Laboratory Procedure Manual

Analyte: **Folate Forms**

Matrix: **Blood**

Method: **Liquid Chromatography Tandem Mass Spectrometry**

Method No: 4015.02

Revised:

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

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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.

Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table for FFMR_K_R:

File Name	Variable Name	SAS Label
FFMR_K_R	LBDRF7SI	Total folate (nmol/L)
	LBXRF1SI	5-Methyl-tetrahydrofolate (nmol/L)
	LBXRF2SI	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-methylene-tetrahydrofolate 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.

B. Test Principle

RBC folate status can be determined directly by measuring folate forms in washed RBCs **[2]** or indirectly by measuring folate forms in a whole blood (WB) lysate **[3]**. The direct measurement requires the addition of exogenous γ-glutamyl hydrolase (exo-GGH) to deconjugate folate polyglutamates to monoglutamates. It also requires the measurement of hemoglobin (Hb) in the RBC lysate to correct for residual moisture in the packed 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 room temperature). To calculate RBC folate, it also requires the measurement of serum folate and hematocrit (Hct) to correct for the serum folate contribution to WB folate and 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) **[4]**. The current method is a modification of a previously published method **[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.

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 room temperature 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 (including this document)	Database analyte code
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

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 10% bleach solution 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 http://intranet.cdc.gov/ossam/workplace-safety/safety-practices/chemical-safety/index.html.

Observe universal precautions (i.e., PPE) during operation of automated liquid handlers (e.g., Hamilton, Caliper-Zephyr); keep instrument doors always locked when it is running; wipe down contaminated decks (10% bleach solution) 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 LIMS

database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See **"4015.02 - SOP Computerization & Data System Management"** for a step-by-step description of data transfer, review, and approval.

(C) For NHANES, data is transmitted electronically on a regular basis (approximately weekly for 3-week turnaround analytes). Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

(D) The 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 **[2]**.

(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 (preferably at -70°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 (preferably at -70°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 and protected from light. After processing, specimens should be frozen and shipped on dry ice by overnight mail. Once received, they should be stored at ≤-20°C until analyzed. Folates are stable for only a few weeks if the specimen is frozen at -20°C. For long-term storage, specimens should be frozen at ≤-70 °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 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.

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

Not applicable for this procedure.

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

A. Reagent Preparation

Prepare all reagents with 0.22 µm filtered (cellulose Nitrate-filters) deionized water with a resistance 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.

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 room temperature for one week.

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 room temperature 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

The ammonium formate buffer described above is used as is to condition the sorbent of the 95-well SPE plate (1.3 mL). Other solvents used to condition the sorbent are 0.5 mL acetonitrile and 0.5 mL of 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 the buffer described above at the time of use.

c) Buffer for folate calibrator mix and ISTD mix: 1% ammonium formate buffer, pH 3.2, with 0.1% ascorbic acid

Add 0.1% ascorbic acid (0.1 g/100 mL).

d) 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 room temperature for one week. Add ascorbic acid powder to a final concentration of 0.1% (0.1 g/100 mL) prior to 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).

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 5 mL of acetic acid (0.5% final concentration).

4) 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 (mixed cellulose esters) 10-mL sterile syringe filter just prior to use.

5) 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).

6) 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 AB Sciex tandem mass spectrometer. This solution is stable at 4 °C for 6 months. For negative ion calibration, PPG standard 3000 and diluent is supplied in the kit by the manufacturer (AB Sciex).

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 B**. **Table II** summarizes diluent information for primary and intermediate stock solutions.

Table II. Diluents used for primary and intermediate stock solutions

Compound	Diluent for primary stock solution I	Diluent for intermediate stock solution II
5-MethylTHF	20 mM phosphate buffer (pH 7.2) [D&F]	0.5% ascorbic acid [D&F]
5-FormylTHF	20 mM phosphate buffer (pH 7.2) [D&F]	0.5% ascorbic acid [D&F]
THF	20 mM phosphate buffer (pH 7.2) [D&F]	1% ascorbic acid [D&F]
5,10-MethenylTHF	1 M HCl [D]	0.5 M HCl with 0.5% 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 sterile filters (mixed cellulose esters)

a) Primary stock solution I (<200 µg/mL)

5-MethylTHF, 5-FormylTHF and THF: These reduced folates are treated the same way. The $^{13}C_5$-labeled compounds are used as internal standards and are also prepared in the same way as described below.

1. Prepare a primary stock solution in a volumetric flask by dissolving an accurately known mass (±0.1 mg) of the pure solid compound in degassed 20 mM phosphate buffer (pH 7.2, containing 0.1% cysteine), 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 to a microcentrifuge vial to determine its concentration by UV spectrophotometry. To the remaining stock solution add ascorbic acid powder to a final concentration of 1%. From the aliquot you removed, prepare two dilutions (e.g., 1/10 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 B). For 5-methylTHF, the ratio of absorbance at 290/245 nm can be monitored (simple reads analysis at each wave length) to ensure that no oxidation takes place. This ratio should exceed 3.3.

3. Prepare a 20 µmol/L intermediate stock solution (see below under b).

4. Aliquot the remainder of the primary stock solution into cryovials (typically 1 mL/vial) that are stored at -70 °C and used approximately every two months to generate a fresh intermediate stock solution. The primary stock solution is stable for at least 2 years.

5,10-MethenylTHF: 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 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 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 to a microcentrifuge vial to determine its concentration by UV spectrophotometry. To the remaining stock solution add ascorbic acid powder to a final concentration of 1%. From the aliquot you removed, prepare two dilutions (e.g., 1/10 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 primary stock solution concentration (**Appendix B**).

3. Prepare a 20 µmol/L intermediate stock solution (see below under b).

4. Aliquot the remainder of the primary stock solution into cryovials (typically 1 mL/vial) that are stored at -70 °C and used approximately every two months to generate a fresh intermediate stock solution. The primary stock solution is stable for at least 2 years.

Folic acid (PGA): The solubility of PGA decreases as the pH decreases from alkaline to acidic **[8]**. 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 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 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 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 B**).

3. Prepare a 20 µmol/L intermediate stock solution (see below under b).

4. Aliquot the remainder of the primary stock solution into cryovials (typically 1 mL/vial) that are stored at -70 °C and used approximately every two months to generate a fresh intermediate stock solution. The primary stock solution is stable for at least 2 years.

MeFox: This 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 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 ~13), 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 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 B**).

3. Prepare a 20 µmol/L intermediate stock solution (see below under b).

4. Aliquot the remainder of the primary stock solution into cryovials (typically 1 mL/vial) that are stored at -70 °C and used approximately every two months to generate a fresh intermediate stock solution. The primary stock solution is stable for at least 2 years.

b) Intermediate stock solution II (20 µmol/L)

Prepare a 20 µmol/L intermediate stock solution for each folate separately by diluting a portion of the primary stock solution in the diluent specified in Table II (typically 10 mL volume). Aliquot the intermediate stock solution into microcentrifuge tubes that are stored at -70 °C and used to generate daily working solutions (typically 40–50 vials at 0.2 mL/vial). This procedure is repeated approximately once every 2 months to generate a fresh intermediate stock solution.

Note:
Fresh individual primary stock solutions I are prepared approximately every 2 years. Individual intermediate stock solutions II 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 13C5-5-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 at -70 °C **[7]**. Buffers and diluents are degassed with nitrogen & filtered (0.45 µm sterile filters (mixed cellulose esters) 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 stock II solutions.

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 II (µL)	100	50	50	50	50	50
1% Ammonium formate buffer with 0.1% ascorbic acid (µL)	650					
Folate conc. (µmol/L) in Mix A	2.0	1.0	1.0	1.0	1.0	1.0

b) Internal standard mix (Mix B)

To avoid pipetting small volumes, the 5 minor folate forms (PGA, 5-formylTHF, THF, and 5,10-methenylTHF, MeFox,) are premixed as follows: 100 µL of each intermediate stock II solutions is mixed together for a total volume of 500 µL and 50 µL of premix is used to prepare the internal standard Mix B.

The internal standard Mix B (total volume of 8 mL) contains a mixture of the exo-GGH enzyme and each internal standard prepared in 0.1% ascorbic acid, as shown in **Table IV**. 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) **[2]**. To optimize enzyme use, and avoid sample dilution, the enzyme is directly added from the original vial to the internal standard mix (200 µL enzyme solution containing 100 µg enzyme in 8 mL Mix B).

Table IV: Information for internal standard mix B

Mix B	**$^{13}C_5$-5-MethylTHF**	**$^{13}C_5$-PGA**	**$^{13}C_5$-5-FormylTHF**	**$^{13}C_5$-5,10-MethenylTHF**	**$^{13}C_5$-THF**	**$^{13}C_5$-MeFox**
Intermediate stock II (µL)	40					
Premix of minor folate forms (µL)		50				
0.1% ascorbic acid (mL)	7.71					
Exo-GGH enzyme (500 µg/mL) (µL)	200					
Labeled folate conc. (nmol/L) in Mix B	100	25	25	25	50	25

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 **[9]**.

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 C**, 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 room temperature; WB is then diluted with 1% ascorbic acid to achieve a 1:11 dilution. WB lysate (usually 700 µL) is aliquoted into 2.0-mL Nalgene cryovials, vials are kept capped and frozen. The QC pools are stored at -70°C and 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 room temperature; 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) is aliquoted into 2.0-mL Nalgene cryovials and vials are capped. The QC pools are stored at -70°C to maintain stability.

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

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 (Whatman)
    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 SPE instrument (Caliper-Zephyr)
    a) 96-well Bond Elute SPE blocks [50 mg phenyl sorbent] (Agilent Technologies)
    b) Captiva 96-well filter plates [0.45 µm PVDF embedded into the well] (Agilent Technologies, Lake Forest, CA) 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 (Chromtech)
    c) PEEK tubing 0.005 and 0.007 ID (Supelco)
    d) HPLC Solvent glass inlet filters, purge frits, gold seal and outlet caps (Agilent Technologies, Lake forest, CA)
    e) Pipette tips (100-1000 µL) for Eppendorf pipette (Brinkmann)
    f) Pipette tips (1000 µL & 10 mL) for Eppendorf pipette (Brinkmann)
    g) Pipette tips (10-100 µL) for Eppendorf pipettes (Brinkmann)
    h) Pipette tips (0.5-10 µL) for Eppendorf pipette (Brinkmann)

i) Combitip plus (500 µL) for Eppendorf repeater pipette (Brinkmann)

j) Positive displacement pipette tips (50 µL , 100 µL, 1000 µL) for Gilson pipettes (Gilson)

k) 30 mm Nunc 1-mL 96-well HPLC plate for 96-well autosampler (Fischer Scientific)

l) Nunc plastic seals for 30 mm 1 mL 96-well HPLC plates (Fischer Scientific)

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

n) 0.45 µm PVDF filters (Millipore)

o) 0.22 µm (cellulose nitrate) water filtration units 500 mL capacity (Corning)

p) 2.0 mL polypropylene cryovials (Nalgene Company)

q) 1.0 mL disposable syringes (Hamilton)

r) Sterile syringe filters (0.45 µm, MF-MCE membrane [mixed cellulose esters], Millipore)

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

4) Folate Standards

a) PGA (Pteroylglutamic acid, free acid or Na_2-salt), 5-methylTHF ([6S]-5CH_3-H_4PteGlu, Ca-or Na_2-salt), 5-formylTHF ([6S]-5CHO-H_4PteGlu, Ca-or Na_2-salt), MeFox ([6S](pyrazino-s-triazine derivative), THF ([6S]-H_4PteGlu, free acid, Ca-or-Na_2-salt) and 5,10-methenylTHF ([6S]-5,10-CH=H_4PteGlu-Cl x HCl salt) (Merck & Cie [formerly Eprova]), Schaffhausen, Switzerland) http://www.merck.ch/en/company/merck_in_switzerland/merck_cie_schaffhouse/merck_cie_schaffhausen.html)

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)

5) Chemicals and Solvents

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

b) Ammonium hydroxide (28–30% as NH_3, ACS reagent – J.T. Baker Brand by Avantor)

c) L-Cysteine (Sigma, Life Science)

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

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

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

g) L-ascorbic acid (vitamin C min 99% purity (Fisher Scientific Co [Spectrum chemicals])

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

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

j) Methanol, acetonitrile HPLC certified solvent (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 that 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.

To provide adequate throughput for this method as well as backup instrumentation during times of repair and maintenance, we utilize multiple LC-MS/MS systems of the AB 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) HP1260 HPLC systems (Agilent)
   Models G1367B HIP 96-well plate thermostatted autosamplers, ALS thermostat G1330B, G1316A thermostated column compartment, G1312A binary pump and G1379B in-line mobile phase degasser

2) AB Sciex 6500 QTrap triple quadrupole mass spectrometer with turboionspray (TIS) as ion source in ESI mode, with Analyst Windows Microsoft software (AB Sciex)

3) Nitrogen generator (model Table-31N) connected to the in house gas supply to supply curtain, exhaust, and source gases to the mass spectrometer instrument (AB Sciex 6500's) in addition to nitrogen gas for the collision cell (Peak Scientific Instruments)

4) Caliper-Zephyr automated 96-probe solid phase extraction system (Perkin Elmer Health Science, Inc.); can also be used for sample transfer for filtration on Captiva 96 well-plate filters

5) Microlab Starlet liquid sample handler for sample preparation and dilution (Hamilton)

6) Syringe pump (Harvard apparatus)

7) Eppendorf repeater pipettor (Brinkmann Instruments Inc.)

8) Eppendorf pipettes 10 µL, 100 µL, 200 µL, 1000 µL, and 10 mL (Brinkmann Instruments Inc.)

9) Positive air displacement pipettes (Pipetman) 50 µL, 100 µL, and 1000 µL (Gilson Inc.)

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

11) Dual syringe continuous dispenser, 600 series (Hamilton Microlab)

12) Daigger Vortex Genie 2 mixer (VWR)

13) Magnetic stirrer (Baxter Scientific Products)

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

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

16) Cary 300 Bio UV/visible spectrophotometer (Agilent Technologies)

17) 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 **[6]**. 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 QCs 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 **[4,5]**. The calibration range is from 1–100 nmol/L for 5-methylTHF and from 0.5–50 nmol/L for all other folate forms. 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). For details, see **"4015.02 - SOP Calibration and Calibration Verification"**. The folate concentration in this material has been determined by consensus value assignment but is mainly representative for the microbiologic assay. 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. When possible, we also participate in other external PT programs, such as the UK NEQAS Haematinics survey. Details can be found in the proficiency testing form. For general information on the handling, analysis, review, and reporting of proficiency testing materials see **"NBB_SOP Proficiency Testing Procedure"**.

In a matrix comparison study using blood samples from 60 donors we found excellent correlation ($r \geq 0.98$) and good agreement (within ±5% for 5-methylTHF, PGA, and non-methyl folate; within ~±15% for MeFox) for RBC folate calculated from 3 matrices: traditional WB lysates (4 h @37°C to allow the endogenous GGH to deconjugate polyglutamates; serum folate and Hct correction), WB lysates incubated with exo-GGH (30 min @ room temperature; serum folate and Hct correction), and RBC lysates incubated with exo-GGH (30 min @ room temperature; Hb and MCHC correction) **[10]** (**Appendix D**). 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 LC-MS/MS results for RBC lysates treated with exo-GGH (30 min @ room temperature) 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 E)**. 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 F**.

B. Instrument Calibration

1) Tandem mass spectrometer

The calibration of the mass spectrometer is scheduled on a semi-annual basis as part of a preventive maintenance program and is performed by service engineer from AB Sciex. If necessary, the analyst can recalibrate by using the calibration standards described below and by following the instructions contained in the operator's manual.

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. Please refer to the User's Manual and the **"4015.02 - SOP MS Tuning & Mass Spectrometer Calibration"** for additional details.

2) 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. Additionally, pipetting accuracy may be checked by using a yellow dye test.

Daily and weekly maintenance of the system is executed through the instrument operated software that checks for deck cleaning, tip waste, and 8 channel tightness. For details see, **"4015.02 - SOP Automated Sample Pipetting, Maintenance, and Verification"**.

3) Caliper-Zephyr SPE instrument

A 96-probe automated SPE instrument is used for sample extraction and cleanup. Once per year a Perkin-Elmer service engineer performs preventive maintenance including arm adjustment and volume verification (10 µL to 1000 µL), and replacement of parts if needed. As a routine maintenance, the analyst performs a weekly cleanup (dry wipe) and silicone lubrication of the 96-probe head. This instrument can, also be used for non-volume-critical solvent transfers.

4) Pipettes (air displacement and positive displacement)

On site calibration is performed annually by a certified company. Every six months, calibration verification is performed by the analyst gravimetrically, using a calibrated analytical balance.

5) Varian UV/vis spectrophotometer

Calibration verification is performed three times per year by participation in the CAP Instrumentation survey.

6) Balances

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

7) 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 QCs, 83 patient samples, and second set of QCs, for a total of 96 samples (96-well plate format). Three levels of blood QCs 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 (QCs and unknown patient samples), folate intermediate stock II solutions (calibrator and internal standard); it takes about 40 min for the samples to reach ambient temperature.

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 (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)

1) The 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 room temperature. Next, the Hamilton is used to add water (calibrators only) and buffer (calibrators and specimens) to the 96-well plate. Finally, the plate is subjected to automated SPE sample extraction and clean-up.

2) For a detailed step-by-step description, see “4015.02 - SOP Automated Sample Pipetting using Hamilton Microlab Starlet”.

   a) Check and restock tip racks.

   b) Fill reagent troughs and put in place.

   c) Put calibrators, QC samples, and unknown patient samples in place.

d) Put internal standard mixture in place.

e) Put a 96-well collection plate (sample destination plate) in place.

f) The pipetting program is executed and dispenses according to the scheme in **Table VI**.

Table VI: Pipetting Scheme

Well	Sample type	Internal standard mix with exo-GGH	Calibrator mix	Sample solvent #1	QC or patient specimen
1	Reagent blank (Double blank)	---	---	400 µL	---
2	Calibrator S0 (blank)	60 µL mix B	---	150 µL	---
3	Calibrator S1	60 µL mix B	150 µL S1		---
4	Calibrator S2	60 µL mix B	150 µL S2		---
5	Calibrator S3	60 µL mix B	150 µL S3		---
6	Calibrator S4	60 µL mix B	150 µL S4		---
7	Calibrator S5	60 µL mix B	150 µL S5		---
8	Low QC – Set 1	60 µL mix B	---		150 µL
9	Medium QC – Set 1	60 µL mix B	---		150 µL
10	High QC – Set 1	60 µL mix B	---		150 µL
11-93	Patient samples	60 µL mix B	---		150 µL
94	Low QC – Set 2	60 µL mix B	---		150 µL
95	Medium QC – Set 2	60 µL mix B	---		150 µL
96	High QC – Set 2	60 µL mix B	---		150 µL

g) After calibrators, IS mix, QC and patient samples are dispensed, the 96-well plate sits at room temperature for 30 min to allow endogenous folate to equilibrate with IS mix and also to allow the exo-GGH to deconjugate folate polyglutamates.

h) After the 30-min incubation, a second pipetting program is executed that dispenses according to the scheme shownin **Table VII**. This prepares the samples for SPE clean-up on the 96-probe instrument (Caliper-Zephyr).

Table VII: Pipetting Scheme

Well	Sample type	Water	Sample solvent #1
1	Reagent blank (Double blank)	150 µL	----
2	Calibrator S0 (blank)	150 µL	190 µL
3	Calibrator S1	150 µL	190 µL
4	Calibrator S2	150 µL	190 µL
5	Calibrator S3	150 µL	190 µL
6	Calibrator S4	150 µL	190 µL
7	Calibrator S5	150 µL	190 µL
8	Low QC – Set 1	---	340 µL
9	Medium QC – Set 1	---	340 µL
10	High QC – Set 1	---	340 µL
11-92	Patient Samples	---	340 µL
93	Low QC – Set 2	---	340 µL
94	Medium QC – Set 2	---	340 µL
95	High QC – Set 2	---	340 µL

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) Incubate at room temperature for 30 min.

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

D. Automated Solid Phase Extraction method

1) A 96-probe SPE instrument (Caliper-Zephyr) 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) For a detailed step-by-step description, see "4015.02 - SOP Automated SPE using 96-probe Caliper Zephyr".

   a) The instrument is prepared by first starting the "Maestro Software" on the desk top and opening the Folate SPE method.

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

   c) Finally, the Folate SPE method is run from the software.

   d) The SPE method performs the following steps:

      - Conditioning: 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)

      - Loading: 500 µL sample is loaded in 4 steps (125 µL x4)

      - Washing: SPE plate is washed in 3 steps with 1.3 mL of solvent #2 (0.5 mL x 2 and 0.3 mL x 1)

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

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

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

2) Captiva filter plate is placed on top of a fresh HPLC collection plate (31 mm Nunc plate).

3) Caliper-Zephyr liquid handler is used to transfer 300 µL of the extracted sample into a fresh Captiva filter plate (Agilent Technologies).

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

5) The HPLC collection plates are sealed with the pre-slit seals and arranged on the HP1200 96-well model autosampler for LC-MS/MS analysis.

F. LC-MS/MS Instrument Preparation

1) The Agilent HPLC system coupled to the AB Sciex MS/MS system is used to quantitate folate vitamers in extracted WB lysates or RBC lysates.

2) For a detailed step-by-step description, see **"4015.02 - SOP LC-MSMS Instrument Preparation"**.

   a) Prior to each run, HPLC lines are purged and the HPLC column is primed with a series of solvents; pressures are recorded.

   b) Methanol:Water (90:10) is used for line purging for ~5min 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. Pressure is recorded.

   c) 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.

   d) The tandem mass spectrometer is prepared. MS/MS method parameters for each folate vitamer and the corresponding internal standards are listed in **Appendix G**:

      - Wiping of orifice plate (methanol damped lint-free Kimwipe)
      - Cleaning of curtain plate (water & methanol)
      - Checking of ion spray needle for any blockage and cleaning if necessary

   e) The appropriate instrument method is loaded and a new batch containing the sample sequence of the current run is created.

   f) Daily instrument checks are conducted:

      - At least 2 sample solvent injections are performed before the actual run is started to verify that the system is working OK.
      - 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.

   g) The sample plate is loaded on the autosampler and the batch is submitted for analysis.

   h) The HPLC rinse method is loaded at the end of the sample batch. It runs isocratic (90:10 [methanol: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.

   i) The HPLC shutdown method is loaded after the HPLC rinse method. It runs isocratic (90:10 [methanol:water]) for 1 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.

G. Processing and Reporting a Run

1) The Applied Biosystems Analyst software is used to review/process a run. A LIMS database is used for additional levels of data review by the analyst, project lead, QA officer, and supervisor and for data reporting.

2) For a detailed step-by-step description, see **"4015.02 - SOP Processing and Integrating a Run".**

(a) Reviewing the run:

- When the batch run is finished acquiring the data, the data is reviewed in Analyst. Chromatograms for each folate form (respective transition) are checked for retention time, peak shapes, peak separation, intensity, and/or potential interferences.

(b) Quantitation and integration of the completed data file:

- Generate a results table using auto integration.
- Review integrations and make any necessary integration corrections either using the manual or auto integration option. Auto integration is preferred over manual integration.
- Import the results file into the LIMS database for further data review - See **"4015.02 - SOP Computerization and Data System Management"**.

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

- The final integrated results can either be directly imported to the LIMS database (typical) or alternatively can be processed by importing into an Excel template sheet for final calculations and interpretation.
- Transfer the peak areas for the analyte and the internal standard for each sample into the appropriate fields in the Excel sheet.
- The calibration curve with slope, intercept, and R2 is automatically generated (linear, not forced through zero, no weighting) based on area ratios.
- The concentrations of QCs and unknowns are automatically calculated using the slope and intercept information.
- The file is saved and maintained for documentation.
- 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).

H. Exporting a Run

The procedure to export a run to a LIMS database is described in section 3 & in **"4015.02 - SOP Computerization and Data System Management"**.

I. 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. 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 and for the sum of the folate forms as RBC tFOL.

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

To obtain a RBC tFOL result, the individual folate form results are added up. 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.

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.

J. System Maintenance

The system maintenance consists mainly of the different prime, purge, and wash cycles described in Section 8 of this document, and performed before and after each run. Column connections are checked for leaks daily and are wiped with a water-moistened tissue if any residues have built up. Solvent bottles are refilled as needed and cleaned on a monthly basis. Filters in the solvent bottles are replaced as needed (typically every 6-8 months). The pre-column filters are replaced after ~200 injections. The curtain plate is cleaned on daily basis first with water, then wiped with lint free Kimwipes dabbed in methanol. The orifice plate is also wiped daily with methanol dabbed lint-free Kimwipes. Preventative maintenance is performed by service engineers on all major equipment (MS/MS, HPLC, Caliper-Zephyr, Hamilton) at least once a year.

K. Special Method Notes

Since the majority of folate in blood occurs in the polyglutamate form which needs to be deconjugated to monoglutames 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 (30 min @ room temperature) in the presence of commercially available exo-gamma glutamyl hydrolase prior to SPE and analysis **[2,10]**. 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.

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 <15 nmol/L (corresponding to an RBC folate of <317 nmo/L at an approximate dilution of 1:22) 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.

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.

B. Bench Quality Controls

Bench QC specimens are prepared from 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 **[14]**.

Three QC pools per run with two or more QC results (replicates) per pool:

1) If all 3 QC run means are within 2 S_m limits and individual results are within 2 S_i limits, accept the run

2) If 1 of the 3 QC run means is outside a 2 S_m limit – reject run if:

    a) 1 3S Rule—Run mean is outside a 3 S_m limit or

    b) 2 2S Rule—Two or more of the three run means are outside the same 2 S_m limit or

    c) 10 X-bar Rule—Current and previous nine run means are on the same side of the characterization mean

3) If one of the 6 QC individual results is outside a 2 S_i limit – reject run if:

    a) Outlier – One individual result is beyond the characterization mean ± 4 S_i or

    b) 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)

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

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

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

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC. The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated periodically. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in a LIMS database. For runs that are not imported into the database (i.e., R&D, troubleshooting, research-type runs), QC results are stored electronically in the analyte-specific folder on the DLS network.

C. Sample QC Criteria

Each individual sample result is checked against established sample QC criteria limits to assure data quality. The method uses the following sample QC criteria. For details see **"4015.02 - SOP Sample QC Criteria"**.

- Calibration curve criteria for R^2
- Calibrator difference to the target and calibration drift between front and back calibrator injections
- Relative retention time (retention time quantitation ion/retention time ISTD)
- ISTD minimum area
- Manual integration flagging

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

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 step-wise 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 generally be made fresh daily and pH should be checked.

(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 room temperature will cause degradation of folates and should be avoided.

(M) Nitrogen gas cylinder for 96-probe SPE system (Caliper-Zephyr) should be periodically monitored for gas. The pressure for out flow should be 40 PSI, and the gas pressure to the instrument at the regulator should be adjusted to 5 PSI. Change the cylinder before the gauge reads 500 PSI.

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

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 **[15]**, while levels <748 nmol/L in women of reproductive age are interpreted as increased risk of neural-tube birth defects in the offspring **[16]**.

Clinical reference ranges reported for RBC folate are 317-1422 nmol/L with the CPB radioassay **[17]**.

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 **[9]**. Pfeiffer et al. also reported microbiologic assay-equivalent reference ranges for pre-fortification (NHANES 1988-1994) and early post-fortification (NHANES 1999-2004) periods **[18]**.

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 LC-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 Westat, abnormal reports are automatically forwarded to the NCHS survey physician for follow-up. For smaller, non-NHANES studies, abnormal values are identified to the study principal investigator. Emails sent concerning abnormal results are maintained by the supervisor for the duration of the study. Most of these studies are epidemiological in nature.

15. Specimen Storage and Handling during Testing

Specimens should be brought and maintained at room temperature during preparation and testing and then returned to frozen storage (typically at -70°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 LC-MS/MS method under some circumstances.

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

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

For NHANES 1999+, all data are reported electronically approximately weekly to Westat who then transfer the results to NCHS. For some smaller studies, electronic copies of a data report are sent and upon request hard copies can be sent as well.

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

A LIMS 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 at least 2 years after results have been publically 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 in a freezer at -70°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 LIMS database.

19. Method Performance Documentation

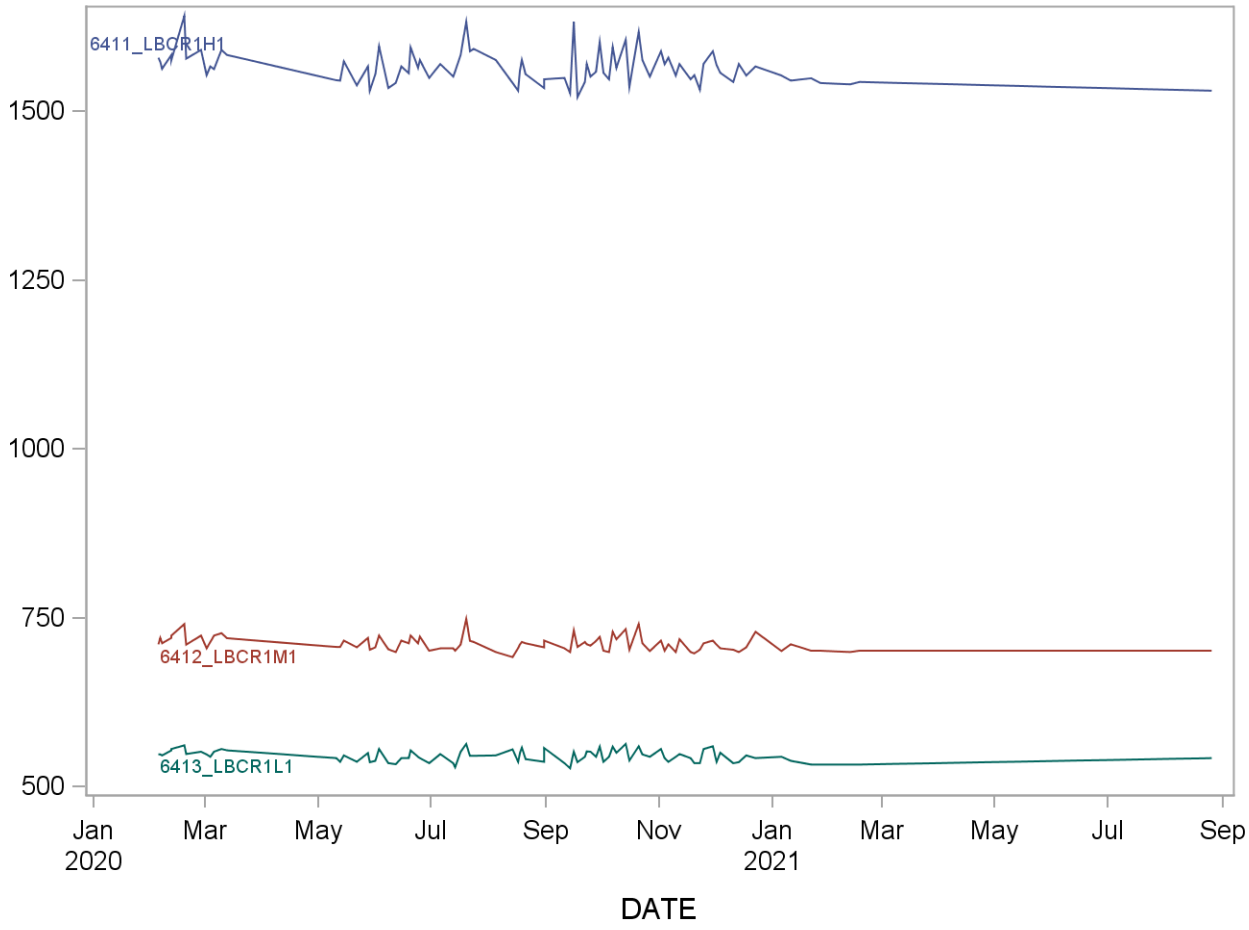
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in **Appendix A**. The signatures attested by the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.

20. Summary Statistics and QC Graph

Please see following pages.

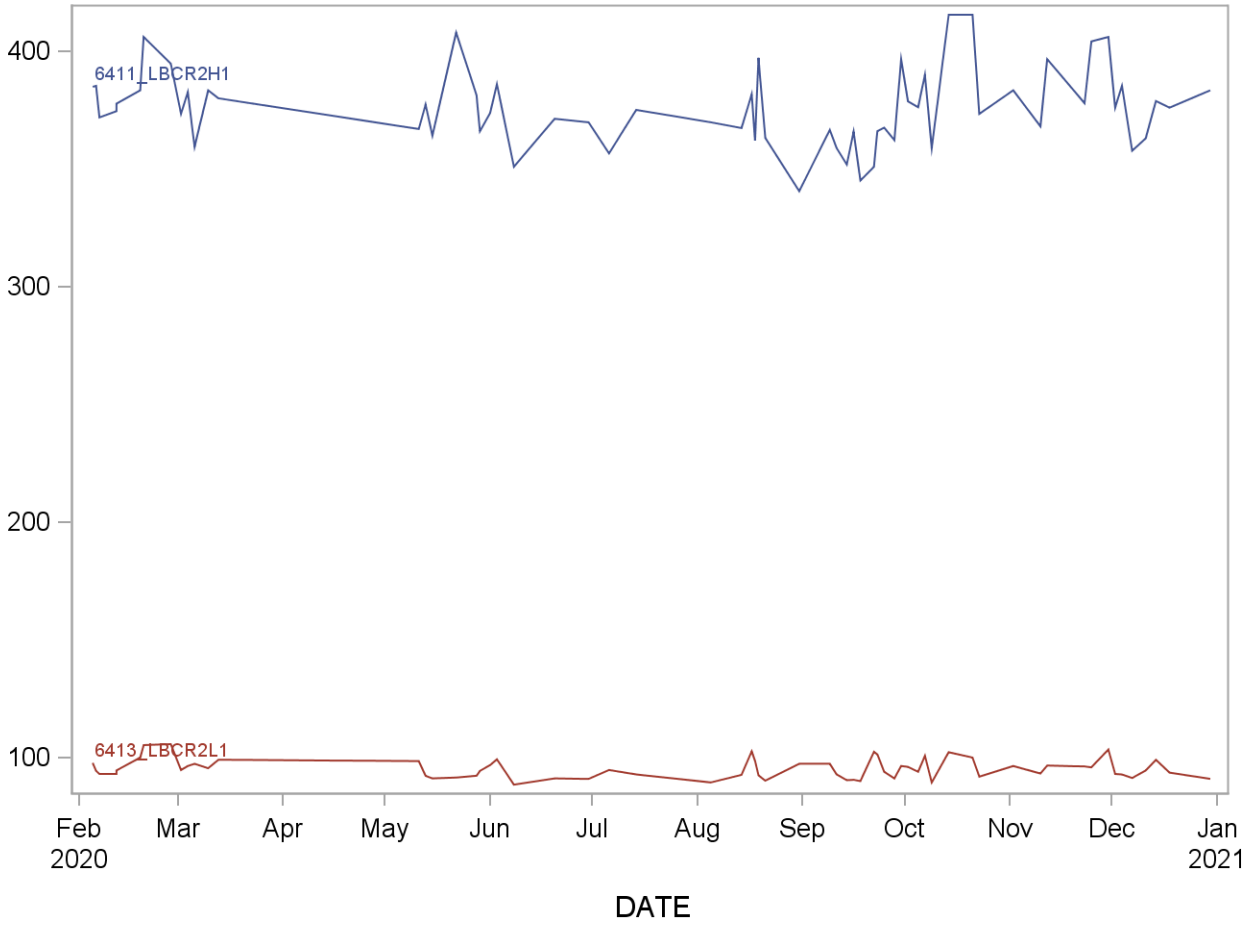
2019-2020 Summary Statistics and QC Chart
LBXRF1 (5-Methyl-tetrahydrofolate, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCR1H1	85	05FEB20	26AUG21	1564.24	24.28	1.6
6413_LBCR1L1	85	05FEB20	26AUG21	544.89	8.74	1.6
6412_LBCR1M1	85	05FEB20	26AUG21	711.27	10.94	1.5



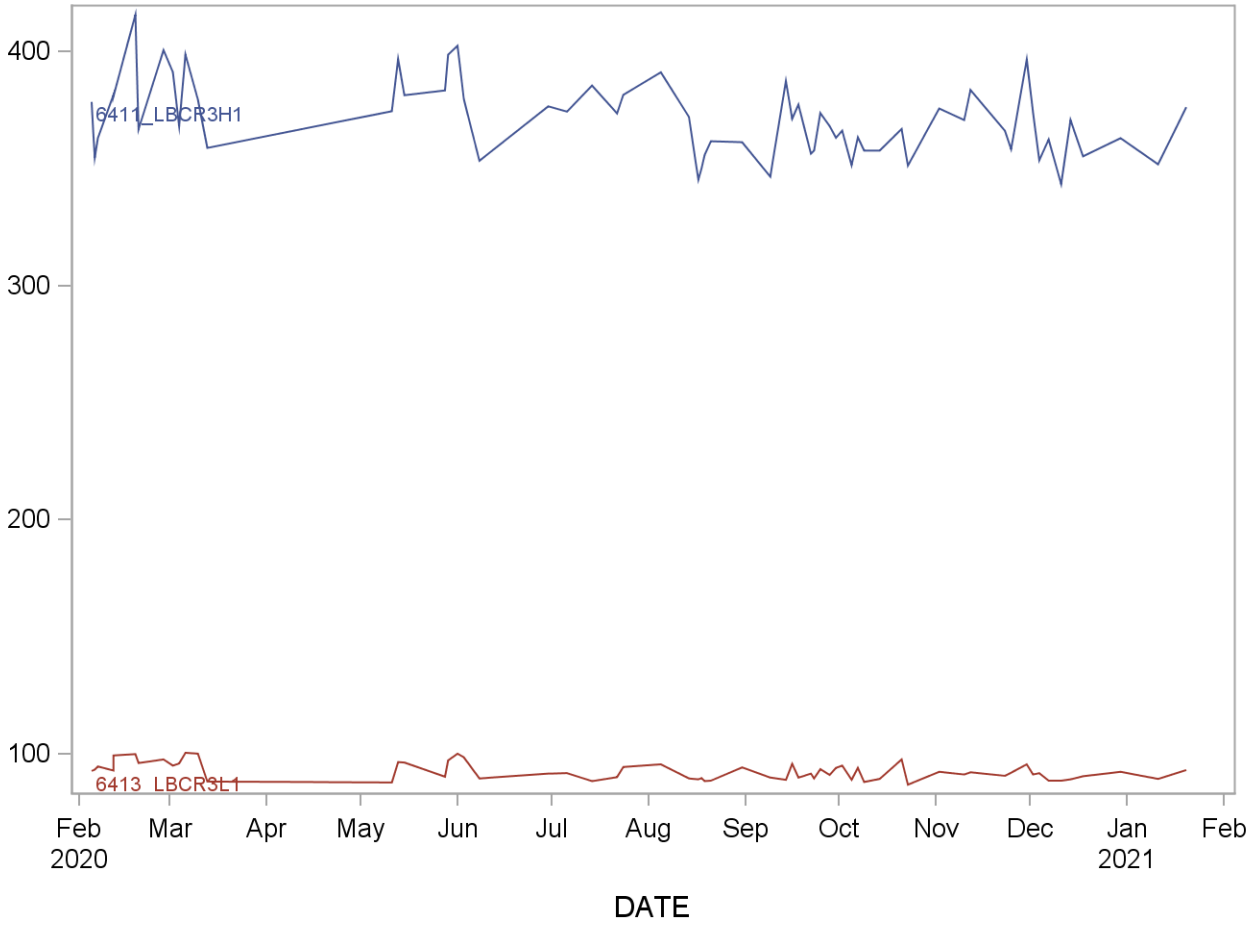
2019-2020 Summary Statistics and QC Chart
LBXRF2 (Folic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCR2H1	63	05FEB20	30DEC20	375.9613	16.3722	4.4
6413_LBCR2L1	63	05FEB20	30DEC20	95.1818	4.1365	4.3



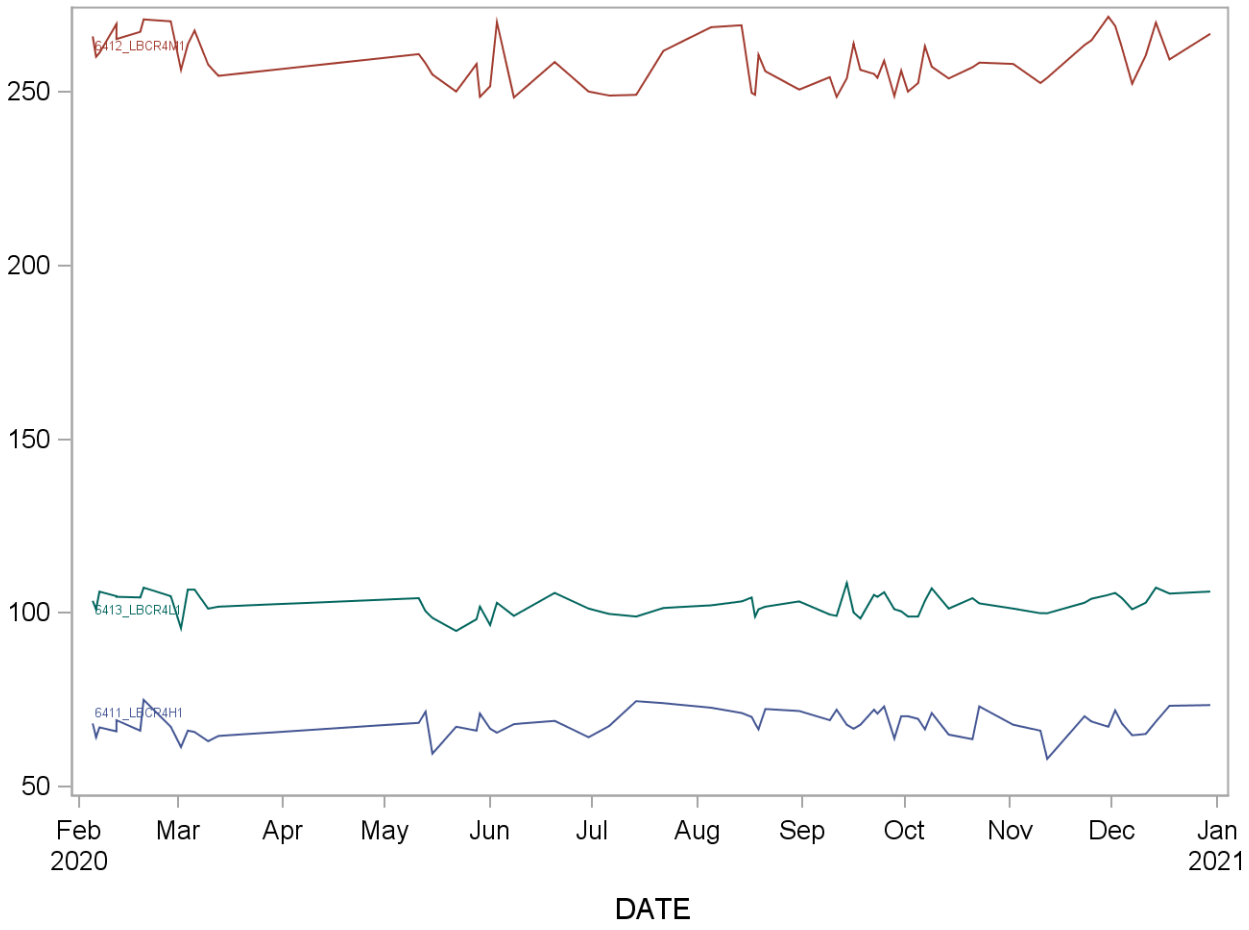
2019-2020 Summary Statistics and QC Chart
LBXRF3 (5-Formyl-tetrahydrofolic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCR3H1	64	05FEB20	20JAN21	371.0280	15.8036	4.3
6413_LBCR3L1	64	05FEB20	20JAN21	92.6175	3.6154	3.9



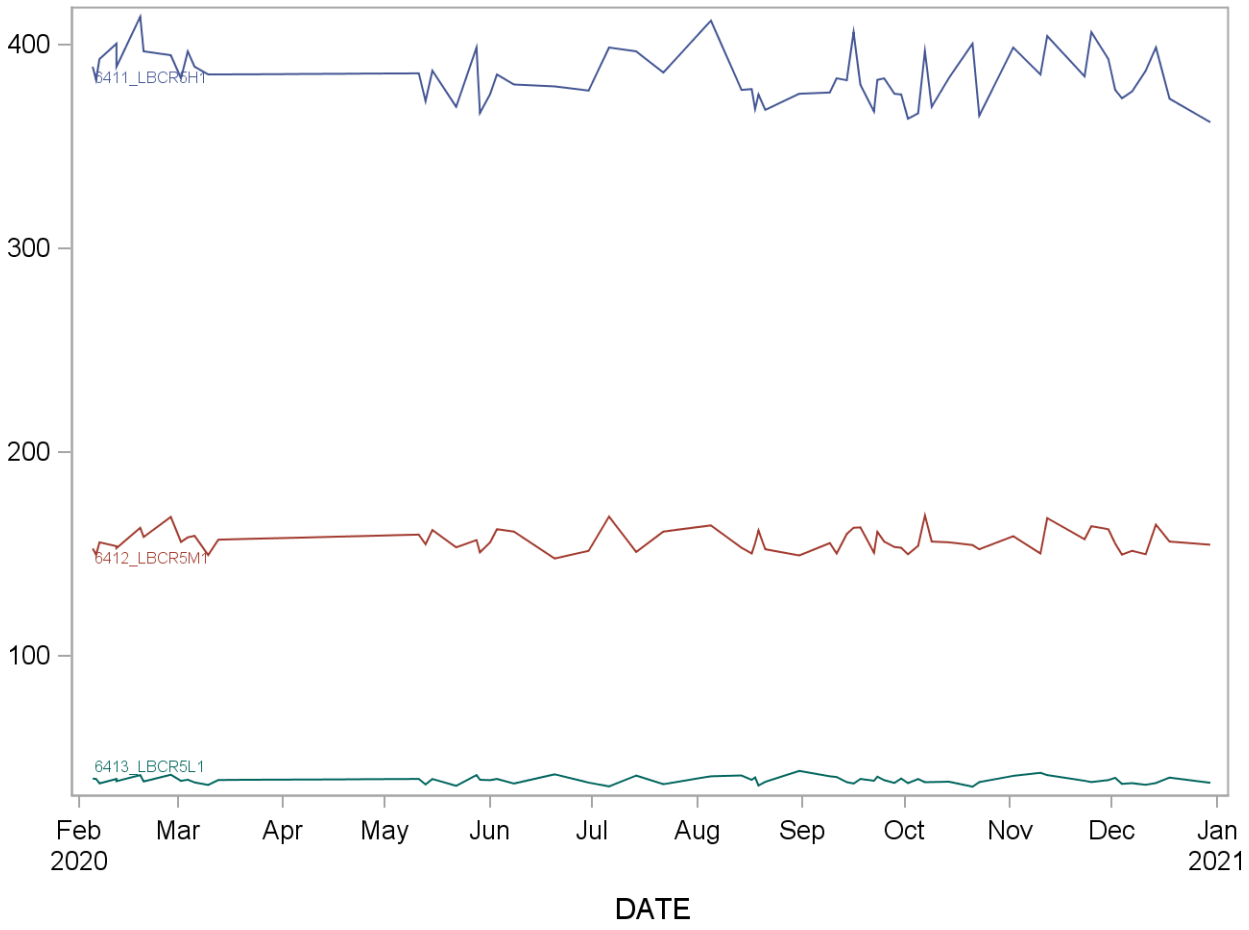
2019-2020 Summary Statistics and QC Chart
LBXRF4 (Tetrahydrofolic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCR4H1	64	05FEB20	30DEC20	68.2329	3.5740	5.2
6413_LBCR4L1	64	05FEB20	30DEC20	102.4133	3.0681	3.0
6412_LBCR4M1	64	05FEB20	30DEC20	258.6155	6.9394	2.7



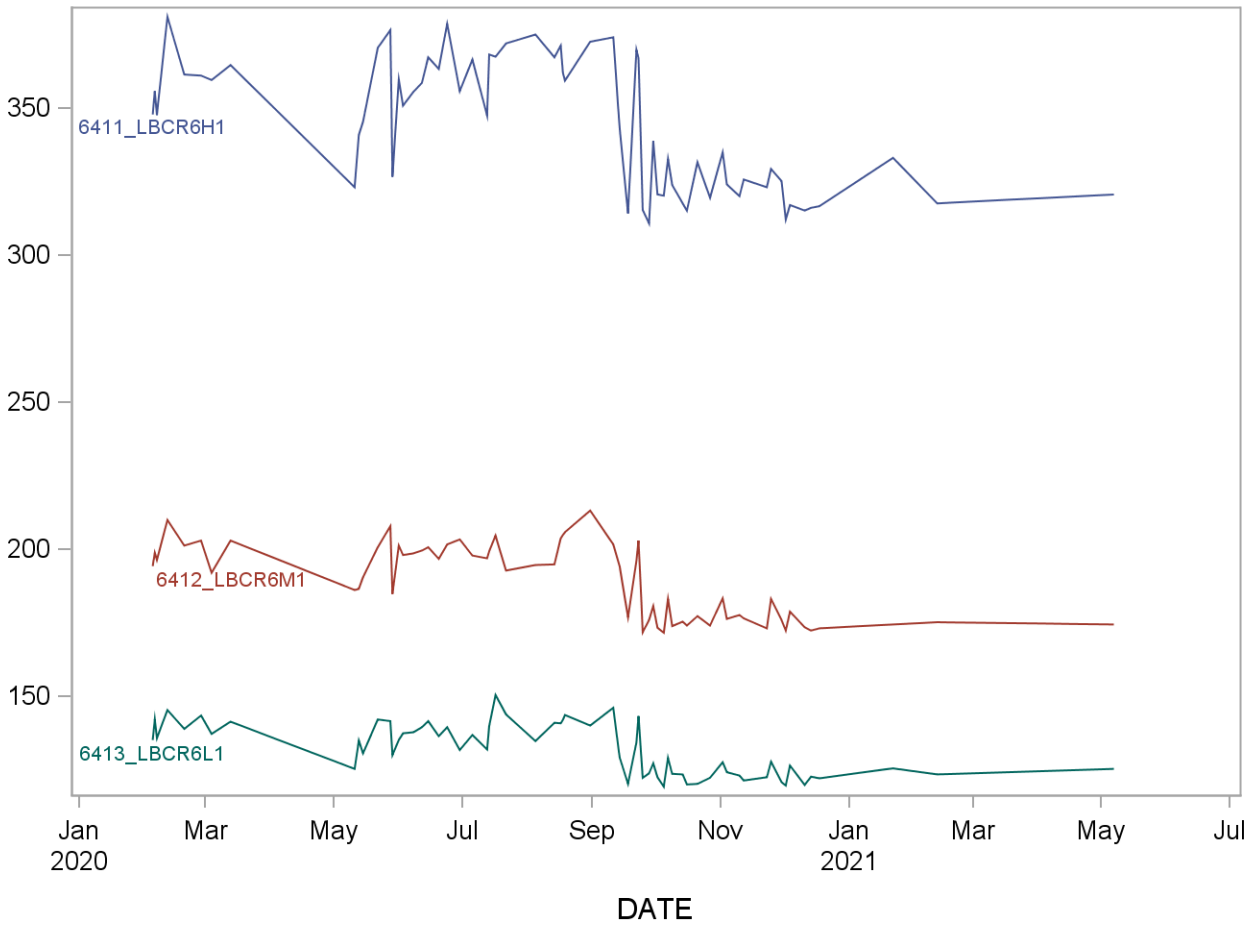
2019-2020 Summary Statistics and QC Chart
LBXRF5 (5,10--Methenyl-tethrofolic acid, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCR5H1	64	05FEB20	30DEC20	384.2797	12.3177	3.2
6413_LBCR5L1	64	05FEB20	30DEC20	38.8907	1.7240	4.4
6412_LBCR5M1	64	05FEB20	30DEC20	156.3795	5.3832	3.4



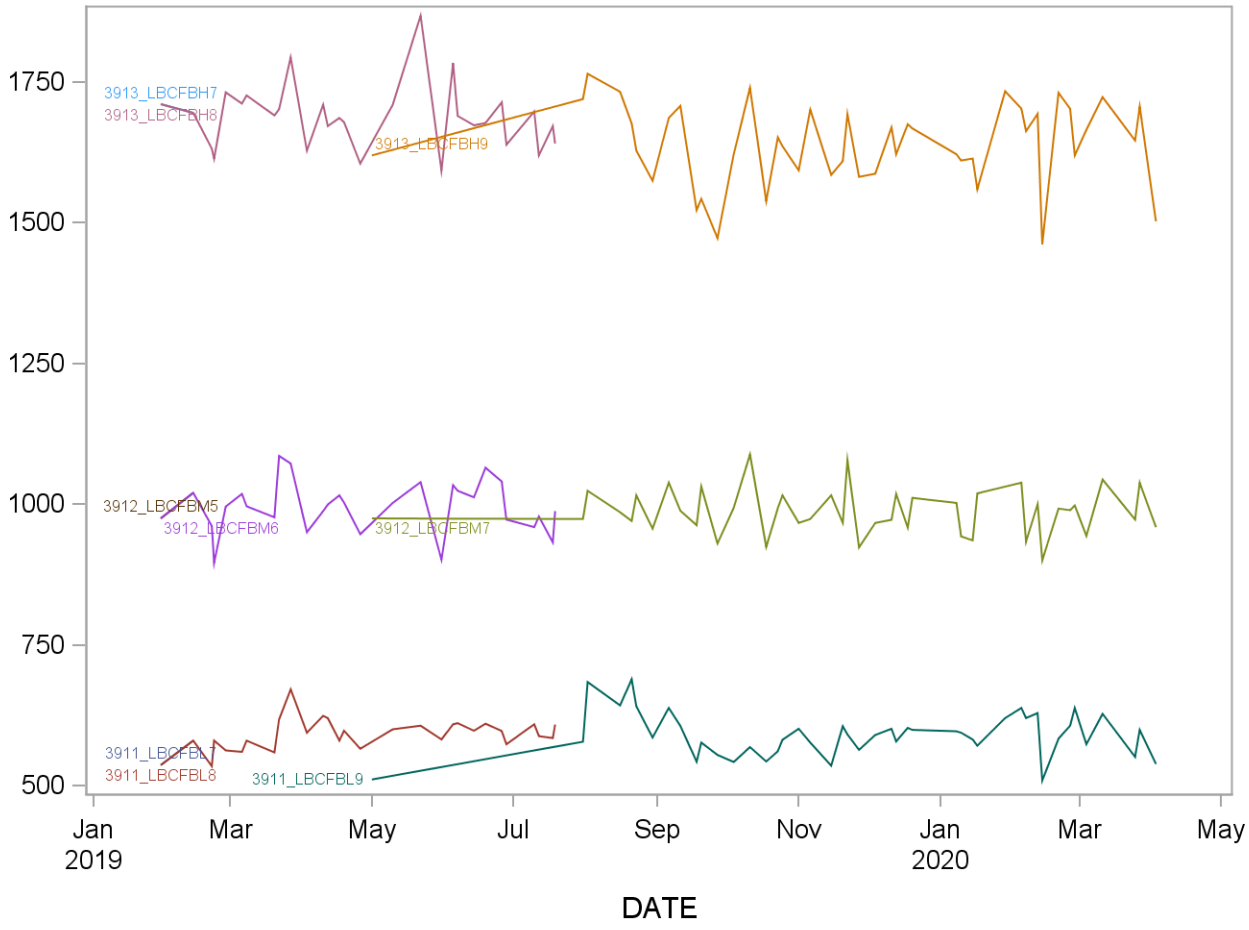
2019-2020 Summary Statistics and QC Chart
LBXRF6 (Mefox oxidation product, RBC (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
6411_LBCR6H1	64	05FEB20	07MAY21	344.265	22.180	6.4
6413_LBCR6L1	64	05FEB20	07MAY21	132.093	8.734	6.6
6412_LBCR6M1	64	05FEB20	07MAY21	189.264	12.595	6.7



2019-2020 Summary Statistics and QC Chart
LBXRBF (Folate, RBC(nmol/L RBC))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
3913_LBCFBH7	2	30JAN19	13FEB19	1702.3	11.0	0.6
3913_LBCFBH8	29	30JAN19	19JUL19	1687.7	59.0	3.5
3911_LBCFBL7	2	30JAN19	13FEB19	558.3	30.8	5.5
3911_LBCFBL8	29	30JAN19	19JUL19	591.0	27.9	4.7
3912_LBCFBM5	2	30JAN19	13FEB19	997.3	32.2	3.2
3912_LBCFBM6	29	30JAN19	19JUL19	994.5	45.7	4.6
3913_LBCFBH9	45	01MAY19	03APR20	1638.8	73.3	4.5
3911_LBCFBL9	45	01MAY19	03APR20	590.3	39.4	6.7
3912_LBCFBM7	45	01MAY19	03APR20	987.7	41.1	4.2



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Acknowledgements

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Appendix A: Method Performance Documentation

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 (%)
	Replicate	Spike concentration	Measured concentration Day 1 (05/23/19)	Measured concentration Day 2 (05/31/19)	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1 (05/23/19)	Measured concentration 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 (%)
	Replicate	Spike concentration	Measured concentration Day 1 (05/23/19)	Measured concentration Day 2 (05/31/19)	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1 (05/23/19)	Measured concentration 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

	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	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

	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	0.04	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				

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**

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 condit 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 condit 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 condit (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 condit QC material stored at -70°C for 1.5 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:	2019-06-13 (HZ:6500)
Analyte:	**5-MethylTHF**

Quality material 1 (MR18531)

	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability
Replicate 1	19.20	18.90	19.30	19.60	20.10	19.16	
Replicate 2	19.70	18.70	19.30	19.50	19.60	18.96	
Replicate 3	19.30	18.90	19.30	19.80	19.60	18.57	
Mean	19.40	18.83	19.30	19.63	19.77	18.90	#DIV/0!
% difference from initial	--	**-2.92**	**-0.52**	**1.20**	**1.89**	--	**#DIV/0!**

Quality material 2 (HR18532)

	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability
Replicate 1	41.40	41.40	41.60	42.70	42.30		
Replicate 2	41.70	41.60	41.40	41.90	42.10		
Replicate 3	41.40	41.50	42.40	42.20	42.70		
Mean	41.50	41.50	41.80	42.27	42.37	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**0.00**	**0.72**	**1.85**	**2.09**	--	**#DIV/0!**

Analyte: Folic acid

Quality material 1 (MR18531)

	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability
Replicate 1	0.25	0.20	0.26	0.23	0.24		
Replicate 2	0.24	0.21	0.19	0.26	0.25		
Replicate 3	0.25	0.25	0.20	0.24	0.23		
Mean	0.25	0.22	0.21	0.24	0.24	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**-10.88**	**-12.38**	**-0.82**	**-3.27**	--	**#DIV/0!**

Quality material 2 (HR18532)

	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability
Replicate 1	9.85	10.00	9.96	10.60	10.20		
Replicate 2	9.90	10.20	9.74	10.20	10.00		
Replicate 3	10.30	9.80	10.30	10.70	9.90		
Mean	10.02	10.00	10.00	10.50	10.03	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**-0.17**	**-0.17**	**4.83**	**0.17**	--	**#DIV/0!**

Analyte: MeFox

Quality material 1 (MR18531)

	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability
Replicate 1	5.04	5.25	5.08	5.17	5.10		
Replicate 2	5.22	4.93	5.08	5.27	5.50		
Replicate 3	5.19	5.20	4.94	5.23	5.17		
Mean	5.15	5.13	5.03	5.22	5.26	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**-0.45**	**-2.27**	**1.42**	**2.07**	--	**#DIV/0!**

Quality material 2 (HR18532)

	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability (1)	Processed sample stability (2)	Initial measurement	Long-term stability
Replicate 1	8.85	9.14	9.16	9.08	9.48		
Replicate 2	9.40	8.94	9.29	9.09	9.19		
Replicate 3	9.31	9.37	9.25	9.24	9.27		
Mean	9.19	9.15	9.23	9.14	9.31	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**-0.40**	**0.51**	**-0.54**	**1.38**	--	**#DIV/0!**

Analyte:	5-FormylTHF						
Quality material 1 (MR18531)							
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability**
Replicate 1	0.22	0.39	0.36	0.27	0.23		
Replicate 2	0.24	0.42	0.32	0.27	0.18		
Replicate 3	0.25	0.43	0.31	0.27	0.26		
Mean	0.24	0.41	0.33	0.27	0.22	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**74.33**	**38.79**	**14.81**	**-5.64**	--	**#DIV/0!**
Quality material 2 (HR18532)							
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability**
Replicate 1	9.72	9.49	9.58	9.84	9.41		
Replicate 2	9.53	9.53	9.64	9.75	9.67		
Replicate 3	9.53	9.33	9.82	10.10	10.20		
Mean	9.59	9.45	9.68	9.90	9.76	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**-1.49**	**0.90**	**3.16**	**1.74**	--	**#DIV/0!**

Comment: In Quality material 1 the concentration of 5-formylTHF is near the LOD; thus larger variability is expected.

Analyte:	Tetrahydrofolate						
Quality material 1 (MR18531)							
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability**
Replicate 1	7.59	6.60	7.05	7.11	7.35		
Replicate 2	7.62	6.87	7.24	7.21	7.36		
Replicate 3	7.65	6.77	7.03	7.28	7.27		
Mean	7.62	6.75	7.11	7.20	7.33	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**-11.46**	**-6.74**	**-5.51**	**-3.85**	--	**#DIV/0!**
Quality material 2 (HR18532)							
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability**
Replicate 1	1.61	1.50	1.55	1.69	1.56		
Replicate 2	1.50	1.66	1.63	1.61	1.47		
Replicate 3	1.48	1.79	1.70	1.67	1.57		
Mean	1.53	1.65	1.63	1.66	1.53	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**7.84**	**6.32**	**8.28**	**0.22**	--	**#DIV/0!**

Analyte:	5,10-MethenylTHF						
Quality material 1 (MR18531)							
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability**
Replicate 1	4.56	4.33	4.55	4.23	4.38		
Replicate 2	4.63	4.01	4.53	4.16	4.25		
Replicate 3	4.25	4.34	4.39	4.54	4.53		
Mean	4.48	4.23	4.49	4.31	4.39	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**-5.65**	**0.22**	**-3.79**	**-2.08**	--	**#DIV/0!**
Quality material 2 (HR18532)							
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability (1)**	**Processed sample stability (2)**	**Initial measurement**	**Long-term stability**
Replicate 1	11.30	11.10	10.70	10.90	10.90		
Replicate 2	11.70	11.00	11.00	10.80	10.90		
Replicate 3	10.80	11.70	10.60	10.70	11.90		
Mean	11.27	11.27	10.77	10.80	11.23	#DIV/0!	#DIV/0!
% difference from initial measurement	--	**0.00**	**-4.44**	**-4.14**	**-0.30**	--	**#DIV/0!**

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: 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	13C5-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	441.4	446.4
MeFox	0.1 N NaOH (pH 13)	280	19,365	6	473.4	482.4

(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.

Additional information can be found in:

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 C: Approximate QC Pool Target Concentrations (nmol/L) for the Various Folate Vitamers and the 5-methylTHF Oxidation Product (MeFox)

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

[&] A WB lysate or RBC lysate TFOL concentration of 11 nmol/L corresponds to a RBC TFOL concentration of 317 nmol/L (based on a Hct of 40% and a molar conversion factor of 2.176 [derived from 5-methylTHF]).

Appendix D: Agreement of blood folate forms among different sample types: Analysis by LC-MS/MS [10][1]

	RBC folate		
Analyte	**Traditional WB lysate[2]**	**WB lysate with exo-GGH[3]**	**RBC lysate with Hb-correction[4]**
	Concentration, nmol/L (95% CI)		
Total folate[5]	1033 (957, 1109)	1087 (1007, 1167)	1071 (988, 1153)
5-MethylTHF	831 (764, 897)	867 (798, 936)	843 (771, 915)
MeFox	147 (136, 158)	165 (152, 178)	172 (158, 186)
Non-methyl folate[6]	53.3 (34, 73)	53.0 (34, 73)	52.9 (35, 71)
	Pearson correlation (95% CI) *vs.* RBF traditional		
Total folate		0.99 (0.99, 0.99)	0.97 (0.96, 0.98)
5-MethylTHF		0.99 (0.99, 0.99)	0.98 (0.96,0.99)
MeFox		0.98 (0.97, 0.99)	0.98 (0.96, 0.99)
Non-methyl folate		0.99 (0.99, 1.00)	0.99 (0.99, 1.00)
	Relative difference (95% CI) *vs.* RBF traditional		
Total folate		5.3% (4.5%, 6.1%)	3.6% (1.8%, 5.3%)
5-MethylTHF		4.4% (3.7%, 5.1%)	1.2% (-0.58%, 3.0%)
MeFox		12% (11%, 14%)	17% (15%, 19%)
Non-methyl folate		-1.5% (-3.4%, 0.4%)	2.3% (-1.9%, 6.4%)
	Weighted Deming regression slope and intercept (95% CI) *vs.* traditional		
Total folate		1.05 (1.02, 1.07) 3.65 (19.1, 26.4)	1.06 (1.01, 1.12) -29.4 (-81.6, 22.8)
5-MethylTHF		1.05 (1.02, 1.07) -2.66 (-19, 14)	1.06 (1.01, 1.12) -40 (-81, 0.59)
MeFox		1.13 (1.08, 1.19) -1.67 (-8.78, 5.43)	1.19 (1.13, 1.27) -4.31 (-13.8, 5.18)
Non-methyl folate		0.99 (0.97, 1.03) -0.41 (-1.35, 0.53)	1.03 (0.96, 1.10) -0.51 (-2.38, 1.37)

[1] n = 60 subjects per sample type

[2] WB lysate incubated for 4h @ 37C; endogenous plasma GGH used to deconjugate polyglutamates; RBC folate results calculated from WB-Lys folate using serum folate correction and Hct

[3] WB lysate treated with recombinant exo-GGH for 30 min @ room temperature; RBC folate results calculated from WB lysate folate using serum folate correction and Hct

[4] RBC lysate treated with recombinant exo-GGH for 30 min @ room temperature; RBC folate results calculated from RBC lysate folate using Hb measured in the RBC-Lys for a non-volumetric correction of RBC content and MCHC to convert back to RBC folate

[5] Total folate (sum of folate forms: 5-methylTHF, MeFox, and non-methyl folate)

[6] Non-methylfolate (sum of THF, 5-formylTHF, and 5,10-methenylTHF)

Appendix E: Method comparison: LC-MS/MS *vs.* microbiologic assay (MBA) [11]

Comparison of red blood cell (RBC) total folate[1]	
Mean total folate ± SD (nmol/L)	
LC-MS/MS[2]: 899 ± 271	*MBA: 1002 ± 271*
Pearson correlation (95% CI) *LC-MS/MS vs MBA: 0.95 (0.92, 0.97)*	
Relative difference (95% CI) *LC-MS/MS vs MBA: -11% (-13%, -8.4%)*	
Weighted Deming regression slope and intercept (95% CI) *LC-MS/MS (y) vs. MBA (x): 0.95 (0.87, 1.02); -51 (-116, 15)*	

[1] Results calculated from RBC lysate using Hb for a non-volumetric correction of RBC content and MCHC to convert back to RBC folate

[2] RBC folate by LC-MS/MS represents total folate as the sum of folate forms (5-methyltetrahydrofolate, folic acid and non-methyl folate)

Appendix F: 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. A total of six 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. Please refer to Chapter 20 of the 2017 DLS Policies and Procedures Manual for further information on ruggedness testing. 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.

Variations in sample preparation

(A) 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.

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

1) pH of ammonium formate buffer (Sample solvent #1)

2) Formic acid concentration in ammonium formate buffer (Sample solvent #1)

3) Ascorbic acid concentration in ammonium formate buffer (Sample solvent #1)

4) Ammonium formate concentration in intermediate wash step during which matrix compounds are eluted but analytes are retained (SPE wash buffer)

5) Ascorbic acid concentration in SPE elution buffer (Sample solvent # 3)

6) Acetic acid concentration in SPE elution buffer (Sample solvent # 3)

7) Exo-GGH concentration in WB lysate or RBC lysate sample for folate polyglutamate deconjugation

(C) Findings:

1) Varying the pH of the ammonium formate sample preparation buffer does not appear to affect folate species results in serum samples.

2) Varying the formic acid concentration in the ammonium formate sample preparation buffer does not appear to affect folate species results in serum samples.

3) Varying the ascorbic acid concentration in the ammonium formate sample preparation buffer does not appear to affect folate species results in serum samples.

4) Varying the ammonium formate concentration in the SPE wash buffer does not appear to affect the folate species results in serum samples.

5) Varying the ascorbic acid concentration in the SPE elution buffer does not appear to affect folate species results in serum samples.

6) Varying the acetic acid concentration in the SPE elution buffer does not appear to affect folate species results in serum samples.

7) 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.

Ruggedness testing for whole blood folate vitamers by LC-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)

Ruggedness testing 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)
1. Variation in ExoGGH levels for deconjugation	5.0 µg/mL lysate	MET: 20 MFO: 5.2 THF: 7.6 MYT: 4.1 FOT[b]: 10 PGA[b]: 10	2. 5 µg/mL lysate	MET: 19 MFO: 4.9 THF: 7.3 MYT: 4.0 FOT[b]: 9.9 PGA[b]: 9.5	10 µg/mL lysate	MET: 20.3 MFO: 5.4 THF: 7.7 MYT: 4.5 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 G: 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.