.44
Between Run	0.7941	0.0882	0.1469	1.00
Total	1.25		0.2582	**1.75**

Quality material 3 (HR-18532_LC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	41.0	42.6	41.81	0.668	0.668	3497
2	41.8	41.9	41.86	0.005	0.005	3504
3	43.0	43.2	43.10	0.003	0.003	3715
4	40.1	42.1	41.10	1.037	1.037	3378
5	42.4	42.5	42.49	0.003	0.003	3610
6	42.8	41.7	42.22	0.325	0.325	3565
7	41.2	42.6	41.90	0.491	0.491	3511
8	41.8	41.5	41.62	0.027	0.027	3465
9	42.5	42.7	42.62	0.018	0.018	3632
10	44.0	42.9	43.42	0.308	0.308	3770
Grand sum	844.259	Grand mean	42.21			
		Std Dev	0.70			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	5.7719	0.5772	0.7597	1.80
Between Run	8.8866	0.9874	0.4529	1.07
Total	14.66		0.8845	**2.10**

Analyte: Folic acid

Quality material 1 (LR-18530_LC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2.40	2.44	2.42	0.000	0.000	11.73
2	2.52	2.37	2.45	0.005	0.005	11.97
3	2.51	2.33	2.42	0.008	0.008	11.69
4	2.25	2.19	2.22	0.001	0.001	9.87
5	2.39	2.41	2.40	0.000	0.000	11.52
6	2.43	2.67	2.55	0.014	0.014	13.01
7	2.43	2.48	2.46	0.001	0.001	12.07
8	2.42	2.52	2.47	0.002	0.002	12.20
9	2.63	2.58	2.60	0.001	0.001	13.53
10	2.46	2.66	2.56	0.010	0.010	13.11
Grand sum	49.088	Grand mean	2.45			
		Std Dev	0.11			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.0858	0.0086	0.0927	3.77
Between Run	0.2035	0.0226	0.0837	3.41
Total	0.29		0.1249	**5.09**

Quality material 3 (HR-18532_LC)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	9.61	9.52	9.56	0.002	0.002	182.9
2	9.69	9.98	9.84	0.021	0.021	193.6
3	9.47	11.39	10.43	0.919	0.919	217.5
4	10.95	12.53	11.74	0.619	0.619	275.6
5	10.43	10.71	10.57	0.019	0.019	223.4
6	10.94	10.59	10.76	0.030	0.030	231.7
7	9.19	11.15	10.17	0.953	0.953	206.8
8	10.94	10.56	10.75	0.036	0.036	231.1
9	10.21	10.32	10.26	0.003	0.003	210.7
10	9.91	10.75	10.33	0.175	0.175	213.4
Grand sum	208.8266	Grand mean	10.44			
		Std Dev	0.59			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	5.5521	0.5552	0.7451	7.14
Between Run	6.2997	0.7000	0.2690	2.58
Total	11.85		0.7922	**7.59**

Analyte:	MeFox												
Quality material 1 (LR-18530_LC)							**Quality material 3 (HR-18532_LC)**						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**	**Run**	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	3.64	3.86	3.75	0.013	0.013	28.11	1	9.82	9.69	9.76	0.004	0.004	190.32
2	3.53	3.59	3.56	0.001	0.001	25.35	2	9.40	9.30	9.35	0.002	0.002	174.85
3	3.56	3.65	3.61	0.002	0.002	26.06	3	8.57	9.26	8.91	0.121	0.121	158.92
4	3.47	3.57	3.52	0.002	0.002	24.75	4	8.76	8.91	8.83	0.006	0.006	156.07
5	3.76	3.75	3.76	0.000	0.000	28.22	5	9.67	9.63	9.65	0.000	0.000	186.32
6	3.77	3.84	3.80	0.001	0.001	28.90	6	9.75	9.49	9.62	0.017	0.017	184.97
7	3.64	3.65	3.65	0.000	0.000	26.61	7	9.32	9.70	9.51	0.035	0.035	180.84
8	3.50	3.47	3.48	0.000	0.000	24.24	8	8.79	9.06	8.93	0.017	0.017	159.33
9	3.54	3.41	3.47	0.004	0.004	24.14	9	9.13	9.39	9.26	0.017	0.017	171.62
10	3.36	3.66	3.51	0.022	0.022	24.65	10	8.60	9.79	9.19	0.352	0.352	169.01
Grand sum	72.2162	**Grand mean**	3.61				**Grand sum**	186.0246	**Grand mean**	9.30			
		Std Dev	0.12						**Std Dev**	0.33			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		**Sum squares**	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.0925	0.0092	0.0962	2.66	**Within Run**	1.1442	0.1144	0.3383	3.64
Between Run	0.2692	0.0299	0.1016	2.81	**Between Run**	1.9937	0.2215	0.2314	2.49
Total	0.36		0.1399	**3.87**	**Total**	3.14		0.4098	**4.41**

Analyte:	5-FormylTHF												
Quality material 1 (LR-18530_LC)							**Quality material 3 (HR-18532_LC)**						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**	**Run**	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	2.60	2.54	2.57	0.001	0.001	13.21	1	10.33	9.88	10.10	0.052	0.052	204.16
2	2.62	2.61	2.61	0.000	0.000	13.66	2	10.04	10.01	10.03	0.000	0.000	201.00
3	2.59	2.52	2.56	0.001	0.001	13.07	3	10.61	10.48	10.55	0.004	0.004	222.39
4	2.46	2.53	2.50	0.001	0.001	12.46	4	9.39	9.78	9.58	0.038	0.038	183.61
5	2.44	2.57	2.50	0.004	0.004	12.54	5	10.00	10.09	10.05	0.002	0.002	201.94
6	2.64	2.64	2.64	0.000	0.000	13.90	6	10.22	10.22	10.22	0.000	0.000	208.98
7	2.52	2.32	2.42	0.010	0.010	11.71	7	9.74	9.96	9.85	0.012	0.012	194.10
8	2.42	2.55	2.48	0.004	0.004	12.33	8	10.25	9.99	10.12	0.017	0.017	204.82
9	2.37	2.67	2.52	0.022	0.022	12.68	9	9.59	9.64	9.62	0.001	0.001	185.00
10	2.44	2.48	2.46	0.000	0.000	12.08	10	10.83	10.99	10.91	0.006	0.006	238.06
Grand sum	50.5051	**Grand mean**	2.53				**Grand sum**	202.0	**Grand mean**	10.10			
		Std Dev	0.07						**Std Dev**	0.40			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		**Sum squares**	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.0878	0.0088	0.0937	3.71	**Within Run**	0.2666	0.0267	0.1633	1.62
Between Run	0.0842	0.0094	0.0170	0.67	**Between Run**	2.8814	0.3202	0.3831	3.79
Total	0.17		0.0952	**3.77**	**Total**	3.15		0.4164	**4.12**

Analyte:	Tetrahydrofolate					
Quality material 1 (LR-18530_LC)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	2.92	2.71	2.82	0.011	0.011	15.90
2	2.79	2.81	2.80	0.000	0.000	15.67
3	2.81	2.62	2.71	0.009	0.009	14.72
4	2.97	2.77	2.87	0.010	0.010	16.45
5	2.92	2.81	2.86	0.003	0.003	16.38
6	2.74	2.85	2.80	0.003	0.003	15.64
7	2.75	2.89	2.82	0.005	0.005	15.94
8	2.71	2.85	2.78	0.005	0.005	15.43
9	2.67	2.73	2.70	0.001	0.001	14.61
10	2.78	2.93	2.86	0.005	0.005	16.32
Grand sum	56.0349	**Grand mean**	2.801745			
		Std Dev	0.06			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.10311954	0.0103	0.1015	3.62
Between Run	0.0602	0.0067	0.0000	0.00
Total	0.16		0.1015	**3.62**

Quality material 2 (MR-18531_LC)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	7.14	7.16	7.15	0.000	0.000	102
2	7.06	7.22	7.14	0.007	0.007	102
3	6.72	7.02	6.87	0.023	0.023	94
4	6.58	7.42	7.00	0.179	0.179	98
5	6.67	6.87	6.77	0.010	0.010	92
6	7.40	7.26	7.33	0.005	0.005	108
7	7.03	7.20	7.12	0.007	0.007	101
8	6.95	6.84	6.90	0.003	0.003	95
9	7.21	7.25	7.23	0.001	0.001	105
10	7.77	7.43	7.60	0.028	0.028	116
Grand sum	142.2162	**Grand mean**	7.11081			
		Std Dev	0.24			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.5242	0.0524	0.2290	3.22
Between Run	1.0723	0.1191	0.1827	2.57
Total	1.60		0.2929	**4.12**

Analyte:	5,10-MethenylTHF					
Quality material 2 (MR-18531_LC)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	4.18	4.27	4.22	0.002	0.002	35.686
2	4.07	4.09	4.08	0.000	0.000	33.250
3	4.37	4.59	4.48	0.012	0.012	40.096
4	4.50	4.20	4.35	0.022	0.022	37.859
5	4.54	4.23	4.39	0.025	0.025	38.464
6	4.51	4.33	4.42	0.009	0.009	39.052
7	4.28	4.03	4.16	0.016	0.016	34.574
8	4.13	3.99	4.06	0.005	0.005	32.960
9	4.32	4.28	4.30	0.000	0.000	37.015
10	4.78	4.47	4.62	0.024	0.024	42.709
Grand sum	86.1	**Grand mean**	4.31			
		Std Dev	0.18			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.2305	0.0230	0.1518	3.52
Between Run	0.5828	0.0648	0.1444	3.35
Total	0.81		0.2095	**4.86**

Quality material 3 (HR-18532_LC)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	10.27	12.11	11.19	0.845	0.845	250.48
2	10.14	9.36	9.75	0.152	0.152	190.12
3	10.83	11.36	11.10	0.069	0.069	246.29
4	10.60	10.47	10.54	0.004	0.004	221.99
5	10.52	10.39	10.46	0.004	0.004	218.72
6	10.15	10.54	10.35	0.039	0.039	214.04
7	11.41	10.42	10.92	0.246	0.246	238.30
8	10.33	9.79	10.06	0.072	0.072	202.32
9	10.19	10.65	10.42	0.054	0.054	217.24
10	10.05	10.57	10.31	0.068	0.068	212.72
Grand sum	210.2	**Grand mean**	10.51			
		Std Dev	0.45			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	3.1041	0.3104	0.5571	5.30
Between Run	3.6648	0.4072	0.2200	2.09
Total	6.77		0.5990	**5.70**

C. Stability

Stability - fill in yellow shaded cells	
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 3 times (2 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 (typicall at room temperature)	
Describe condition:	QC material (not yet processed) stored at room temperature for 3 hrs, then processed for analysis
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler	
Describe condition:	(1): Processed samples were kept in the autosampler overnight prior to analysis next day
	(2): Processed samples were kept at -20°C for 3 days (over the weekend) prior to analysis
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 material stored at -70°C for up to 5 years
All stability sample results should be within **±15%** of nominal concentration	
Method name:	**Folate Forms by LC-MS/MS**
Method #:	**4015**
Matrix:	**RBC**
Units:	**nmol/L**
Run date:	**2019-08-15 (HZ:6500)**

Analyte:	5-MethylTHF							
Quality material 1 (LR18530)	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability (~1.5 yr)**	**Long-term stability (5 yr)**
Replicate 1	14.67	13.89	14.07	14.87	14.36	14.59	14.70	14.30
Replicate 2	14.71	14.08	13.99	14.36	14.65	14.43	14.90	14.50
Replicate 3	14.44	13.82	13.57	15.69	14.58	14.43	14.90	14.6
Mean	**14.60**	**13.93**	**13.87**	**14.97**	**14.53**	**14.48**	**14.83**	**14.47**
% difference from initial measurement	--	**-4.63**	**-5.00**	**2.52**	**-0.51**	--	**2.43**	**-0.10**
Quality material 2 (MR18531)	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability (~1.5 yr)**	**Long-term stability (5 yr)**
Replicate 1	19.56	18.20	19.08	19.34	18.03	19.16	19.20	19.20
Replicate 2	18.68	18.14	18.67	19.31	19.77	18.96	19.50	18.90
Replicate 3	18.71	18.59	18.58	19.21	19.29	18.57	19.40	19.00
Mean	**18.98**	**18.31**	**18.78**	**19.28**	**19.03**	**18.90**	**19.37**	**19.03**
% difference from initial measurement	--	**-3.54**	**-1.08**	**1.59**	**0.26**	--	**2.49**	**0.73**
Quality material 3 (HR18532)	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability (~1.5 yr)**	**Long-term stability (5 yr)**
Replicate 1	40.85	40.57	40.10	40.76	41.19	42.54	42.50	41.70
Replicate 2	40.08	40.02	40.17	40.07	41.06	41.82	42.90	41.50
Replicate 3	41.84	40.81	39.84	41.45	41.28	43.09	43.00	41.50
Mean	**40.92**	**40.47**	**40.04**	**40.76**	**41.18**	**42.48**	**42.80**	**41.57**
% difference from initial measurement	--	**-1.12**	**-2.16**	**-0.39**	**0.62**	--	**0.75**	**-2.16**

Analyte:	Folic acid							
Quality material 1 (LR18530)	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability (~1.5 yr)**	**Long-term stability (5 yr)**
Replicate 1	2.58	2.45	2.40	2.62	2.61	2.53	2.63	2.57
Replicate 2	2.37	2.53	2.69	2.58	2.58	2.54	2.64	2.44
Replicate 3	2.70	2.46	2.35	2.49	2.39	2.58	3.08	2.68
Mean	**2.55**	**2.48**	**2.48**	**2.56**	**2.53**	**2.55**	**2.78**	**2.56**
% difference from initial measurement	--	**-2.91**	**-2.80**	**0.49**	**-0.93**	--	**9.04**	**0.4**
Quality material 3 (HR18532)	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability (~1.5 yr)**	**Long-term stability (5 yr)**
Replicate 1	10.24	9.60	9.52	9.67	10.11	10.73	11.20	9.95
Replicate 2	10.38	9.43	10.00	10.03	9.59	10.28	11.30	9.68
Replicate 3	9.95	9.87	10.06	10.60	9.61	10.21	11.40	10.00
Mean	**10.19**	**9.63**	**9.86**	**10.10**	**9.77**	**10.41**	**11.30**	**9.88**
% difference from initial measurement	--	**-5.47**	**-3.25**	**-0.87**	**-4.14**	--	**8.60**	**-5.08**

Analyte:	MeFox							
Quality material 1 (LR18530)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	3.25	3.13	3.35	3.40	3.40	3.49	3.21	3.21
Replicate 2	3.09	3.15	3.33	3.27	3.40	3.63	3.23	3.21
Replicate 3	3.19	3.13	3.30	3.22	3.40	3.54	3.29	3.27
Mean	3.18	3.14	3.32	3.30	3.40	3.55	3.24	3.23
% difference from initial measurement	--	-1.37	4.56	3.81	6.97	--	-8.74	-9.12
Quality material 2 (MR18531)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	4.97	4.89	4.63	4.86	4.91	4.94	4.49	4.49
Replicate 2	4.66	4.65	4.69	4.74	4.71	4.97	4.58	4.52
Replicate 3	4.72	4.61	4.66	4.64	4.70	4.91	4.70	4.57
Mean	4.78	4.72	4.66	4.75	4.77	4.94	4.59	4.53
% difference from initial	--	-1.39	-2.53	-0.76	-0.22	--	-7.08	-8.36
Quality material 3 (HR18532)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	8.59	8.56	8.07	8.58	8.32	8.90	8.36	8.17
Replicate 2	8.41	7.88	8.34	8.56	8.46	8.95	8.54	8.19
Replicate 3	8.38	8.31	8.55	8.68	8.22	9.08	8.37	8.15
Mean	8.46	8.25	8.32	8.61	8.34	8.98	8.42	8.17
% difference from initial measurement	--	-2.48	-1.65	1.71	-1.48	--	-6.19	-9.01

Analyte:	5-FormylTHF							
Quality material 1 (LR18530)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	2.38	2.49	2.42	2.44	2.45	2.45	2.44	2.56
Replicate 2	2.53	2.36	2.30	2.47	2.53	2.43	2.47	2.45
Replicate 3	2.56	2.38	2.39	2.53	2.46	2.54	2.30	2.43
Mean	2.49	2.41	2.37	2.48	2.48	2.48	2.40	2.48
% difference from initial measurement	--	-3.15	-4.90	-0.39	-0.30	--	-2.92	0.18
Quality material 2 (MR18531)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	0.23	0.32	0.37	0.31	0.28	0.21	0.21	0.212
Replicate 2	0.21	0.33	0.27	0.27	0.27	0.21	0.19	0.213
Replicate 3	0.23	0.31	0.35	0.32	0.26	0.21	0.20	0.214
Mean	0.23	0.32	0.33	0.30	0.27	0.21	0.20	0.21
% difference from initial measurement	--	41.92	44.76	32.57	19.31	--	-4.53	2.19
Quality material 3 (HR18532)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	10.00	9.45	9.84	9.81	9.39	10.31	10.00	9.94
Replicate 2	9.60	9.21	9.96	10.05	9.70	10.37	10.20	9.91
Replicate 3	9.62	9.40	9.59	10.01	9.82	10.77	9.89	9.57
Mean	9.74	9.35	9.80	9.96	9.64	10.48	10.03	9.81
% difference from initial measurement	--	-3.95	0.62	2.22	-1.04	--	-4.32	-6.45

Analyte:	Tetrahydrofolate							
Quality material 1 (LR18530)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	2.42	2.48	2.12	2.61	2.50	2.80	2.73	3.06
Replicate 2	2.54	2.38	2.51	2.46	2.14	2.72	2.84	2.85
Replicate 3	2.65	2.19	2.52	2.57	2.32	2.86	2.72	2.85
Mean	2.54	2.35	2.38	2.55	2.32	2.79	2.76	2.92
% difference from initial measurement	--	-7.34	-5.99	0.39	-8.47	--	-1.00	4.62
Quality material 2 (MR18531)	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability (~1.5 yr)	Long-term stability (5 yr)
Replicate 1	7.14	6.19	5.76	6.69	6.81	6.79	6.92	7.57
Replicate 2	6.48	6.01	6.11	6.63	6.71	6.93	7.03	7.39
Replicate 3	6.46	6.21	6.71	6.76	6.56	7.03	7.18	7.63
Mean	6.69	6.14	6.19	6.69	6.69	6.92	7.04	7.53
% difference from initial measurement	--	-8.32	-7.53	-0.01	-0.01	--	1.84	8.87

Note: The concentrations of some minor analytes in quality materials are near the LOD or at LOQ; thus, larger variability is not unusual.

D. LOD, Specificity, and Fit for Intended Use

LOD, specificity and fit for intended use - fill in yellow shaded cells

Method name:	Folate Forms by LC-MS/MS
Method #:	4015
Matrix:	RBC
Units:	nmol/L

Analytes	Mass Spectrometer Instrument (multiple runs)	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
5-MethylTHF	AB Sciex 6500 QTrap Systems	0.09	yes	yes
Folic acid		0.05	yes	yes
5-FormylTHF		0.07	yes	yes
Tetrahydrofolate		0.13	yes	yes
5, 10-MethenylTHF		0.11	yes	yes
MeFox		0.09	yes	yes

Appendix B: Job Aids

A. General

1) JA-4015-G-01-Calibration and Calibration Verification

a) **Calibration**

This assay is calibrated daily by a 5-point calibration curve (linear and 1/x2 weighted). Calibration is performed with the following concentrations:

- 5-MethylTHF at 1, 2, 4, 20, 100 nmol/L
- PGA, 5-formylTHF, MeFox, THF, and 5,10-methenylTHF at 0.5, 1, 2, 10, 50 nmol/L

The **reportable range** for this method is LOD – high calibrator. Because the daily calibration range starts at a concentration slightly higher than the LOD, the expanded calibration range LOD – high calibrator is verified periodically. However, previous versions of this method (#4001 and #4012) were calibrated from 0 – high calibrator and generated comparable results throughout the calibration range to method #4014 (previous version). Furthermore, for the main analyte 5-methylTHF, all results obtained so far have far exceeded the LOD. LODs for folate forms in blood analyzed by SCIEX 6500 are:

Analytes	LOD (nmol/L) WB-Lysate	LOQ (nmol/L) WB-Lysate	LOD (nmol/L) RBC-Lysate	LOQ (nmol/L) RBC-Lysate
5-MethylTHF (MET)	0.13	0.43	0.09	0.30
Folic acid (PGA)	0.14	0.46	0.05	0.17
5-FormylTHF (FOT)	0.20	0.66	0.07	0.23
Tetrahydrofolate (THF)	0.25	0.83	0.13	0.43
5,10-MethenylTHF (MYT)	0.20	0.66	0.11	0.36
MeFox (MFO)	0.10	0.33	0.09	0.30

5-methylTHF instrument results <11 nmol/L (equivalent to ~317 nmol/L RBC folate at an approximate specimen dilution of 1:30 to 1:35) should be reanalyzed for confirmation (critical limit).

Results >High Cal (>100 nmol/L for 5-methylTHF and >50 nmol/L for other folate forms) should be diluted with 0.1% ascorbic acid and reanalyzed.

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 these conditions are met with the calibration procedure of this assay, and therefore no additional calibration verification is required by CLIA.

Calibration verification is required by the DLS Policies and Procedures manual at least twice a year. For this assay we typically process and analyze available reference material at least once per month.

c) **Periodic calibration verification activities**

Analysis of international standard reference material (SRM) multiple times per year can be used to satisfy calibration verification requirements.

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)

Assigned content of 13 ng/AMPOULE (28.3 nmol/L) whole blood folate (consensus value from microbiological assays and radioassays) when reconstituted in 1 mL DI water. We characterized this material in 2005 by LC-MS/MS and found a total folate concentration of 22.0 nmol/L, made up of 19.7 nmol/L 5-methylTHF (CV 3.45%) and 2.34 nmol/L 5-formylTHF (CV 4.43%). However, 5-formylTHF was later found to be an oxidation product of 5-methylTHF (MeFox); MeFox is biologically inactive and is not measured by microbiologic assay. The MeFox concentration in this material is ~3 nmol/L.

NIBSC 95/528 SRM is lyophilized material shipped at ambient temperature. It's recommended to store unopened ampoules at ≤ -20°C. After reconstitution we aliquot small amounts in cryovials and keep under deep frozen conditions (-50°C to -90°C). Analyze reconstituted material as unknown against working standards. Monitor whether the yearly mean measured concentration is stable.

d) **Verification of expanded calibration range to cover LOD to lowest calibrator**

Additional aqueous calibration materials between the LOD and the lowest calibration from the routine calibration curve (5-methylTHF: 1.0–100 nmol/L; other folate forms: 0.5–50 nmol/L) are tested periodically to verify the reportable range. Triplicate calibrators are prepared for the entire calibration range and carried through the entire sample preparation procedure. QC samples are analyzed together with the additional calibrators:

Analytes	Concentration 1 (nmol/L)	Concentration 2 (nmol/L)	Concentration 3 (nmol/L)
MET	0.125	0.250	0.50
PGA	0.063	0.125	0.25
MFO	0.063	0.125	0.25
FOT	0.063	0.125	0.25
MYT	0.063	0.125	0.25
THF	0.063	0.125	0.25

Two calibration curves, the routine, and the expanded curve (5-methylTHF: 0.125–100 nmol/L; other folate forms: 0.063–50 nmol/L) are evaluated for slope, intercept, R^2. Slopes are expected to be interchangeable (overlapping 95% CI) and R^2 are expected to be >0.99. Calculated results for the QC samples using the two calibration curves are expected to agree within ±5% for concentrations >1 nmol/L, within ±15% for concentrations between the LOQ and ≤1 nmol/L and within ±30% for concentrations <LOQ.

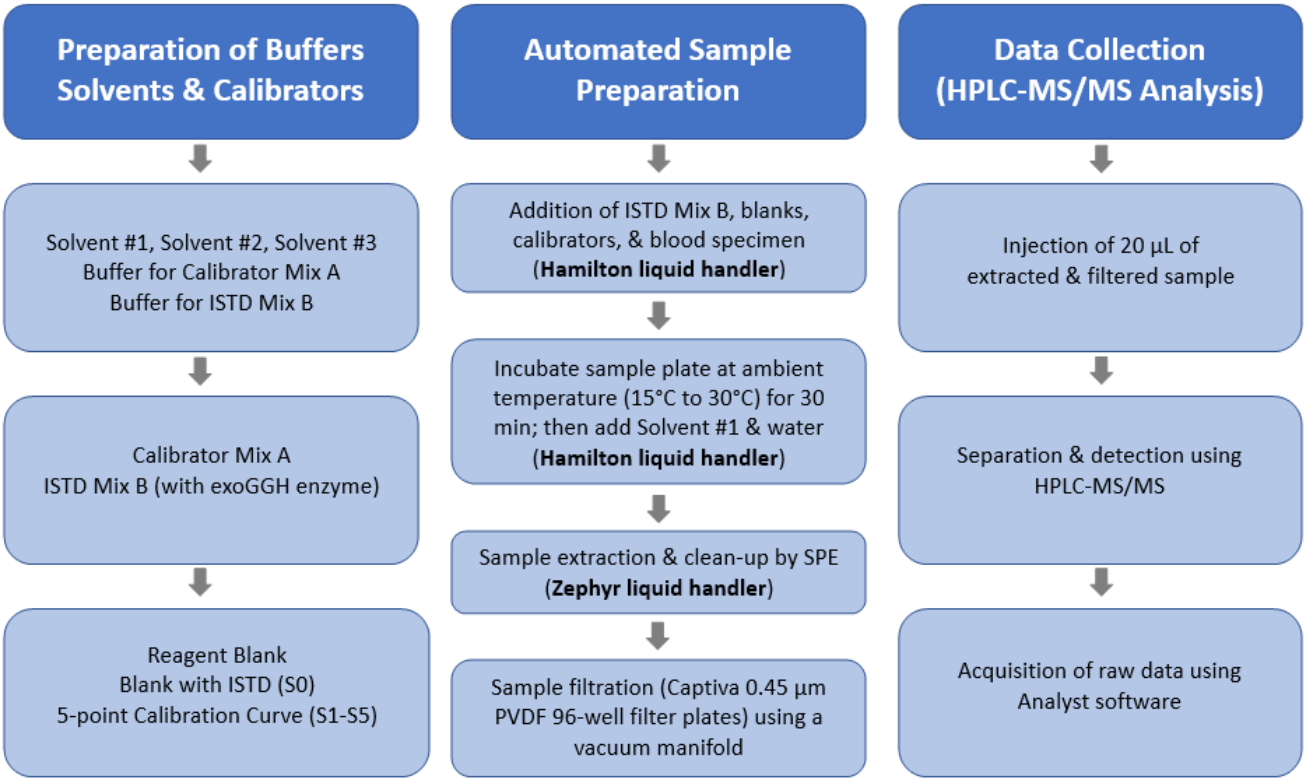
e) **Verification of new calibrator lot**

Each new calibrator lot needs to be verified for accuracy and consistency and approved by the Technical Supervisor-Lab Chief (TS-LC) prior to use for data reporting. The concentration of each new standard stock I is assigned based on UV-Vis absorbance and lower level stock solutions and working calibrator lots are prepared from this stock. The concentration of each new calibrator lot is verified by performing at least one analytical run where two levels (2 replicates/level) of the new calibrator lot (typically S4 and S5) are measured as unknowns using the current calibrator lot. As a minimum, the run includes the bench QC samples and it may also include SRM materials. The General Supervisor-Team Lead (GS-TL) reviews the instrument data for run issues, checks for contamination, calculates the difference between target *vs* measured for each new calibrator level when analyzed as unknown, calculates the difference for each QC and/or SRM from target using current calibrator lot, and compiles a data summary for TS-LC review and approval. TS-LC reviews data (verifies for expected area ratios and target concentrations; verifies QC and/or SRM results are within expected variability, determines whether the new calibrator analyzed as unknown performed as expected (within ± 5% of the current calibrator), and whether additional runs are needed to reach a valid conclusion, then signs the calibration lot verification report prior to using the new calibrator lot for routine use.

Calibrator lot to lot variability monitored over 15 years for 6 different folate forms was small and within acceptable criteria (≤5%): MET: 100% ±2.8%; PGA: 101% ±4.6%; MFO: 99% ±5.1%; FOT: 99% ±3.9%; THF: 99% ±5.1%; MYT: 99% ±4.8%.

B. Reagents & Standards

1) JA-4015-R&S-01-Sample Preparation Flow Chart



2) JA-4015-R&S-02-Reagent Tracking

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

a) **Reagent Preparation**

Date of reagent preparation is logged on reagent bottle or tags attached to reagent bottles. The reagent bottle also has a label indicating for how long the reagent can be used (e.g., Expires 1 month after preparation). Chemicals used to prepare the reagent are documented on reagent tracking sheets in the laboratory. The following information is included on the reagent tracking sheet:

(1) Name of chemical, supplier, catalog number, and lot number

(2) Method and reagent(s) in which it is used

(3) CHaTS barcode (if available)

(4) Date received, started, and stopped use

(5) Any important notes regarding these chemicals

b) **Frequently Prepared Reagents**

(1) 1% Ammonium formate buffer (pH 3.2)

(2) 0.05% Ammonium formate buffer (pH 3.4)

(3) Elution solvent (49% water, 40% methanol, 10% acetonitrile, 1% acetic acid with 0.5% ascorbic acid)

(4) HPLC mobile phase (49.5% water, 40% methanol, 10% acetonitrile, and 0.5% acetic acid)

(5) HPLC rinse & shutdown solvent (90% methanol and 10% water)

(6) 0.1% and 0.5% Ascorbic acid (used as powder)

c) **Infrequently Prepared Reagents**

(1) 0.1 M Phosphate buffer pH 7.2 (using potassium phosphate monobasic and dibasic salts)

(2) 1 M Hydrochloric acid (HCl)

(3) 0.5 M Hydrochloric acid (HCl)

(4) 0.1N Sodium hydroxide (NaOH)

(5) Cysteine (~0.1%)

d) **Standards Preparation**

(1) Stock Solutions:

All calibrators and internal standards (ISTDs) specified in the APM originate from single-analyte reagents. Information available on chemicals used to prepare stock solutions is tracked in individual written or electronic records (network shared drive) generated when the stock solution is prepared.

(2) Intermediate Standard Solutions:

Intermediate standard and ISTD solutions are prepared by diluting primary stocks as specified in the APM. Aliquots of working standards and ISTD solutions for single use are kept deep frozen (-50°C to -90°C). Information available on the stock solutions used to

prepare the working standard solutions is tracked in individual electronic records (network shared drive) generated when the working standard is prepared.

e) **Quality Control Materials**

Chemicals and other materials (e.g., human serums, antioxidants) used to prepare quality control materials are tracked in individual written or electronic records (network shared drive) generated when the material is prepared. Quality control materials are aliquoted for single use and kept deep frozen (-50°C to -90°C).

Chemicals may be used to amend (i.e., spike) analyte into a quality control material. In these cases, available information on the chemicals used is tracked in the record (either written or electronic).

f) **Other Materials**

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

C. Instruments

1) JA-4015-I-01-Instrument Comparison and System Verification

a) **Instrument Comparison**

When a method is analyzed on multiple instruments, an initial instrument comparison must be conducted to establish analytical comparability. All calibrators, quality control materials, and at least 30 samples 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 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.

b) **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, 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:

(1) 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).

(2) 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).

(3) 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:

(1) Summarize the results in a spreadsheet that contains the raw data, the data analyses, and the summary information and request review by the supervisor.

(2) 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. Save the signed PDF to electronic QA Manual.

2) JA-4015-I-02-Hamilton Microlab STARlet Liquid Handler Maintenance and Calibration Verification using the Volume Field Verification (VFV) Kit

a) **Daily Maintenance**

(1) Load instrument software method to execute the daily maintenance for deck and waste – Click **[Yes]** & continue**.** Empty tip waste; click **[OK]** to continue; (avoid clicking on 'Cancel' - aborts the daily maintenance)

(2) Execute the 1000 µL channel tightness check; ensure 8 teaching needles are installed. Click **[Yes]** & continue. Execute the 1000 µL channel cLLD check; ensure 8 teaching needles are installed. Click **[Yes]** and continue until maintenance method is completed.

b) **Weekly Maintenance**

(1) Load instrument software to execute the daily maintenance for deck & waste – Click **[Yes]**

(2) Using auto-load all racks are moved & put back (can empty deck manually) – Click **[OK]** & continue. Open Front cover, clean the deck – Click **[OK]** & continue

(3) Open front cover, empty & clean the tip waste – Click **[OK]** & continue

(4) Check the laser scanner window (clean using lint free cloth or Q tips lightly soaked in 70% ethanol) – Click **[OK]**

(5) Upon prompting: Execute the 1000 µL channel tightness check; ensure 8 teaching needles are installed – Click **[Yes]** & continue. Execute the 1000 µL channel cLLD check; ensure 8 teaching needles are installed – Click **[Yes]** and continue until maintenance method is completed.

c) **Instrument calibration and volume verification (2 times/year) using Volume Field Verification (VFV) Kit**

(1) Load instrument software to execute the daily maintenance for deck and waste – Click **[Yes]**

(2) Open method & at deck layout find tip carrier and weighing unit locations

(3) Double click Microlab STAR Method Editor icon → **[File]** →**[Open]** →Liquid Class Developer→ Use "***Ultimate Liquid Class Validation. Method"***

(4) On worktable check the tip racks for respective tips (300 µL, 1000 µL etc.) are at appropriate deck locations (Carrier-Tip _CAR_480_A00,), restock if needed.

(5) Following manufacturer's instructions assemble the weighing unit & its components; plug in the corresponding cables from the weighing unit to the balance control; from the balance control to the computer and power source.

(6) Place appropriate test liquid in the given vial; put it on the weighing unit. Go to the "**Microlab Star Run"** method icon on desktop; double click and open a workstation window.

(7) Open **[File],** locate the target file (same as step 1) → double click to open method→ Click **"Start"** →"**Initialize"** will occur automatically → Prompt window appears "**Test Type Choice**"→ **Validation** → **Select "test & tip type"** → In tip count window → Look at "**Labware"** for tip size → Ensure that **"First"** and the **"Last"** standard volume tip counts are correct → Click **"OK"** → Run starts at position 1 → Select **"liquid type"** → Select **"liquid class to test"** → Check "**liquid transfer information"**. Enter density volume (µL), liquid density (g/mL); program calculates total times transferred/ channel→ Select **"default pipetting settings"** Click **"Yes"** → For Anti-Droplet Control (ADC) Select **"No"** → For tip re-

use Select **"No"** → Enter aspiration/dispense submerge depths → Select **"OK"** → Select aspiration/dispense "mix volume & number of mix cycles" → Enter appropriate values for these selections & click **"OK"** → Tips are picked up automatically & weights of the set liquid volume measured with each tip during respective aspirating/ dispensing steps.

(8) Auto-generated data are reviewed on instrument computer; results imported to network (summarized & saved for reference).

D. Data Review

1) JA-4015-DR-01-Computerization and Data System Management

a) **Sample Identification**

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 sample collectors.

b) **Data Collection**

Instrument raw data files and respective batch files collected using Analyst software (SCIEX 6500) are stored on mass spectrometer workstation.

c) **Data Back-up**

Raw batch files and data files from instrument computers are generally transferred via ISLE, KVM-switch, or encrypted thumb drive to NBB shared network drive.

d) **Peak Review and Integration**

The raw data (calibration curve data, patient data; QC data) are processed into results using the Quantitation Wizard software (Analyst); results are saved and reviewed by team lead for peak integrations, chromatograms, slope/intercept and R^2. Finally, the processed and reviewed result files are saved in the import data file folder on the shared network drive.

e) **Data Import**

The result files containing patient data as well as QC data stored on the shared network drive are further exported into the STARLIMS Database for QC and statistical evaluation.

f) **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 ‘4015 (Blood Folate Vitamers LCMSMS)’
- Click on ‘Add’ and select the Instrument
- Run# and Equipment ID will be populated
- [0] Run Instrument Macro – select the excel 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 which replaces the null with “|” (a pipe tab)
- [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 bench QC (SAS) information in Run Comments column
- Click on 'Manage Attachments' and upload the data review checklist for the run
- Click 'Finish Results' located under the test workflow steps and notify Project Lead

Level II – Project Lead:

- Double click the STARLIMS icon on desktop
- Under 'Run-based Tasks', select 'Run Approval'
- Choose 'Show Pending Tests' and select '4015 (Blood Folate Vitamers LCMSMS)'
- Review analyst run sheet, data review checklist, peak integration, and Sample QC evaluation
- Review and confirm Run QC evaluation
- Assess blind QC results - click on 'Blind QC Results Only' tab, 'Assess Blind QC', 'Final Result' and 'Use Default Characterization Sets', 'Proceed to Next Step', '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 v2', 'OK', '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 may 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 the blind QC report and final data review checklist for the run
- In Run Approval tab, click Release Run and notify QA Officer (level III review)

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
- Results set reportable, released, and reported by QA officer

2) JA-4015-DR-02-Processing and Reporting a Run

a) **Reviewing the run:**

(1) After a batch or run is acquired, the data are reviewed in Analyst software. Chromatograms for all folate forms are checked for retention time, peak shape, peak separation, intensity, and any potential interferences.

(2) Data are typically processed on a network computer. Batch and result files are copied from the instrument computer onto the network drive for data processing.

b) **Quantitation and integration of the data file in Analyst software:**

(1) Under the left bar menu, select “Quantitate” and open “Quantitation Wizard”.

(2) In the quantitation wizard menu:

- Under “Available Data Files” select the data file for processing, under “Available Samples” select the number of individual samples for processing, use the arrow key (=>) to transfer the samples under “Selected Samples”, and click “Next”
- Open the dropdown list next to “Setting to Use” and select “Summary Table”, select “None” under the “Default Query” setting, and click “Next”
- Select “Choose Existing Method”, open the dropdown list next to “Method” and select an existing method (e.g., FOL_RBCQuant.qmf or FOL_WBQuant.qmf), and then click “Finish” to process data file.

(3) An auto integrated results table is generated.

(4) In the top bar menu, select “File” and “Save As”. Save the quantitation result file (.rbd) using the following format: “FolateRBCmmddyy” or “FolateWBmmddyy”, where mmddyy (month/date/year) is the sample preparation date.

(5) Review the integration of each sample (blanks, calibrators, QC, and unknowns).

(6) Run and update signal-to-noise for each analyte.

(7) Check calibration curve accuracy:

- Accuracy from target value for S1 should be within 30% for all analytes
- Accuracy from target value for S2-S5 should be within 15% for major analytes
- Accuracy from target value for S2-S5 should be within 20% for minor analytes

(8) Review integration parameters (minimum peak height & peak width, RT window, noise percent, smoothing width, peak splitting factor, etc.) and make necessary integration corrections using either manual or auto integration options. Note: auto integration is preferred over manual integration.

(9) Integration parameter changes can be applied to all samples by right click on the integrated peak and select “Update Method”.

(10) Check QC results for limits and accuracy.

(11) Save any changes to the results table.

(12) When data review and integration is completed, the saved Analyst “rdb” file on the network is imported to LIMS database:

- In Analyst results table, right click and select “Table Settings” then select “Serum Results for Import”.
- In the top bar menu, select “File” then select “Export” and save the results in a designated folder on the network drive.

3) JA-4015-DR-03-STARLIMS Data Review and Criteria

Processed instrument results are imported into STARLIMS database for data review and sample QC evaluation. The quality of individual test result within run is subjected to the sample QC criteria and action is taken based on the flagged result.

a) For flagged results on calibration curve see details on **Table 1**.

b) For flagged results on relative retention time see details on **Table 2**.

c) Evaluate bench QC and blind QC:

(1) Bench QC evaluation is performed by Level I (Analyst)

- If pass → set Run QC "Pass"
- If fail → set Run QC "Fail"; set code 61; repeat the entire run

(2) Blind QC evaluation is performed by Level II (Project Lead)

- If pass → no action; continue with reporting
- If fail (of 4 if 1 or 2 QC are [<or >3SD]) → repeat 10% of samples from the run (including blind QC) to confirm results

d) For flagged results on patient sample see details on **Table 3**.

(1) Pass (codes: 0, 33, 37, or 97) – result passed all criteria and can be reported

(2) Warn, Check, and Fail – result needs to be reviewed or repeated to confirm

e) Check sample reproducibility

When sample is repeated, multiple results will be shown in "Set Final" Wizard window. The "Retest Delta" is difference between the results and should be within ±15%. Set final on the result that meets the sample QC criteria.

Table 1. Calibration Curve and Blank Sample QC Criteria				
Analyte	**Flagged Result**	**Result Status**	**DLS Comment Code**	**Action**
MET, PGA, MFO, FOT, THF & MYT	Numeric Blank Value in Reagent Blank (RB) and Blank (S0)	Warn	None	Check instrument result: • for all analytes & repeat the run if RB contaminated • for area in MET, PGA and MFO from 'S0' • Area in FOT, THF, & MYT expected from 'S0'/override & enter code"0"
	Calibrator (S1) diff. to target: >30%	Check	0	Check instrument result; repeat the run
	Calibrator drift (within-run): >15%	Warn	0	Check instrument result; check % diff. from target value
MET, PGA & MFO	Calibrator (S2-S5) diff. to target: >15%	Check	0	Check instrument result; repeat the run
	Calibrator drift (within-run): >15%	Warn	0	Check instrument result; check % diff. from target value
FOT, THF & MYT	Calibrator (S2) diff. to target: >20%	Check	0	Check instrument result; repeat the run
	Calibrator drift (within-run): >20%	Warn	0	Check instrument result; check % diff. from target value
	Calibrator (S3-S5) diff. to target: >15%	Check	0	Check instrument result; repeat the run
	Calibrator drift (within-run): >15%	Warn	0	Check instrument result; check % diff. from target value
MET, MFO, FOT & MYT	R^2 <0.98	Fail	26	Check instrument result; repeat the run
	R^2 0.98 >= to <0.99	Check	None	Check calibration result; enter code "0"
PGA, & THF	R^2 <0.95	Fail	26	Check instrument result; repeat the run
	R^2 0.95>= to <0.98	Check	None	Check calibration result; enter code "0"

Table 2. Relative Retention Time Sample QC Criteria				
Analyte	**Flagged Result**	**Result Status**	**DLS Comment Code**	**Action**
MET & PGA	Result ≥LOQ & Rel. RT: ≤0.980 or ≥1.02	Fail	26	Check instrument result/repeat the run
	Results <LOQ & Rel. RT: ≤0.980 or ≥1.02	Check	None	Check instrument result/resolve or repeat
MFO	Results ≥LOQ & Rel. RT: ≤0.985 or ≥1.02	Fail	26	Check instrument result/repeat the run
	Result <LOQ & Rel. RT: ≤0.985 or ≥1.02	Check	None	Check instrument result/resolve or repeat
FOT	Results ≥LOQ & Rel. RT: ≤0.985 or ≥1.018	Fail	26	Check instrument result/repeat the run
	Results <LOQ & Rel. RT: ≤0.985 or ≥1.018	Check	None	Check instrument result/resolve or repeat
THF	Results ≥LOQ & Rel. RT: ≤0.963 or ≥1.06	Fail	26	Check instrument result/repeat the run
	Results <LOQ & Rel. RT: ≤0.963 or ≥1.06	Check	None	Check instrument result/resolve or repeat
MYT	Results ≥LOQ & Rel. RT: ≤0.982 or ≥1.02	Fail	26	Check instrument result/repeat the run
	Results <LOQ & Rel. RT: ≤0.982 or ≥1.02	Check	None	Check instrument result/resolve or repeat

Table 3. Patient Sample QC Criteria				
Analytes	**Flagged Result**	**Result Status**	**DLS Comment Code**	**Action**
MET	Null, 0 or No Peak	Fail	26	Check instrument result; repeat/confirm

	<LOD	Fail (Result 1) / Warn (Result 2)	26/37	Check instrument result; repeat/confirm
	<LOQ	Incomplete (Result 1) / Warn (Result 2)	33	Check instrument result; repeat/confirm
	≥LOD <11 nmol/L	Incomplete (Result 1) / Warn (Result 2)	33	Check instrument result; repeat/confirm
	≥11 nmol/L & ≤100 nmol/L	Pass	0	None
	>100 nmol/L	Fail	26	Check instrument result; dilute/repeat
	≥11 nmol/L & ≤100 nmol/L; Retest # ≥1 & dilution >1.0	Pass	97	None
MFO	Null, 0 or No Peak	Check	37	Check instrument result; repeat/confirm
	<LOD	Check	37	Check instrument result; repeat/confirm
	<LOQ	Warn	0	Check instrument result
PGA, MFO, FOT, THF, & MYT	≥LOD & ≤ 50 nmol/L	Pass	0	None
	>50 nmol/L	Fail	26	Check instrument result; dilute/repeat
	≥LOD & ≤ 50 nmol/L Retest # ≥1 & dilution >1.0	Pass	97	None
PGA, FOT, THF & MYT	Null, 0 or No Peak	Pass	37	None
	<LOD	Pass	37	None
	Signal/Noise ratio <3, & Raw result >LOD	Warn	0	Check instrument result & confirm S/N ratio is correct; check dilution factor (if >1.0); report undiluted result
MET, PGA, MFO, FOT, THF, & MYT	**Retest Delta: ≥ 0.85** 2nd result within ±15% of 1st result	Pass	0/33/37	None
	2nd result not within ±15% of 1st result	Pass	0/33/37	Check instrument result; repeat 3rd time
MET, PGA, MFO, FOT, THF, & MYT	Manual Integration	Check	None	Check instrument result; update code “0”

4) JA-4015-DR-04-Out-of-Control Corrective Action

a) QC performance is evaluated by SAS - run comments updated appropriately (pass/fail)

b) SAS QC failed analytes are investigated, and appropriate corrective action measures as indicated are applied.

c) Verify calibrator response, area ratios, slope/Intercept, R2 (>0.989) of the analyte in question and other analytes.

d) Verify QC status of other analytes in the run. Verify if there were problems with sample preparation and extraction; check chromatograms, peak shape, integration, relative retention time, MS instrument response, or interferences; apply appropriate code.

e) Verify if the issue is caused due to an error in one of the calibrators and eliminating such calibrator from the curve can correct the issue; discuss and seek approval from supervisor.

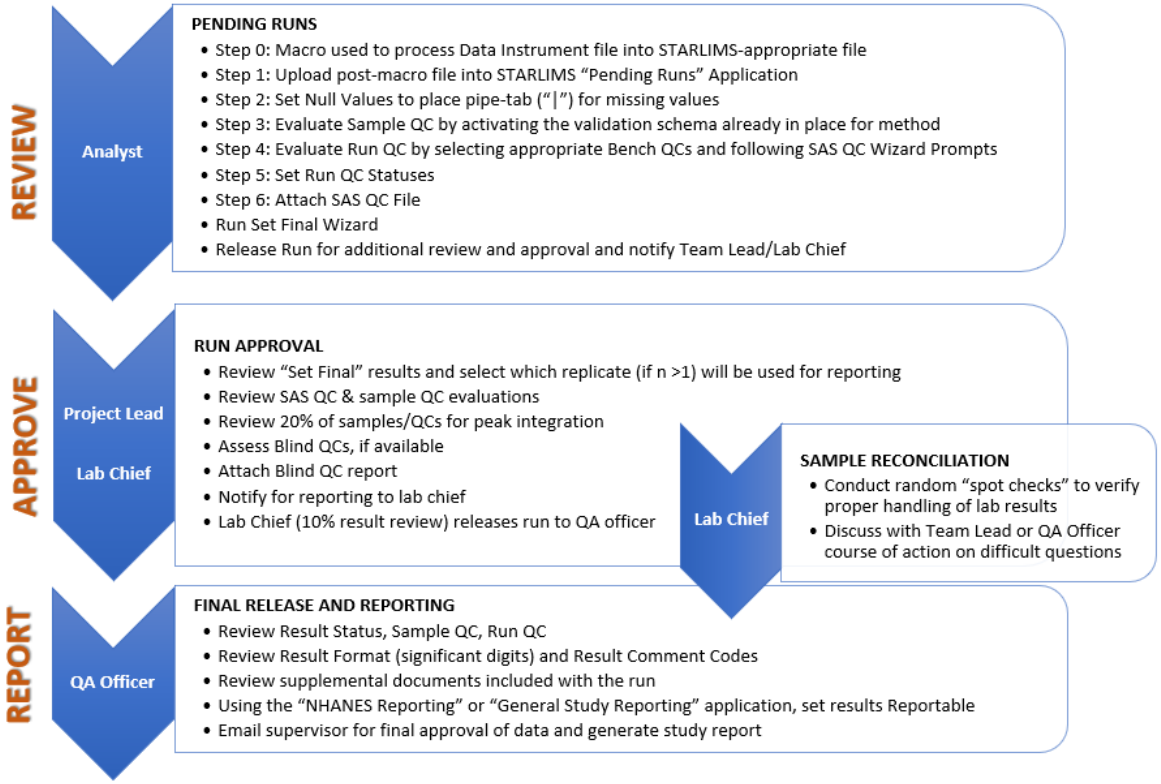
f) Verify if QC in question passed for other analytes; if appropriate, eliminate failed QC and re-run SAS; update comments (pass/fail); discuss and seek approval from supervisor.

g) If the QC failure is true (potential statistical issue); apply code 61 and repeat samples in next run.

h) Enter appropriate comments in STARLIMS database (User fields 1 & 2).

5) JA-4015-DR-05-STARLIMS Data Review Flowchart

The following instruction reflect general steps for data review, approve, sample reconciliation, and report in STARLIMS.



Appendix C: Information on Absorption Maxima, Absorption Coefficients, 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)	
					Unlabeled	$^{13}C_5$-Labeled
5-MethylTHF	20 mM phosphate buffer with 0.1% cysteine (pH 7.2)	290	31,700	1	459.46	464.46
5-FormylTHF	20 mM phosphate buffer with 0.1% cysteine (pH 7.2)	285	37,200	2	473.44	478.44
THF	20 mM phosphate buffer with 0.1% cysteine (pH 7.2)	298	25,000	3	445.43	450.43
5,10-MethenylTHF	1 M HCl (pH 0)	288 & 348	13,500 & 26,500	3–5	455.45	460.45
Folic acid (PGA)	20 mM phosphate buffer (pH 7.2)	282 & 346	27,600 & 7,200	3	441.40	446.40
MeFox	0.1 N NaOH (pH 13)	280	19,365	6	473.40	482.40

a) **References for UV-spectrophotometric determination of folate concentration:**

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

(2) Uyeda K, Rabinowitz JC. J. Biol. Chem. 1965;240:1701.

(3) Rabinowitz JC. In: Boyer PD, Lardy H, and Myrbaeck K (eds.). The Enzymes, vol. 2. 2nd ed., Academic Press, New York 1960. p. 185.

(4) Rabinowitz JC. In: Colowick SP and Kaplan NO (eds.). Methods in Enzymology, vol. 6, Academic Press, New York 1963. p. 814.

(5) Huennekens FM, Ho PPK, Scrimgeour KG. In: Colowick SP and Kaplan NO (eds.). Methods in Enzymology, vol. 6, Academic Press, New York 1963, p. 806.

(6) Personal communication with Jean-Pierre Knapp at Merck Cie, March 2012.

(7) 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.

b) **Formulas to calculate the concentration of folate stock solution based on molar absorptivity:**

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

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

with ε max = molar extinction coefficient and conc = concentration

Example calculation for folic acid (PGA):

Absorbance	Dilution	ε max	MW
0.661	10	27600	441.4

Concentration (ppm or µg/mL) = 0.661 x 10 x 1000 x 441.4/27600 = 105.7

Concentration (µmol/L) = 0.661 x 10 x 1000 x1000 /27600 = 239.5

c) **Conversion factors from conventional (ng/mL) to SI units (nmol/L) for different folate forms:**

	5-MethylTHF	**5-FormylTHF**	**PGA**	**THF**	**5,10-MethenylTHF**	**MeFox**
Unlabeled form	2.176	2.112	2.266	2.245	2.196	2.112
13C5-labeled form	2.153	2.09	2.24	2.22	2.172	2.09

Appendix D: Approximate QC Pool Target Concentrations (nmol/L) for the Various Folate Vitamers and the 5-methylTHF Oxidation Product (MeFox) (whole blood lysate or RBC lysate basis)

QC level	5-MethylTHF	5-FormylTHF	PGA	THF	5,10-MethenylTHF	MeFox	TFOL
Low	5*	1	1	1	1	1	11[&]
Medium	15	2.5	2.5	2.5	2.5	2.5	25
High	25	5	5	5	5	5	50

*As low as possible

Note 1: A WB lysate tFOL concentration of ~11 nmol/L corresponds to a WB tFOL concentration of 127 nmol/L and a RBC tFOL concentration of 317 nmol/L (based on a Hct of 40%.

Note 2: A RBC lysate tFOL concentration of ~9.6 nmol/L corresponds to a RBC tFOL concentration of 317 nmol/L (based on a practical dilution factor of 1:33; the theoretical sample dilution is 1:22, however washed RBCs contain only ~70-75% of RBCs and ~25-30% of saline, which increases the dilution factor by ~1.5-fold to ~1:33.

Appendix E: Agreement of blood folate forms among different sample types: Analysis by LC-MS/MS

	Concentration[1] (mean ± SD), nmol/L			Relative difference, % (95% CI)
Analytes	**WB-Lysate[2]**	**RBC-Lysate[3]**	**WB-Lysate + exoGGH[4]**	**RBC-Lysate *vs* WB-Lysate**
5-MethylTHF	831 ± 258	843 ± 276	867 ± 268	1.22 (-0.58 to 3.02)
Non-methyl folate	53.3 ± 74.4	52.9 ± 70.7	53.0 ± 75.6	2.27 (-1.91 to 6.44)
$TFOL_{Excluding\ MeFox}$	886 ± 255	899 ± 271	922 ± 263	1.37 (-0.43 to 3.18)
MeFox	147 ± 44.1	172 ± 53.5	165 ± 50.8	16.8 (14.8 to 18.8)

[1] n = 60 subjects per sample type

[2] WB-Lysate samples were conventionally prepared by 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, WB-Lysate aliquots were incubated for 4 h at 37°C (in duplicate over 2 days)

[3] RBC-Lysate samples were washed RBC diluted ½ with saline prior to 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, RBC-Lysate aliquots were treated with exoGGH enzyme (5 µg/mL of lysate) for 30 min at ambient temperature (in duplicate over 2 days)

[4] exoGGH, exogenous gamma-glutamyl hydrolase; WB-Lysate samples were conventionally prepared by 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, exoGGH was added to WB-Lysate aliquots (5 µg enzyme/mL sample added to the internal standard mixture), and samples were incubated for 4 h at 37°C (in duplicate over 2 days)

Note: for more information, see reference **[11]**

Appendix F: Method comparison: LC-MS/MS *vs.* microbiologic assay (MBA)

Sample Matrix	Descriptive Statistic	HPLC-MS/MS$_{\text{Excluding MeFox}}$[1]	HPLC-MS/MS$_{\text{Including MeFox}}$[1]	MBA[1]
WB-Lysate[2]	Mean±SD, nmol/L	886±255	1033±295	1089±300
	Median (IQR), nmol/L	893 (724 to 996)	1028 (855 to 1165)	1109 (893 to 1205)
	Pearson correlation *r*	0.963	0.970	
	Relative difference (95% CI)[3], %	-18.6 (-20.3 to -16.9)	-5.12 (-6.85 to -3.40)	
	Weighted Deming regression slope (95% CI)	0.824 (0.767 to 0.882)	0.958 (0.899 to 1.017)	
	Weighted Deming regression intercept (95% CI)	-12.81(-71.51 to 45.9)	-11.4 (-69.7 to 46.9)	
RBC-Lysate[4]	Mean±SD, nmol/L	899±271	1071±319	1037±295
	Median (IQR), nmol/L	900 (739 to 1019)	1051 (852 to 1186)	1052 (839 to 1180)
	Pearson correlation *r*	0.971	0.975	
	Relative difference (95% CI)[3], %	-13.5 (-15.2 to -11.8)	3.13 (1.36 to 4.89)	
	Weighted Deming regression slope (95% CI)	0.901 (0.845 to 0.958)	1.066 (1.005 to 1.127)	
	Weighted Deming regression intercept (95% CI)	-36.5 (-91.2 to 18.1)	-35.4(-91.9 to 21.2)	

[1]Total folate by HPLC-MS/MS is the sum of folate forms (5-methylTHF, 5-formylTHF, THF, 5,10-methenylTHF, excluding or including MeFox); MBA, microbiologic assay

[2] WB-Lysate samples (*n*=60); conventionally prepared as follows: whole blood aliquot from each sample diluted 1/11 with 1% ascorbic acid, then frozen; prior to analysis by HPLC-MS/MS, WB-Lysate aliquots were incubated for 4 h at 37°C; WB-Lysate aliquots for MBA were not incubated prior to analysis; aliquots processed and analyzed as duplicates over 2 days by each assay

[3]Calculated as HPLC-MS/MS minus MBA divided by MBA (expressed as percent) and then averaged across all 60 samples

[4]RBC-Lysate samples (*n*=60); washed RBC samples diluted approximately ½ with saline prior to 1/11 dilution with 1% ascorbic acid, then frozen; prior to analysis, RBC-Lysate aliquots were treated with exoGGH enzyme (5 µg/mL of lysate) for 30 min at ambient temperature; aliquots processed and analyzed as duplicates over 2 days by each assay

Note: for more information, see reference **[11]**

Appendix G: Ruggedness Testing

Folate is an important nutrient involved in one carbon cellular metabolism. WB or RBC folates are measured to determine folate status. We use an isotope-dilution tandem mass spectrometric method in multiple reaction-monitoring mode (MRM) coupled with liquid chromatography (LC-MS/MS) for quantitative measurements of 5 different folate forms.

A previous version of this method (method 4001) using WB lysates has undergone a series of in-house **ruggedness testing** experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. At least five parameters judged to affect the accuracy of the method most likely 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. Because the current method 4015 can also use RBC lysates and we have demonstrated equivalent results with both matrices, we only evaluated one additional ruggedness parameter, namely the addition of commercially available exo-GGH for folate polyglutamate deconjugation, because this is a new step in method 4015. Please refer to the DLS Policies and Procedures Manual for further information on ruggedness testing.

A. Variations in sample preparation

(1) Principle: The buffers used for sample preparation, solid-phase extraction and analyte elution use formic acid, ammonium hydroxide (to adjust pH), ascorbic acid (as antioxidant), and acetic acid (acid modifier). The changes in buffer pH, concentration of formic acid, ascorbic acid, and acetic acid are critical for analyte and/or sample matrix recovery during sample preparation and solid-phase extraction and will affect analyte sensitivity and potentially affect the results.

(2) Proposal: To vary and test the sample preparation and solid-phase extraction conditions.

- pH of ammonium formate buffer (solvent #1)
- Formic acid concentration in ammonium formate buffer (solvent #1)
- Ascorbic acid concentration in ammonium formate buffer (solvent #1)
- Ammonium formate concentration in intermediate wash step during which matrix compounds are eluted but analytes are retained (SPE wash buffer)
- Ascorbic acid concentration in SPE elution buffer (solvent # 3)
- Acetic acid concentration in SPE elution buffer (solvent # 3)
- Exo-GGH concentration in WB lysate or RBC lysate sample for folate polyglutamate deconjugation

(3) Findings:

- Varying the pH of the ammonium formate sample preparation buffer does not appear to affect folate species results in blood samples.
- Varying the formic acid concentration in the ammonium formate sample preparation buffer does not appear to affect folate species results in blood samples.
- Varying the ascorbic acid concentration in the ammonium formate sample preparation buffer does not appear to affect folate species results in blood samples.
- Varying the ammonium formate concentration in the SPE wash buffer does not appear to affect the folate species results in blood samples.
- Varying the ascorbic acid concentration in the SPE elution buffer does not appear to affect folate species results in blood samples.

- Varying the acetic acid concentration in the SPE elution buffer does not appear to affect folate species results in blood samples.
- Varying the concentrations of exo-GGH does not appear to affect RBC folate polyglutamate deconjugation for most folate forms. Using lower exo-GGH (2.5 µg/mL lysate) gave slightly lower results (diff: <10%) for some analytes compared to method specified concentration (5.0 µg/mL lysate); while using higher exo-GGH (10.0 µg /mL lysate) gave comparable results to method specified concentrations.

B. Ruggedness testing results for whole blood folate vitamers by HPLC-MS/MS

Factor	Method specifies	Results[a] (nmol/L)	Lower level	Results[a] (nmol/L)	Higher level	Results[a] (nmol/L)
1. pH of ammonium formate buffer (sample solvent #1)	3.2	METL: 6.3 FOTL: 2.9 PGAL: 3.2 THFL: 5.2 MYTL: 9.4	3.0	METL: 6.2 FOTL: 3.0 PGAL: 2.9 THFL: 4.0 MYTL: 9.2	3.4	METL: 6.2 FOTL: 3.2 PGAL: 3.3 THFL: 5.0 MYTL: 8.3
2. Formic acid concentration in ammonium formate buffer (sample solvent #1)	1%	METL: 6.3 FOTL: 3.2 PGAL: 3.2 THFL: 6.6 MYTL: 8.1	0.8%	METL: 6.0 FOTL: 3.3 PGAL: 3.1 THFL: 5.5 MYTL: 7.8	1.2%	METL: 6.1 FOTL: 3.4 PGAL: 3.2 THFL: 8.2 MYTL: 7.6
3. Ascorbic acid in ammonium formate buffer (sample solvent #1)	0.5%	METL: 6.2 FOTL: 2.8 PGAL: 3.3 THFL: 5.6 MYTL: 8.6	0.3%	METL: 6.2 FOTL: 2.9 PGAL: 3.4 THFL: 6.1 MYTL: 8.6	0.7%	METL: 6.1 FOTL: 2.9 PGAL: 3.5 THFL: 6.0 MYTL: 8.4
4. Ammonium formate concentration in SPE wash buffer	0.05%	METL: 6.1 FOTL: 3.1 PGAL: 3.4 THFL: 5.7 MYTL: 8.5	0.04%	METL: 6.0 FOTL: 3.1 PGAL: 3.3 THFL: 5.9 MYTL: 8.6	0.06%	METL: 6.1 FOTL: 3.1 PGAL: 3.3 THFL: 6.8 MYTL: 8.5
5. Ascorbic acid concentration in SPE elution buffer	0.5%	METL: 5.9 FOTL: 2.6 PGAL: 2.7 THFL: 5.4 MYTL: 7.0	0.3%	METL: 5.9 FOTL: 2.7 PGAL: 3.0 THFL: 6.6 MYTL: 7.7	0.7%	METL: 5.8 FOTL: 2.7 PGAL: 2.7 THFL: 6.1 MYTL: 7.4
6. Acetic acid concentration in SPE elution buffer	1%	METL: 6.1 FOTL: 3.0 PGAL: 3.0 THFL: 5.5 MYTL: 8.7	0.8%	METL: 5.9 FOTL: 2.8 PGAL: 3.1 THFL: 5.6 MYTL: 8.7	1.2%	METL: 5.8 FOTL: 2.7 PGAL: 3.1 THFL: 5.9 MYTL: 8.3

[a] Results are shown for the medium QC sample.

Abbreviations for folate vitamers in WB lysate: METL (5-methylTHF); PGAL (Folic acid); FOTL (5-formylTHF), THFL (tetrahydrofolate); MYTL (5,10-methenylTHF).

C. Ruggedness testing results of exo-GGH for RBC polyglutamate folate deconjugation

Factor	Method specifies	Results[a] (nmol/L)	Lower level	Results[a] (nmol/L)	Higher level	Results[a] (nmol/L)
Variation in ExoGGH levels for deconjugation	5.0 µg/mL lysate	MET: 20 MFO: 5.2 THF: 7.6 MYT: 4.1	2. 5 µg/mL lysate	MET: 19 MFO: 4.9 THF: 7.3 MYT: 4.0	10 µg/mL lysate	MET: 20.3 MFO: 5.4 THF: 7.7 MYT: 4.5

		FOT[b]: 10 PGA[b]: 10		FOT[b]: 9.9 PGA[b]: 9.5		FOT[b]: 10.3 PGA[b]: 9.6

[a]Results are shown for the Medium QC sample for all analytes except FOT and PGA.

[b]Results are shown for the High QC sample for FOT & PGA.

Abbreviations for folate vitamers in WB lysate or RBC lysate: MET (5-methylTHF); MFO (MeFox); THF (tetrahydrofolate); MYT (5,10-methenylTHF); FOT (5-formylTHF); PGA (Folic acid).

Appendix H: Typical MRM Method Parameters (Analysis in Positive Ion Mode)

Analyte (Transition)	Tr (min)	*DP (V)	CE (V)	CXP (V)	EP (V)
5-MethylTHF (*m/z* 460.2 → *m/z* 313.2)	2.37	90	28	13	10
$^{13}C_5$-5-MethylTHF (*m/z* 465.2 → *m/z* 313.2)	2.37	90	28	13	10
PGA (*m/z* 442.2 → *m/z* 295.2)	3.17	70	18	13	10
$^{13}C_5$-PGA (*m/z* 447.2 → *m/z* 295.2)	3.17	70	19	13	10
MeFox (*m/z* 474.4 → *m/z* 284.2)	3.22	90	48	13	10
$^{13}C_5$-MeFox (*m/z* 479.4 → *m/z* 284.4)	3.22	90	48	13	10
5-FormylTHF (*m/z* 474.4 → *m/z* 299.2)	3.16	80	43	13	10
$^{13}C_5$-5-FormylTHF (*m/z* 479 → *m/z* 299.2)	3.16	80	43	13	10
THF (*m/z* 446.2 → *m/z* 299.2)	2.33	85	25	13	10
$^{13}C_5$ THF (*m/z* 451.1 → *m/z* 299.1)	2.33	85	25	13	10
5,10-MethenylTHF (*m/z* 456.1 → *m/z* 412.2)	2.30	155	41	13	10
$^{13}C_5$ -5,10-MethenylTHF (*m/z* 461.1 → *m/z* 416.2)	2.30	155	42	13	10

Tr (min) retention time; DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; EP, Enterance potential; The general instrument parameters used for LC/MS/MS detection and quantitation of all four analytes in multiple reaction mode (MRM) were as follows: resolution Q1 and Q3: unit; dwell time: 110 msec; ion spray voltage: 5500 V; source temperature: 450 ℃; curtain gas: 35 psi; gas 1: 55 psi; gas 2: 60 psi; CAD gas: 8 psi

*DP voltages are subject to change with instrument sensitivity. These voltages can be optimized in Q1 & Q3 based on analyte response.