



Laboratory Procedure Manual

Analyte: **Folate Forms**

Matrix: **Serum**

Method: **Liquid Chromatography Tandem Mass Spectrometry**

Method No: 4013.04

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as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
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Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

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

| File Name | Variable Name | SAS Label |
|-----------|---------------|---|
| FOLFMS_J | LBDFOTSI | Serum total folate (nmol/L) |
| | LBDFOT | Serum total folate (ng/mL) |
| | LBXSF1SI | 5-Methyl-tetrahydrofolate (nmol/L) |
| | LBDSF2SI | Folic acid (nmol/L) |
| | LBXSF3SI | 5-Formyl-tetrahydrofolate (nmol/L) |
| | LBXSF4SI | Tetrahydrofolate (nmol/L) |
| | LBXSF5SI | 5,10-Methenyl-tetrahydrofolate (nmol/L) |
| | LBXSF6SI | 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 childbearing 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.

Serum folate is an indicator of short-term status. The measurement of folate forms circulating in serum may further elucidate the role of folate vitamers relative to various health outcomes. The measurement of total folate (tFOL), which is the sum of the individual folate forms, provides information on the folate status of the individual. The primary circulating folate vitamer in serum is 5-methyltetrahydrofolic acid (5-methylTHF), while the actual bioactive form of folate is tetrahydrofolic acid (THF). Pteroylglutamic acid (PGA, also called folic acid) is primarily derived from supplements and fortified foods. If the intake of PGA exceeds 200 µg per meal, unmetabolized PGA may appear in serum.

B. Test Principle

Five folate forms, 5-methylTHF, PGA, THF, 5-formyltetrahydrofolic 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) [2]. This is a modification of a previously published method [3, 4] to add the measurement of an oxidation product [5] and to scale down the amount of specimen needed and increase the sample throughput [2]. The assay is performed by combining specimen (150 µL serum) with ammonium formate buffer and an internal standard mixture. Sample extraction and clean-up is performed by automated 96-probe solid phase extraction (SPE) using 96-well phenyl SPE plates and 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/x² weighting. The following analytes are quantified (Table I):

Table I: Analytes and abbreviations

| Compound | Abbreviation | |
|--|-----------------------|-----------------------|
| | Scientific literature | 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 | FOL3 |

2. Safety Precautions

Consider all serum 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 **"4013.04_SOP Computerization and Data System Management"** for a step-by-step description of data transfer, review, and approval.
- (C) For NHANES, data is transmitted electronically on a regular basis (approximately weekly for 3-week turnaround analytes). Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.
- (D) The 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) We recommend that specimen donors fast prior to specimen collection, but fasting is not required.
- (B) Serum folate assays are performed on fresh or frozen serum. Ascorbic acid (0.5%) is sometimes added to serum prior to storage to improve folate stability.
- (C) A 500- μ L serum specimen is required to allow for repeat analysis and for automated sample pipetting; a volume of 150 μ L is required for analysis.
- (D) Serum specimens may be collected with regular red-top Vacutainers. The appropriate amount of serum is dispensed into a Nalgene cryovial or other plastic screw-capped vial labeled with the participant's ID.
- (E) Specimens collected in the field should be kept 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 $\leq -20^{\circ}\text{C}$ until analyzed. Folates are stable for only a few weeks if the specimen is frozen at -20°C . For longer storage, specimens should be frozen at $\leq -70^{\circ}\text{C}$. Up to three short (2 h) freeze-thaw cycles cause only minor ($<10\%$) folate degradation [6].
- (F) Specimens should generally arrive frozen. Specimens received at ambient temperature are rejected. Refrigerated samples may be used provided they are brought promptly from the site where the blood was collected. Results from hemolyzed serum specimens should be interpreted with caution because they may have falsely elevated values. Specimens exposed to light for longer than 8 h may undergo 10–20% folate degradation [7]. Therefore, specimens intended for folate analysis should be processed and stored frozen promptly if analysis is not to be performed within 8 h of collection.
- (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 serum or plasma should be transferred into a sterile Nalgene cryovial labeled with the participant's ID; 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 $\text{M}\Omega/\text{cm}$. 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. If the ascorbic acid is used to dilute folate stock solutions, degas 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 ¹³C₅-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 ¹³C₅-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 $\mu\text{mol/L}$ 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 $-70\text{ }^{\circ}\text{C}$ 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 ($\mu\text{mol/L}$) at neutral (or alkaline) pH and ensure buffering when working at slightly acidic pH at much lower PGA concentrations (nmol/L). The $^{13}\text{C}_5$ -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 ($\pm 0.1\text{ mg}$) of the pure solid compound in degassed 20 mM phosphate buffer (pH 7.2), targeting a final concentration of $\sim 50\text{ }\mu\text{g/mL}$ (e.g., 5 mg in 100 mL). Vortex briefly to help dissolve the contents. Keep the volumetric flask in a luke-warm water bath ($\sim 30\text{ }^{\circ}\text{C}$) for $\sim 20\text{ min}$ to ensure complete solubility. Keep the volumetric flask at ambient temperature for $\sim 20\text{ min}$ 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 $\mu\text{mol/L}$ 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 $-70\text{ }^{\circ}\text{C}$ 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 $^{13}\text{C}_5$ -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 ($\pm 0.1\text{ mg}$) of the pure solid compound in degassed 0.1 N NaOH (pH ~ 13), targeting a final concentration of $\sim 100\text{ }\mu\text{g/mL}$ (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 $\mu\text{mol/L}$ 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 $-70\text{ }^{\circ}\text{C}$ 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 vials 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 [5].

Buffers & 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 100 µL of premix is used to prepare the internal standard Mix B.

The internal standard Mix B (total volume of 16 mL) contains a mixture of each internal standard prepared in 1% ammonium formate buffer, pH 3.2, with 0.1% ascorbic acid, as shown in **Table IV**.

Table IV: Information for internal standard mix B

| Mix B | ¹³ C ₅ - 5-MethylTHF | ¹³ C ₅ - PGA | ¹³ C ₅ - 5-FormylTHF | ¹³ C ₅ - MeFox | ¹³ C ₅ - THF | ¹³ C ₅ - 5,10-MethenylTHF |
|---|---|---------------------------------------|---|---|---------------------------------------|--|
| Intermediate stock II (μL) | 80 | | | | | |
| Premix of minor folate forms (μL) | 100 | | | | | |
| 1% Ammonium formate buffer with 0.1% ascorbic acid (mL) | 15.82 | | | | | |
| Labeled folate conc. (nmol/L) in Mix B | 100 | 25 | 25 | 25 | 25 | 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 serum tFOL are 7–10 (low), 25 (medium), and 50 nmol/L (high). The low QC aims to be close to the deficiency cutoff value of 7 nmol/L. The high QC aims to be in the top third of the population distribution (75th percentile is ~50 nmol/L). Because the United States has mandatory food fortification with folic acid, it is difficult to find donors with low serum folate levels and the prevalence of clinical folate deficiency is <1% in the US population.

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. 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.

The serum is pooled and pools are filtered through gauze before being dispensed to remove fibrinogen. Serum (usually 800 μL) is aliquoted into 2.0-mL Nalgene cryovials, capped, and frozen. The QC pools are stored at -70 °C and are stable for at least 3 years. Ascorbic acid (0.5%) can be added to the serum pools to ensure even better long-term folate 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 that 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 μ m 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 μ m (Phenomenex)
- b) 0.5 μ m 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 μ L) for Eppendorf pipette (Brinkmann)
- f) Pipette tips (1000 μ L & 10 mL) for Eppendorf pipette (Brinkmann)
- g) Pipette tips (10-100 μ L) for Eppendorf pipettes (Brinkmann)
- h) Pipette tips (0.5-10 μ L) for Eppendorf pipette (Brinkmann)
- i) Combitip plus (500 μ L) for Eppendorf repeater pipette (Brinkmann)
- j) Positive displacement pipette tips (50 μ L , 100 μ L, 1000 μ L) 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) 0.45 µm water filtration units 500 mL capacity (Nalgene)
- p) 2.0 mL polypropylene cryovials (Nalgene Company)
- q) 1.0 mL disposable syringes (Hamilton)
- r) 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 Na₂-salt), 5-methylTHF ([6S]-5CH₃-H₄PteGlu, Ca-or Na₂-salt), 5-formylTHF ([6S]-5CHO-H₄PteGlu, Ca-or Na₂-salt), MeFox ([6S](pyrazino-s-triazine derivative), THF ([6S]-H₄PteGlu, free acid, Ca-or-Na₂-salt) and 5,10-methenylTHF ([6S]-5,10-CH=H₄PteGlu-Cl x HCl salt) (Merck & Cie [formerly Eprova]), Schaffhausen, Switzerland
http://www.merck.ch/en/company/merck_in_switzerland/merck_cie_schaffhouse/merck_cie_schaffhausen.html
- b) ¹³C₅-PGA, ¹³C₅-5-methylTHF, ¹³C₅-5-formylTHF, ¹³C₅-MeFox, ¹³C₅-THF and ¹³C₅-5,10-methenylTHF (Merck & Cie)

(5) Chemicals and Solvents

- a) Ammonium hydroxide (28–30% as NH₃, Mallinckrodt Chemicals)
- b) L-Cysteine (Sigma, Life Science)
- c) Potassium phosphate dibasic and monobasic salts (Fisher Scientific Co)
- d) Formic acid (>95%) reagent grade (Sigma Aldrich)
- e) Acetic acid (99%) reagent grade (Fisher Scientific Co)
- f) L-ascorbic acid (vitamin C min 99% purity (Fisher Scientific Co[Spectrum chemicals])
- g) Hydrochloric acid (36.5-38%) (JT Baker)
- h) Water, 18 MΩ/cm, HPLC grade (Aqua Solutions)
- i) Methanol, acetonitrile HPLC certified solvent (Honeywell/Burdick & Jackson Laboratories)
- j) 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 thermostatted column heater, 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 1.6.2 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 μ L, 100 μ L, 200 μ L, 1000 μ L, and 10 mL (Brinkmann Instruments Inc.)
- (9) Positive air displacement pipettes (Pipetman) 50 μ L, 100 μ L, and 1000 μ L (Gilson Inc.)
- (10) Digiflex CX (ICN Biomedicals, Inc. Diagnostics Division)
- (11) Dual syringe continuous dispenser, 600 series (Hamilton Microlab)
- (12) Galaxy mini table top microcentrifuge (VWR Scientific Products)
- (13) Daigger Vortex Genie 2 mixer (VWR)
- (14) Magnetic stirrer (Baxter Scientific Products)
- (15) pH meter (Corning Pinnacle 530 or Accumet XL150 (pH/MV; Fisher Scientific)
- (16) Analytical Balance Model AG104 (Mettler Instrument Corp.)
- (17) Cary 300 Bio UV/visible spectrophotometer (Agilent Technologies)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

In-house studies showed that aqueous calibration provides equivalent results to calibration in serum and slopes for the two calibration curves (serum vs. water) were less than $\pm 5\%$ different for all folate forms [2]. 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 [2, 3]. 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 international reference materials. For details, see “**4013.04_SOP Calibration and Calibration Verification**”.

In 2005, the National Institute of Standards and Technology (NIST) released a new three-level standard reference material (SRM) for homocysteine and folate in human serum, SRM 1955. This material was characterized by various mass-spectrometry-based methods used at NIST and the CDC. Because of the good agreement between the NIST and CDC methods for 5-methylTHF and PGA, NIST used the CDC results as part of the value assignment process. Good agreement was also found for tFOL between the CDC LC-MS/MS and microbiologic assay (level 1: 6.0 vs. 5.6; level 2: 13 vs. 14; level 3: 41 vs. 44).

In 2011, NIST released a one-level standard reference material for metabolites in human plasma, SRM 1950. This material was characterized by various mass-spectrometry-based methods used at NIST and the CDC. The CDC LC-MS/MS procedure matched the certified value [uncertainty] for 5-methylTHF (26.7 vs. 26.9 [0.70] nmol/L) and the reference value for PGA (4.03 vs. 3.42 [1.02] nmol/L).

In 2006, the National Institute for Biological Standards and Control (NIBSC) issued the first WHO certified reference material (lyophilized, one level) for folate in human serum (03/178, established 2004). The folate concentration in this material has been certified by LC-MS/MS through measurements provided by NIST and CDC.

We participate in external proficiency testing programs from the College of American Pathologists (CAP): K Ligand survey (3 times per year) and LN5 Calibration Verification Ligand survey (2 times per year). When possible, we also participate in other external proficiency testing program such as the UK NEQAS Haematinics survey (twice a year). 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**”.

Information on method characteristics has been published [2–5]. 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 D**.

We have performed in-house comparisons of the LC-MS/MS assay and the microbiologic assay, the latter still considered an accurate “reference point” for total folate (tFOL). For serum samples, there is excellent correlation and agreement between the two assays [2, 9]. The microbiologic assay produces results that are within $\pm 10\%$ of the LC-MS/MS results.

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 "**4013.04_SOP Tuning and mass calibration of mass spectrometer**" 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 μ L and 1000 μ L. 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, "**4013.04_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 μ L to 1.0 mL), 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 serum 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 serum 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 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 from cryovials into a 96-well plate that is then subjected to automated SPE sample extraction and clean-up.
- (2) For a detailed step-by-step description, see **“4013.04_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** for a final volume of 0.55 mL.

- g) After the pipetting is completed, the sample plate is removed, covered with the 96-well plastic seal, and incubated at room temperature for 20 min prior to SPE for the internal standards to equilibrate with the endogenous folates.

Table VI: Pipetting Scheme

| Well | Sample type | Internal standard mix | Calibrator mix | Sample solvent #1 | Water | QC or patient specimen |
|-------|------------------------------|-----------------------|----------------|-------------------|--------|------------------------|
| 1 | Reagent blank (Double blank) | --- | --- | 400 µL | 150 µL | --- |
| 2 | Calibrator S0 (blank) | 60 µL mix B | --- | 340 µL | 150 µL | --- |
| 3 | Calibrator S1 | 60 µL mix B | 150 µL S1 | 190 µL | 150 µL | --- |
| 4 | Calibrator S2 | 60 µL mix B | 150 µL S2 | 190 µL | 150 µL | --- |
| 5 | Calibrator S3 | 60 µL mix B | 150 µL S3 | 190 µL | 150 µL | --- |
| 6 | Calibrator S4 | 60 µL mix B | 150 µL S4 | 190 µL | 150 µL | --- |
| 7 | Calibrator S5 | 60 µL mix B | 150 µL S5 | 190 µL | 150 µL | --- |
| 8 | Low QC – Set 1 | 60 µL mix B | --- | 340 µL | --- | 150 µL |
| 9 | Medium QC – Set 1 | 60 µL mix B | --- | 340 µL | --- | 150 µL |
| 10 | High QC – Set 1 | 60 µL mix B | --- | 340 µL | --- | 150 µL |
| 11-93 | Patient samples | 60 µL mix B | --- | 340 µL | --- | 150 µL |
| 94 | Low QC – Set 2 | 60 µL mix B | --- | 340 µL | --- | 150 µL |
| 95 | Medium QC – Set 2 | 60 µL mix B | --- | 340 µL | --- | 150 µL |
| 96 | High QC – Set 2 | 60 µL mix B | --- | 340 µL | --- | 150 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) To construct a 5-point calibration curve, follow the pipetting scheme shown in **Table VI**. A reagent blank that contains only reagents and a blank that contains reagents and only the internal standard mix (S0) is included in each run.
- (3) QC and patient samples (vial 8 and beyond): add 340 µL solvent #1, 60 µL of internal standard mix (mix B) and 150 µL specimen for a final volume of 0.55 mL.
- (4) Incubate the sample plate at room temperature for 20 min for the internal standards to equilibrate with the endogenous folates prior to 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 “**4013.04_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 serum.
- (2) For a detailed step-by-step description, see **"4013.04_SOP LC-MS/MS 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 E**:
 - 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 **"4013.04_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 **"4013.04_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/x²weighted).

H. Exporting a Run

The procedure to export a run to a LIMS database is described in section 3 and in “**4013.04_SOP Computerization and Data System Management**”.

I. Calculations

Serum results for each folate species are directly reported as nmol/L. To obtain a serum tFOL result, the individual results from each folate form (generally excluding MeFox; see special method notes) have to be added up. If a vitamer 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 forms is missing, the serum tFOL result is also missing.

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

Serum tFOL folate may or may not include MeFox, depending on the study and investigator request. It is still of scientific debate whether MeFox is only generated *in vitro* or may already be present *in vivo* [10]. Including MeFox into the tFOL may slightly overestimate folate status, while excluding it may slightly underestimate status, however the difference between the two approaches is rather small (~5%) [10]. The 3 minor analytes THF, 5-formylTHF, and 5,10-methenylTHF, particularly the latter two, are usually <LOD in serum specimens, however they still have to be measured to accurately capture serum tFOL and they can reach concentrations >1 nmol/L in some samples [10].

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 results <7 nmo/L (3 ng/mL) 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 serum pools, 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 [11].

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 $\pm 4 S_i$ 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 “**4013.04_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) Hemolyzed serum samples may give falsely elevated 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)

Clinical reference ranges reported for serum folate are 11–36 nmol/L with the microbiologic assay and 7–36 with chemiluminescence assays [12].

Post-fortification reference ranges for the U.S. population generated with the microbiologic assay for NHANES 2005–2010 are shown below [13].

Serum folate: 12.7–104 nmol/L (2.5th–97.5th percentile; $n = 23,528$)

In our hands, the LC/MS/MS method and the microbiologic assay give relatively comparable results [9]. Pfeiffer et al. also reported microbiologic assay-equivalent reference ranges for pre-fortification (NHANES 1988–1994) and early post-fortification (NHANES 1999–2004) periods, as well as reference ranges by population subgroups for all three time periods [13].

Reference ranges for individual folate vitamers based on LC-MS/MS were determined using data from NHANES 2011–2012 [10]. While these values are of interest to researchers, they cannot be interpreted clinically. Serum tFOL levels <7 nmol/L (3 ng/mL) represent a negative folate balance and are usually indicative of inadequate folate intake [14].

14. Critical Call Results (“Panic Values”)

Any NHANES samples with serum tFOL levels <7 nmol/L (3 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 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 plasma or serum 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 serum 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 serum is retained for at least 2 years after results have been publically released; at that point, samples with sufficient volume (>0.2 mL) are returned to NHANES and samples with insufficient volume may be autoclaved.

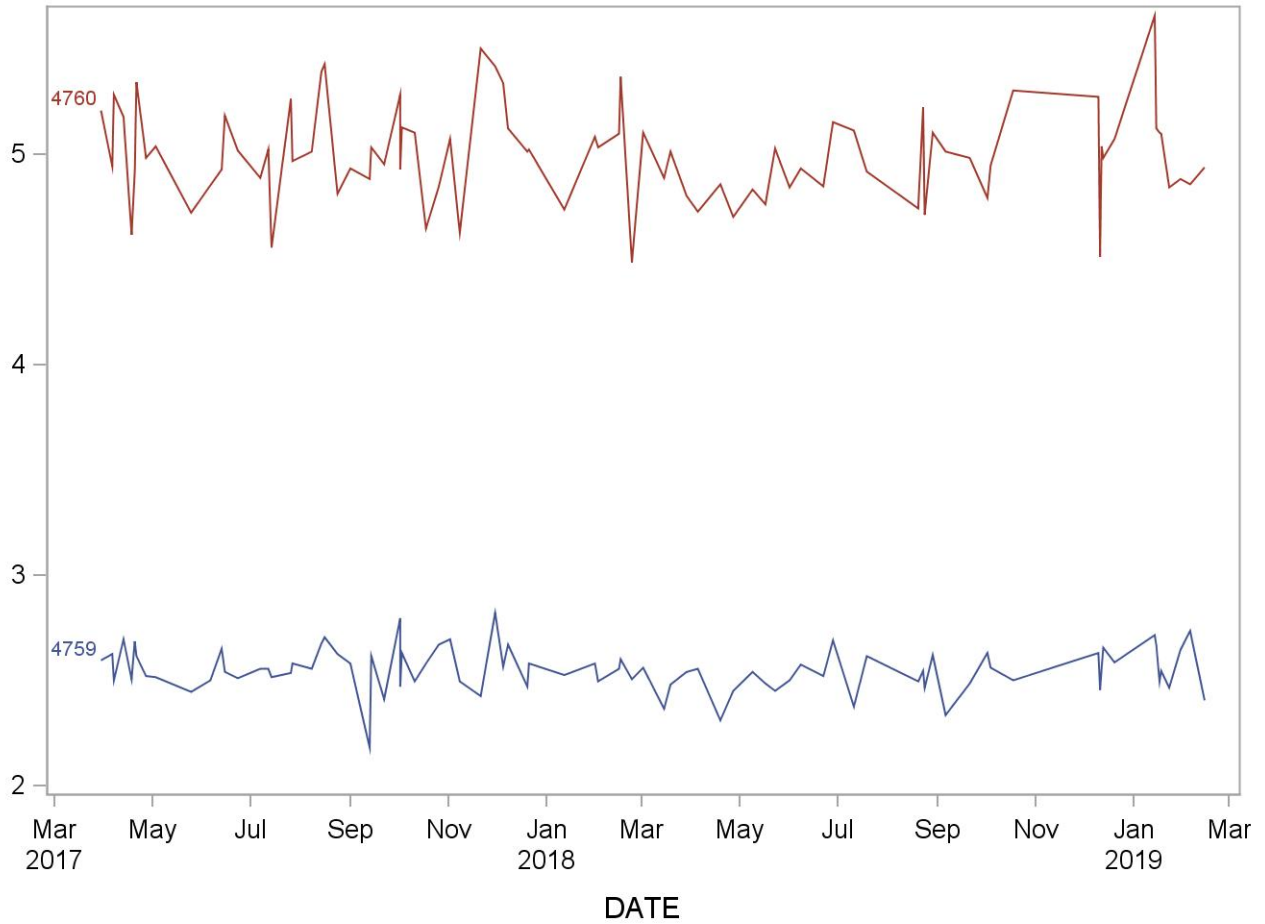
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70°C. The specimen ID is read off of the vial by a barcode reader 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. Summary Statistics and QC Graphs

Please see following pages.

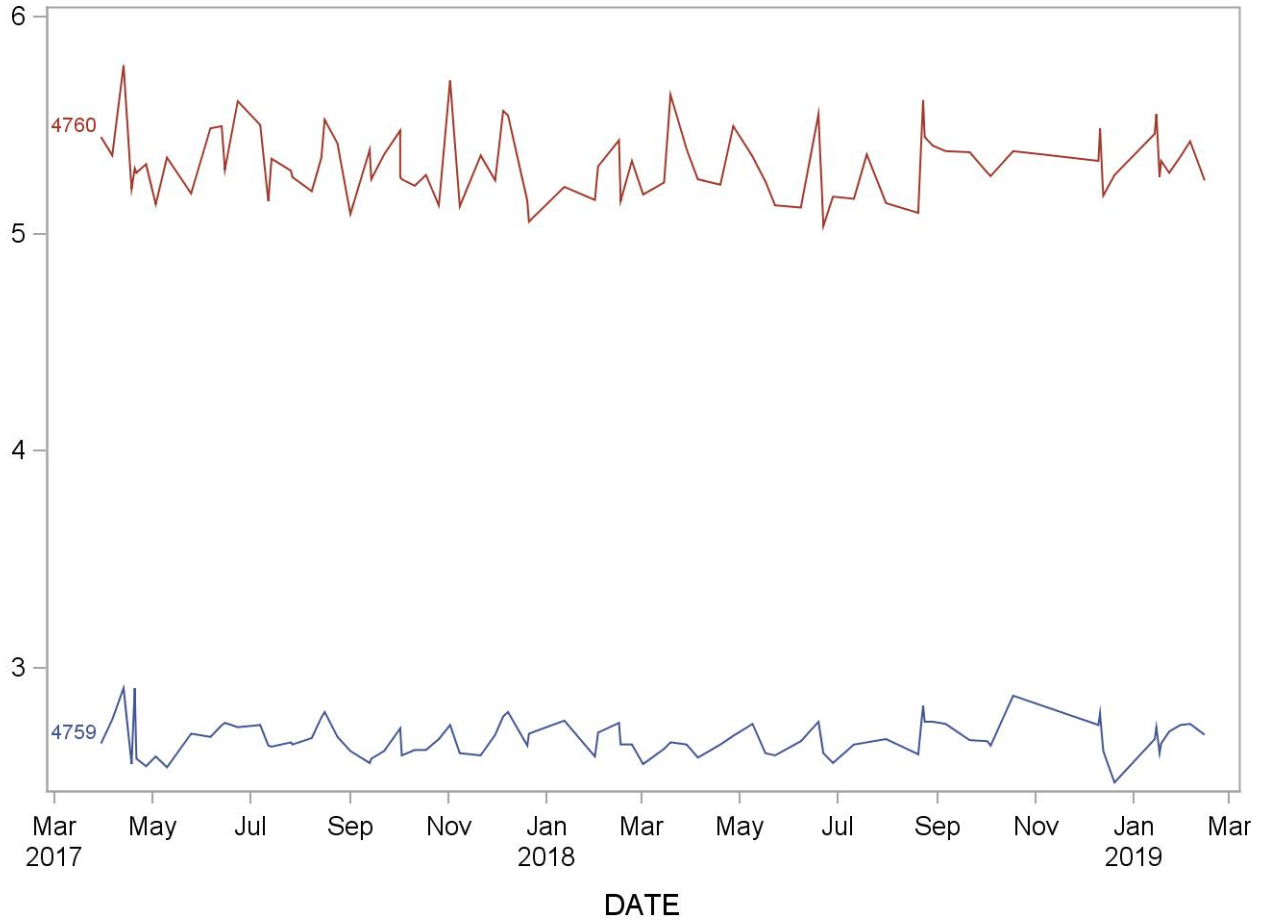
2017-2018 Summary Statistics and QC Chart for 5,10-Methyl-tetrahydrofolate (nmol/L)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------|----|------------|----------|--------|--------------------|--------------------------|
| 4760 | 85 | 30MAR17 | 14FEB19 | 4.9966 | 0.2264 | 4.5 |
| 4759 | 85 | 30MAR17 | 14FEB19 | 2.5506 | 0.1049 | 4.1 |



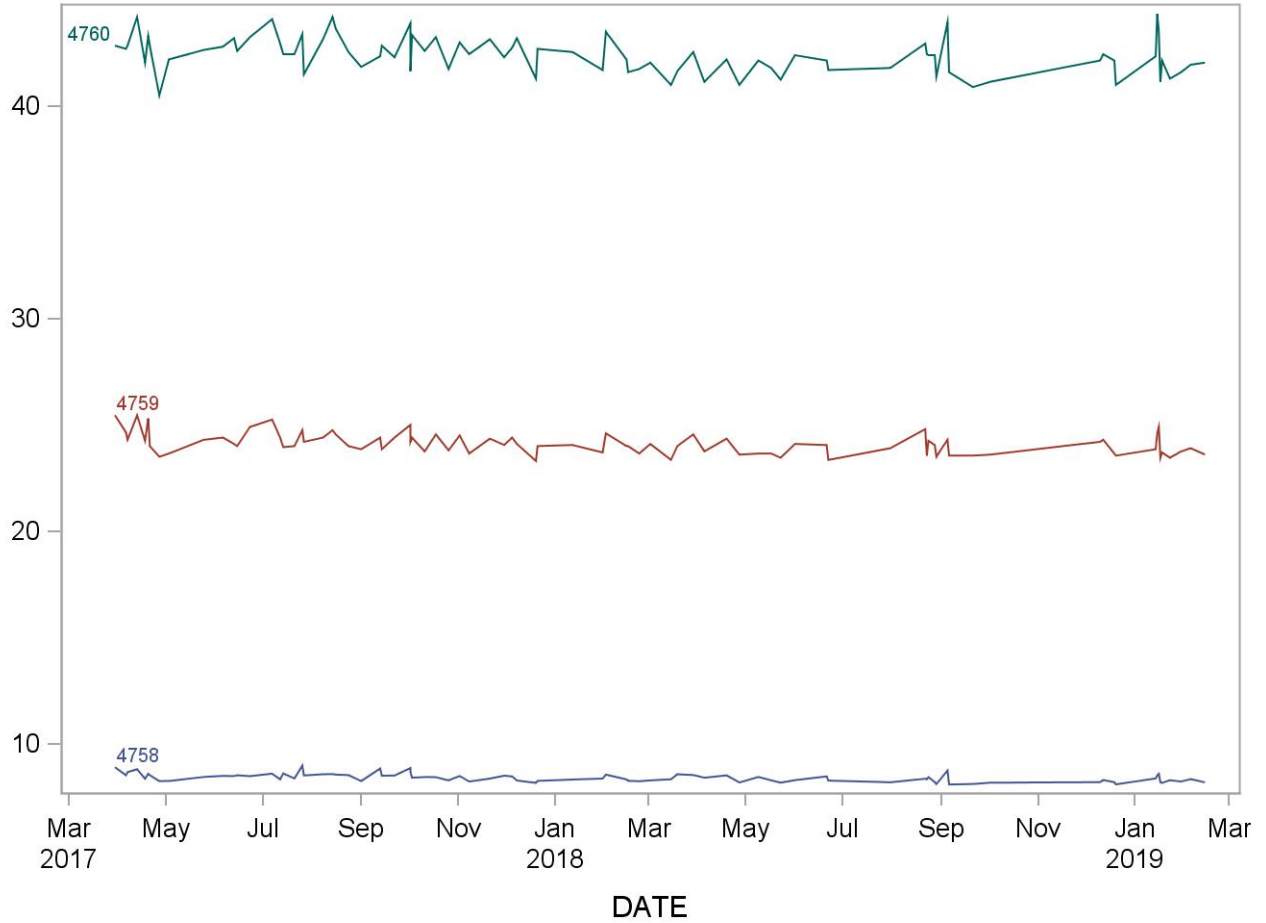
2017-2018 Summary Statistics and QC Chart for 5-Formyl-tetrahydrofolate (nmol/L)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------|----|------------|----------|--------|--------------------|--------------------------|
| 4760 | 85 | 30MAR17 | 14FEB19 | 5.3224 | 0.1542 | 2.9 |
| 4759 | 85 | 30MAR17 | 14FEB19 | 2.6737 | 0.0817 | 3.1 |



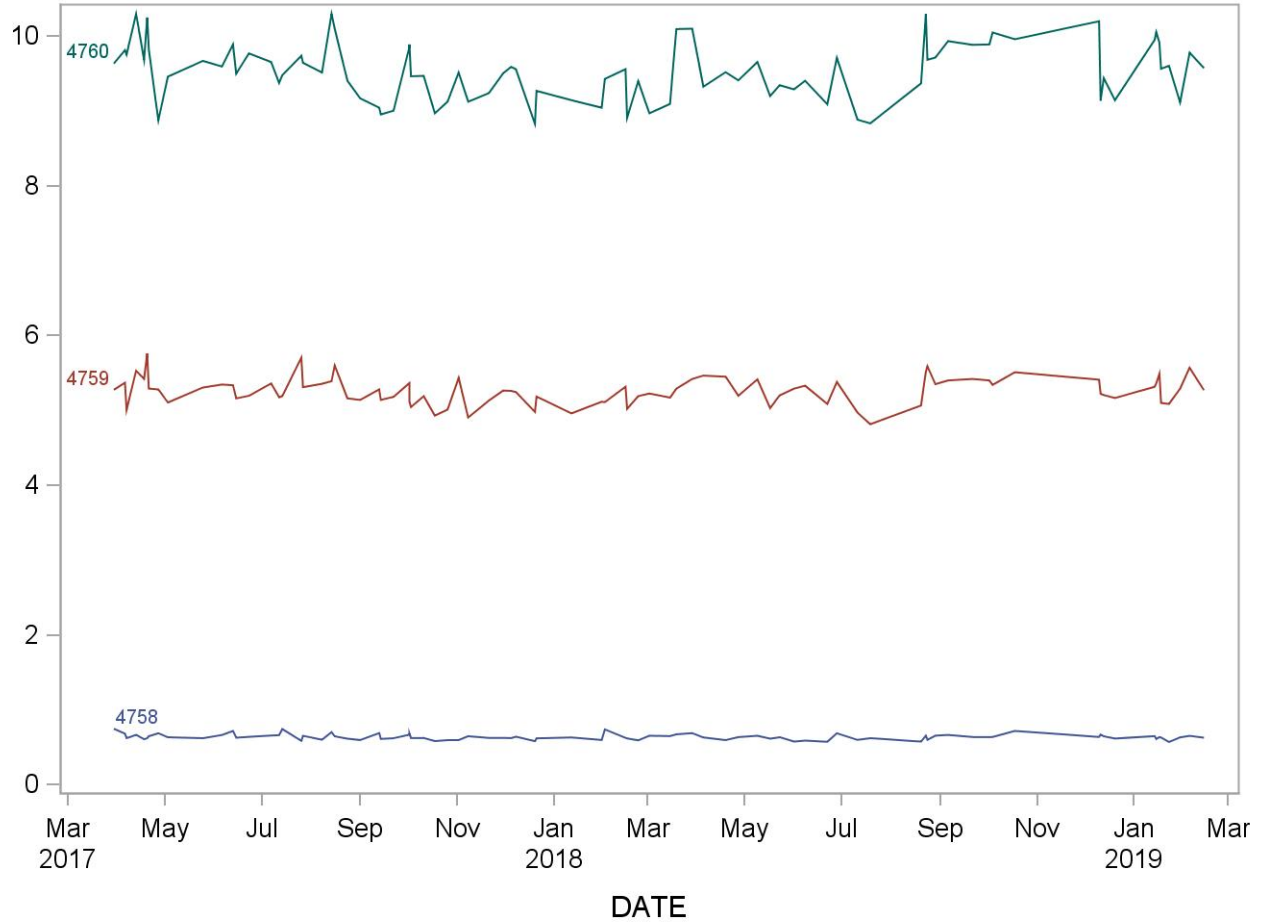
2017-2018 Summary Statistics and QC Chart for 5-Methyl-tetrahydrofolate (nmol/L)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------|----|------------|----------|---------|--------------------|--------------------------|
| 4760 | 84 | 30MAR17 | 14FEB19 | 42.3881 | 0.8482 | 2.0 |
| 4758 | 84 | 30MAR17 | 14FEB19 | 8.3887 | 0.1944 | 2.3 |
| 4759 | 84 | 30MAR17 | 14FEB19 | 24.1065 | 0.4967 | 2.1 |



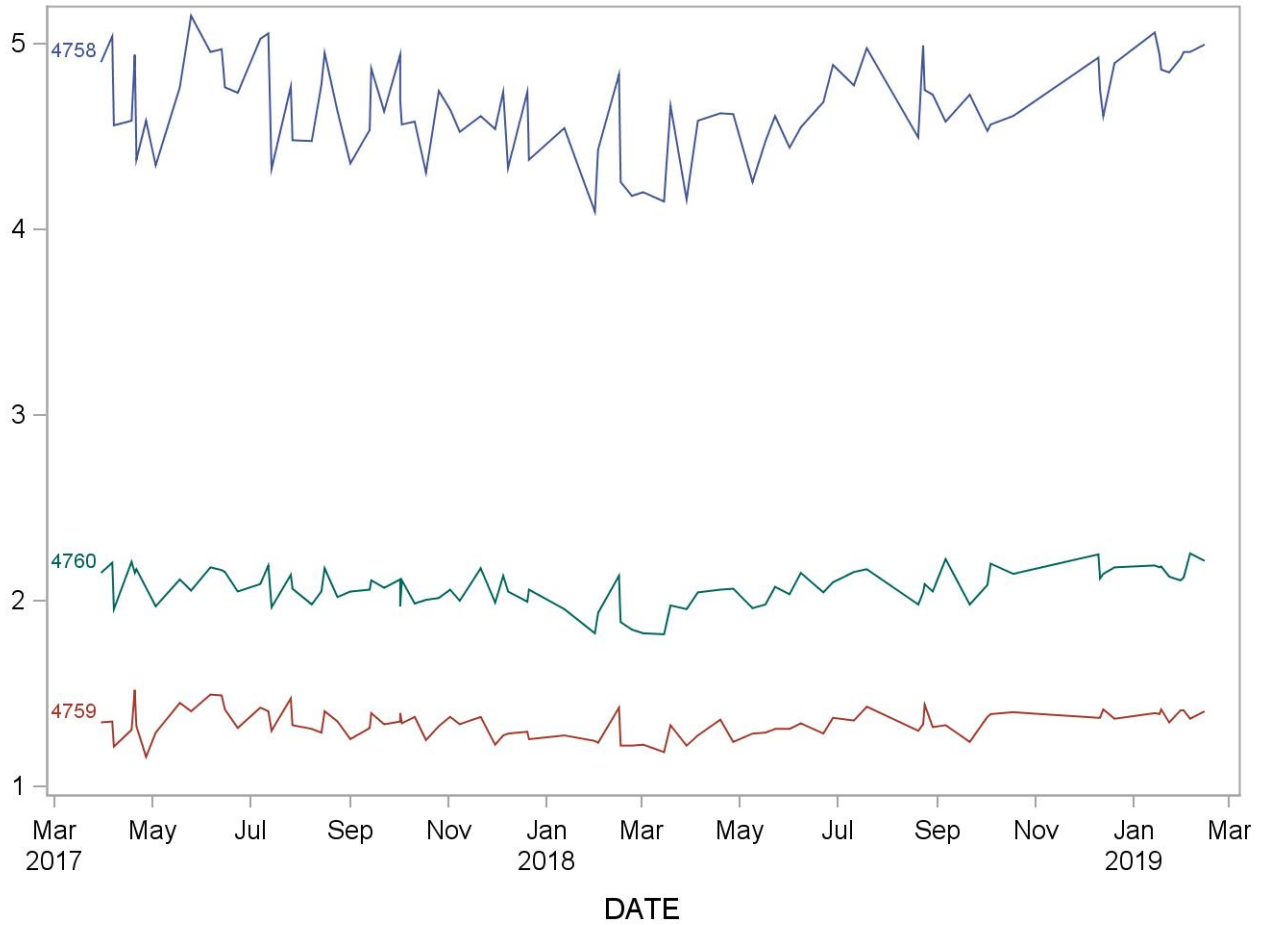
2017-2018 Summary Statistics and QC Chart for Folic acid (nmol/L)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------|----|------------|----------|--------|--------------------|--------------------------|
| 4760 | 84 | 30MAR17 | 14FEB19 | 9.5300 | 0.3811 | 4.0 |
| 4758 | 84 | 30MAR17 | 14FEB19 | 0.6351 | 0.0387 | 6.1 |
| 4759 | 84 | 30MAR17 | 14FEB19 | 5.2611 | 0.1818 | 3.5 |



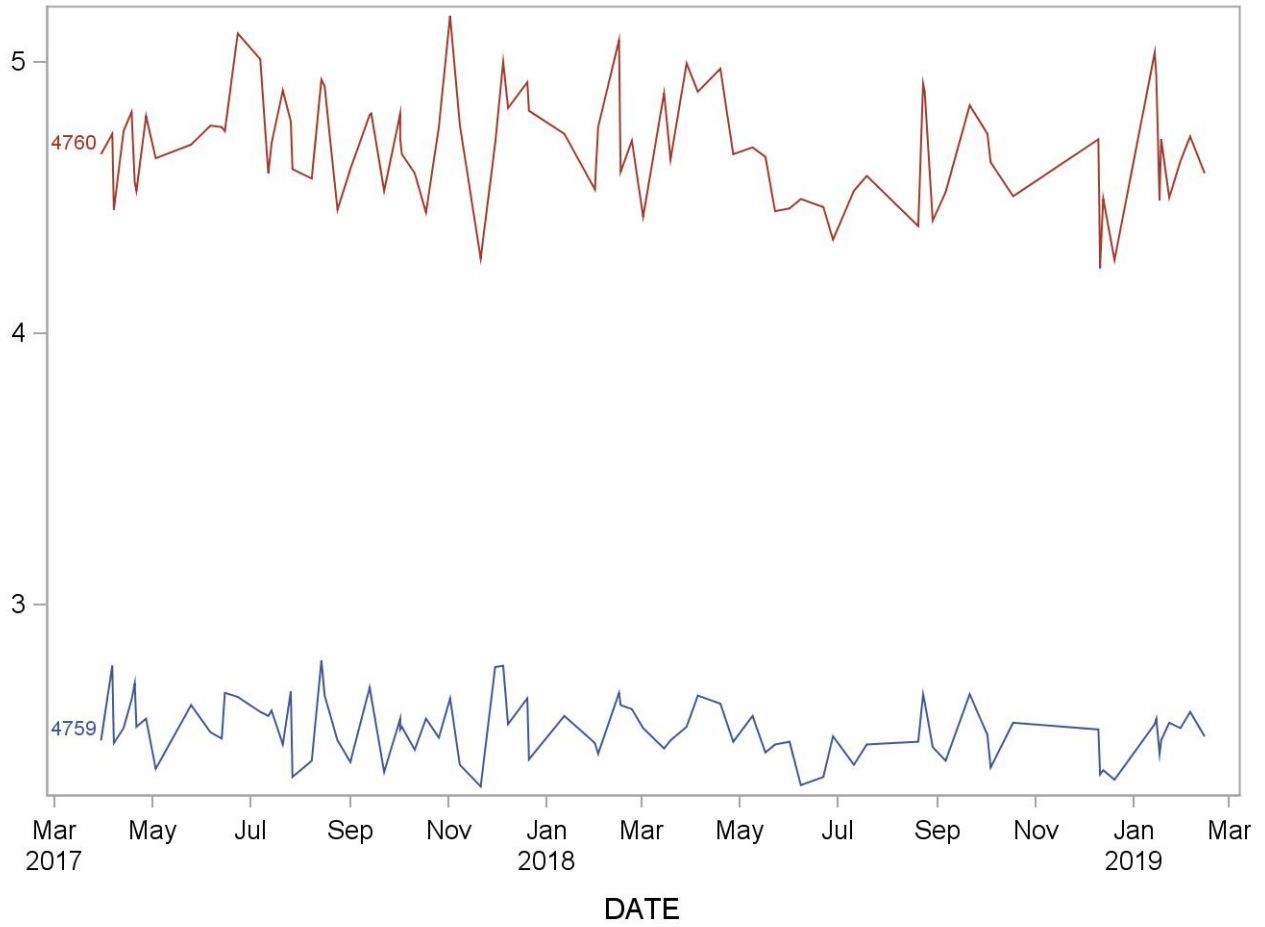
2017-2018 Summary Statistics and QC Chart for Mefox oxidation product (nmol/L)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------|----|------------|----------|--------|--------------------|--------------------------|
| 4760 | 84 | 30MAR17 | 14FEB19 | 2.0727 | 0.0996 | 4.8 |
| 4758 | 84 | 30MAR17 | 14FEB19 | 4.6615 | 0.2475 | 5.3 |
| 4759 | 84 | 30MAR17 | 14FEB19 | 1.3374 | 0.0742 | 5.6 |



2017-2018 Summary Statistics and QC Chart for Tetrahydrofolate (nmol/L)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------|----|------------|----------|--------|--------------------|--------------------------|
| 4760 | 85 | 30MAR17 | 14FEB19 | 4.6872 | 0.1992 | 4.2 |
| 4759 | 85 | 30MAR17 | 14FEB19 | 2.5432 | 0.1086 | 4.3 |



20. 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.**

References

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- (13) Pfeiffer CM, Hughes JP, Lacher DA, Bailey RL, Berry RJ, Zhang M, Yetley EA, Rader JI, Sempos CT, Johnson CL. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988-2010. *J Nutr.* 2012;142:886–93.
- (14) Institute of Medicine, Food and Nutrition Board. *Dietary reference intakes: Thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline.* Washington, D.C.: National Academy Press; 1998.

Acknowledgements

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Appendix A: Method Performance Documentation

| Accuracy compared to Reference Material - fill in yellow shaded cells | | | | | | | | | | | | |
|---|--------------------------|---------------|------------------------|-------|-------|-------|-------|------|------|--------|------|-----------------------------------|
| Mean concentration should be within $\pm 15\%$ of the nominal value except at 3^*LOD , where it should be within $\pm 20\%$ | | | | | | | | | | | | |
| Method name: | Folate Forms by LC-MS/MS | | | | | | | | | | | |
| Method #: | 4013.04 | | | | | | | | | | | |
| Matrix: | Serum | | | | | | | | | | | |
| Units: | nmol/L | | | | | | | | | | | |
| Reference material: | NIST & NIBSC materials | | | | | | | | | | | |
| Analyte: | Folate Forms | | | | | | | | | | | |
| 5-MethylTHF | | | Measured concentration | | | | | | | | | Difference from nominal value (%) |
| Reference material | Replicate | Nominal value | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Mean | SD | CV (%) | | |
| NIST SRM 1955 Level 1 | 1 | 4.26 | 4.36 | 4.75 | 4.78 | 4.97 | 4.66 | 4.71 | 0.21 | 4.46 | 10.6 | |
| | 2 | | 4.37 | 4.75 | 4.78 | 4.99 | 4.72 | | | | | |
| NIST SRM 1955 Level 2 | 1 | 9.73 | 10.00 | 10.39 | 10.50 | 11.00 | 10.58 | 10.5 | 0.32 | 3.02 | 7.7 | |
| | 2 | | 10.02 | 10.33 | 10.52 | 10.84 | 10.62 | | | | | |
| NIST SRM 1955 Level 3 | 1 | 37.1 | 37.30 | 39.34 | 37.71 | 39.01 | 38.14 | 38.2 | 0.80 | 2.08 | 2.9 | |
| | 2 | | 36.98 | 38.79 | 37.96 | 38.89 | 37.62 | | | | | |
| NIST SRM 1950 | 1 | 26.93 | 27.59 | 28.90 | 27.83 | 29.65 | 28.77 | 28.7 | 0.80 | 2.77 | 6.6 | |
| | 2 | | 28.16 | 28.93 | 28.46 | 30.22 | 28.47 | | | | | |
| NIBSC 03-178 | 1 | 9.75 | 10.65 | 10.00 | 10.40 | 10.05 | 10.06 | 10.2 | 0.25 | 2.42 | 4.2 | |
| | 2 | | 10.40 | 10.05 | 10.16 | 9.87 | 9.95 | | | | | |

| 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 #: | 4013.04 | | | | | | | | | | | |
| Matrix: | Serum | | | | | | | | | | | |
| Units: | nmol/L | | | | | | | | | | | |
| Analyte: | 5-MethylTHF | | | | | | | | | | | |
| Replicate | Sample 1 | | | | | Sample 2 | | | | | Mean recovery (%) | SD (%) |
| | Spike concentration | Day 1 (12/07/17) | Day 2 (12/08/17) | Mean | Recovery (%) | Spike concentration | Day 1 (12/07/17) | Day 2 (12/08/17) | Mean | Recovery (%) | | |
| Sample | 0 | 12.5 | 11.8 | 12.40 | 96 | 0 | 17.5 | 17.1 | 17.45 | 96 | 2.3 | |
| | 2 | 12.8 | 12.3 | | | 17.9 | 17.4 | | | | | |
| | 3 | 12.8 | 12.2 | | | 17.6 | 17.2 | | | | | |
| Sample + Spike 1 | 5 | 17.5 | 16.8 | 17.22 | 96 | 5 | 22.1 | 21.5 | 22.08 | 93 | | |
| | 2 | 17.5 | 17.0 | | | 22.6 | 21.8 | | | | | |
| | 3 | 17.6 | 16.9 | | | 22.3 | 22.2 | | | | | |
| Sample + Spike 2 | 10 | 22.1 | 21.8 | 22.15 | 98 | 10 | 26.8 | 27.1 | 27.10 | 97 | | |
| | 2 | 22.5 | 21.7 | | | 27.4 | 26.7 | | | | | |
| | 3 | 23.0 | 21.8 | | | 27.2 | 27.4 | | | | | |
| Sample + Spike 3 | 20 | 32.0 | 31.4 | 32.15 | 99 | 20 | 36.0 | 34.9 | 36.20 | 94 | | |
| | 2 | 33.2 | 31.5 | | | 37.0 | 35.5 | | | | | |
| | 3 | 33.2 | 31.6 | | | 37.2 | 36.6 | | | | | |

Folate Forms
NHANES 2017-2018

| Analyte: | | MeFox | | | | | | | | | | | | |
|------------------|---|---------------------|------------------------|------------------|------|--------------|---------------------|------------------------|-------|------|--------------|-------------------|--------|------|
| Replicate | | Sample 1 | | | | | Sample 2 | | | | | Mean recovery (%) | SD (%) | |
| | | Spike concentration | Measured concentration | | Mean | Recovery (%) | Spike concentration | Measured concentration | | Mean | Recovery (%) | | | |
| | | | Day 1 (12/07/17) | Day 2 (12/08/17) | | | Day 1 (12/07/17) | Day 2 (12/08/17) | | | | | | |
| Sample | 1 | 0 | 4.37 | 4.05 | 4.2 | | 0 | 5.52 | 5.56 | 5.64 | | 85 | 1.8 | |
| | 2 | | 4.68 | 3.95 | | | | | 5.67 | | | | | 5.49 |
| | 3 | | 4.23 | 3.86 | | | | | 5.48 | | | | | 6.10 |
| Sample + Spike 1 | 1 | 5 | 8.86 | 7.89 | 8.5 | 86 | 5 | 9.84 | 9.74 | 9.8 | 83 | | | |
| | 2 | | 8.39 | 8.68 | | | | | 9.33 | | | | | 9.79 |
| | 3 | | 8.53 | 8.66 | | | | | 10.30 | | | | | 9.77 |
| Sample + Spike 2 | 1 | 10 | 11.8 | 12.6 | 12.7 | 85 | 10 | 14.0 | 14.5 | 14.1 | 84 | | | |
| | 2 | | 12.5 | 13.4 | | | | | 13.8 | | | | | 13.3 |
| | 3 | | 12.7 | 13.2 | | | | | 14.3 | | | | | 14.5 |
| Sample + Spike 3 | 1 | 20 | 22.8 | 21.6 | 21.9 | 88 | 20 | 21.7 | 22.4 | 22.8 | 86 | | | |
| | 2 | | 21.3 | 22.1 | | | | | 22.1 | | | | | 24.1 |
| | 3 | | 21.6 | 21.9 | | | | | 23.1 | | | | | 23.1 |

| Analyte: | | Folic acid | | | | | | | | | | | | |
|------------------|---|---------------------|------------------------|------------------|------|--------------|---------------------|------------------------|------|------|--------------|-------------------|--------|------|
| Replicate | | Sample 1 | | | | | Sample 2 | | | | | Mean recovery (%) | SD (%) | |
| | | Spike concentration | Measured concentration | | Mean | Recovery (%) | Spike concentration | Measured concentration | | Mean | Recovery (%) | | | |
| | | | Day 1 (12/07/17) | Day 2 (12/08/17) | | | Day 1 (12/07/17) | Day 2 (12/08/17) | | | | | | |
| Sample | 1 | 0 | 0.33 | 0.43 | 0.41 | | 0 | 0.55 | 0.62 | 0.60 | | 107 | 2.8 | |
| | 2 | | 0.38 | 0.44 | | | | | 0.56 | | | | | 0.64 |
| | 3 | | 0.32 | 0.54 | | | | | 0.57 | | | | | 0.65 |
| Sample + Spike 1 | 1 | 5 | 5.60 | 5.34 | 5.6 | 103 | 5 | 5.85 | 5.81 | 5.8 | 105 | | | |
| | 2 | | 5.35 | 5.74 | | | | | 6.04 | | | | | 5.64 |
| | 3 | | 5.50 | 5.78 | | | | | 6.01 | | | | | 5.63 |
| Sample + Spike 2 | 1 | 10 | 11.1 | 11.3 | 11.2 | 108 | 10 | 11.0 | 11.2 | 11.4 | 108 | | | |
| | 2 | | 10.9 | 11.6 | | | | | 11.5 | | | | | 11.5 |
| | 3 | | 10.9 | 11.6 | | | | | 11.4 | | | | | 12.0 |
| Sample + Spike 3 | 1 | 20 | 22.4 | 22.5 | 22.4 | 110 | 20 | 20.8 | 21.8 | 21.5 | 105 | | | |
| | 2 | | 23.1 | 22.3 | | | | | 21.6 | | | | | 21.3 |
| | 3 | | 21.8 | 22.5 | | | | | 20.9 | | | | | 22.8 |

| Analyte: | | Tetrahydrofolate | | | | | | | | | | | | |
|------------------|---|---------------------|------------------------|------------------|-------|--------------|---------------------|------------------------|-------|-------|--------------|-------------------|--------|-------|
| Replicate | | Sample 1 | | | | | Sample 2 | | | | | Mean recovery (%) | SD (%) | |
| | | Spike concentration | Measured concentration | | Mean | Recovery (%) | Spike concentration | Measured concentration | | Mean | Recovery (%) | | | |
| | | | Day 1 (12/07/17) | Day 2 (12/08/17) | | | Day 1 (12/07/17) | Day 2 (12/08/17) | | | | | | |
| Sample | 1 | 0 | 0.39 | 0.18 | 0.27 | | 0 | 0.62 | 0.37 | 0.45 | | 97 | 1.4 | |
| | 2 | | 0.25 | 0.26 | | | | | 0.44 | | | | | 0.39 |
| | 3 | | 0.33 | 0.18 | | | | | 0.55 | | | | | 0.31 |
| Sample + Spike 1 | 1 | 5 | 4.92 | 5.04 | 5.11 | 97 | 5 | 5.25 | 5.48 | 5.16 | 94 | | | |
| | 2 | | 4.83 | 5.40 | | | | | 4.94 | | | | | 5.08 |
| | 3 | | 5.19 | 5.27 | | | | | 4.69 | | | | | 5.54 |
| Sample + Spike 2 | 1 | 10 | 9.76 | 9.83 | 9.95 | 97 | 10 | 9.58 | 10.30 | 10.33 | 99 | | | |
| | 2 | | 9.72 | 10.10 | | | | | 9.84 | | | | | 11.20 |
| | 3 | | 9.79 | 10.50 | | | | | 9.95 | | | | | 11.10 |
| Sample + Spike 3 | 1 | 20 | 19.40 | 19.90 | 19.73 | 97 | 20 | 19.30 | 20.70 | 19.85 | 97 | | | |
| | 2 | | 18.80 | 20.70 | | | | | 19.20 | | | | | 20.50 |
| | 3 | | 19.40 | 20.20 | | | | | 18.80 | | | | | 20.60 |

| Analyte: | | 5-FormylTHF | | | | | | | | | | | | |
|------------------|---------------------|------------------------------------|---------------------|------|---------------------|--------------|---------------------|------------------------------------|------|------|----|--------------|-------------------|--------|
| Replicate | Spike concentration | Sample 1 Measured concentration | | | | Recovery (%) | Spike concentration | Sample 2 Measured concentration | | | | Recovery (%) | Mean recovery (%) | SD (%) |
| | | Day 1 (12/07/17) | Day 2 (12/08/17) | Mean | Day 1 (12/07/17) | | | Day 2 (12/08/17) | Mean | | | | | |
| Sample | 1 | 0 | 0.14 | 0.14 | | | 0 | 0.14 | 0.14 | | | | | |
| | 2 | | 0.14 | 0.14 | 0.14 | | | 0.14 | 0.14 | 0.14 | | | | |
| | 3 | | 0.14 | 0.14 | | | | 0.14 | 0.14 | | | 97 | 2.4 | |
| Sample + Spike 1 | 1 | 5 | 5.14 | 4.72 | | | 5 | 4.8 | 4.81 | | | | | |
| | 2 | | 4.67 | 5.42 | 5.00 | 97 | | 4.98 | 4.79 | 4.85 | 94 | | | |
| | 3 | | 4.88 | 5.14 | | | | 4.76 | 4.94 | | | | | |
| Sample + Spike 2 | 1 | 10 | 9.92 | 10.5 | | | 10 | 8.96 | 10.3 | | | | | |
| | 2 | | 9.64 | 10.2 | 10.09 | 100 | | 8.96 | 9.7 | 9.58 | 94 | | | |
| | 3 | | 10.1 | 10.2 | | | | 9.34 | 10.2 | | | | | |
| Sample + Spike 3 | 1 | 20 | 19.6 | 20.6 | | | 20 | 18.7 | 19.5 | | | | | |
| | 2 | | 19.8 | 21.0 | 20.1 | 100 | | 18.7 | 20.1 | 19.5 | 97 | | | |
| | 3 | | 19.6 | 20.0 | | | | 18.8 | 20.9 | | | | | |

| Analyte: | | 5,10-MethenylTHF | | | | | | | | | | | | |
|------------------|---------------------|------------------------------------|---------------------|-------|---------------------|--------------|---------------------|------------------------------------|-------|-------|-----|--------------|-------------------|--------|
| Replicate | Spike concentration | Sample 1 Measured concentration | | | | Recovery (%) | Spike concentration | Sample 2 Measured concentration | | | | Recovery (%) | Mean recovery (%) | SD (%) |
| | | Day 1 (12/07/17) | Day 2 (12/08/17) | Mean | Day 1 (12/07/17) | | | Day 2 (12/08/17) | Mean | | | | | |
| Sample | 1 | 0 | 0.14 | 0.14 | | | 0 | 0.14 | 0.14 | | | | | |
| | 2 | | 0.14 | 0.14 | 0.14 | | | 0.14 | 0.14 | 0.14 | | | | |
| | 3 | | 0.14 | 0.14 | | | | 0.14 | 0.14 | | | 108 | 2.1 | |
| Sample + Spike 1 | 1 | 5 | 6.07 | 5.17 | | | 5 | 5.24 | 5.13 | | | | | |
| | 2 | | 5.79 | 5.45 | 5.52 | 108 | | 5.81 | 5.11 | 5.48 | 107 | | | |
| | 3 | | 5.42 | 5.21 | | | | 5.75 | 5.84 | | | | | |
| Sample + Spike 2 | 1 | 10 | 10.50 | 10.80 | | | 10 | 11.40 | 10.50 | | | | | |
| | 2 | | 11.00 | 11.50 | 10.92 | 108 | | 11.30 | 9.80 | 10.77 | 106 | | | |
| | 3 | | 11.00 | 10.70 | | | | 10.50 | 11.10 | | | | | |
| Sample + Spike 3 | 1 | 20 | 23.9 | 21.5 | | | 20 | 23.9 | 21.0 | | | | | |
| | 2 | | 22.0 | 21.8 | 22.6 | 112 | | 21.4 | 21.6 | 22.0 | 109 | | | |
| | 3 | | 24.3 | 21.9 | | | | 22.1 | 21.7 | | | | | |

| Precision - fill in yellow shaded cells | | | | | | | | | | | | | |
|---|--------------------------|---------------|---------|-----------------|-------|----------|------------------------------|-------------|---------------|---------|-----------------|-------|----------|
| Total relative standard deviation should be $\leq 15\%$ (CV $\leq 15\%$) | | | | | | | | | | | | | |
| Method name: | Folate Forms by LC-MS/MS | | | | | | | | | | | | |
| Method #: | 4013.04 | | | | | | | | | | | | |
| Matrix: | Serum | | | | | | | | | | | | |
| Units: | nmol/L | | | | | | | | | | | | |
| Run dates: | 2017-03-09 to 2017-03-21 | | | | | | | | | | | | |
| Analyte: | 5-MethylTHF | | | | | | | | | | | | |
| Quality material 1 (MS16431) | | | | | | | Quality material 2 (HS16432) | | | | | | |
| 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 | 24.5 | 23.6 | 24.05 | 0.202 | 0.202 | 1157 | 1 | 42.8 | 41.7 | 42.25 | 0.302 | 0.302 | 3570 |
| 2 | 25.2 | 24.3 | 24.75 | 0.202 | 0.202 | 1225 | 2 | 44.4 | 43.2 | 43.80 | 0.360 | 0.360 | 3837 |
| 3 | 23.9 | 24.4 | 24.15 | 0.063 | 0.063 | 1166 | 3 | 42.6 | 42.1 | 42.35 | 0.063 | 0.063 | 3587 |
| 4 | 24.9 | 25.0 | 24.95 | 0.003 | 0.003 | 1245 | 4 | 44.0 | 43.2 | 43.60 | 0.160 | 0.160 | 3802 |
| 5 | 24.4 | 24.2 | 24.30 | 0.010 | 0.010 | 1181 | 5 | 43.1 | 42.8 | 42.95 | 0.022 | 0.023 | 3689 |
| 6 | 24.1 | 24.0 | 24.05 | 0.003 | 0.003 | 1157 | 6 | 42.9 | 41.9 | 42.40 | 0.250 | 0.250 | 3596 |
| 7 | 25.2 | 25.0 | 25.10 | 0.010 | 0.010 | 1260 | 7 | 43.6 | 44.1 | 43.85 | 0.063 | 0.063 | 3846 |
| 8 | 24.2 | 24.2 | 24.20 | 0.000 | 0.000 | 1171 | 8 | 42.9 | 42.4 | 42.65 | 0.063 | 0.063 | 3638 |
| 9 | 24.7 | 25.2 | 24.95 | 0.063 | 0.063 | 1245 | 9 | 43.9 | 44.1 | 44.00 | 0.010 | 0.010 | 3872 |
| 10 | 24.7 | 24.7 | 24.70 | 0.000 | 0.000 | 1220 | 10 | 43.4 | 43.6 | 43.50 | 0.010 | 0.010 | 3785 |
| Grand sum | 490.4 | Grand mean | 24.52 | | | | Grand sum | 862.7 | Grand mean | 43.14 | | | |
| | | Std Dev | 0.41 | | | | | | Std Dev | 0.69 | | | |
| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | | | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | |
| Within Run | 1.1100 | 0.1110 | 0.3332 | 1.36 | | | Within Run | 2.6050 | 0.2605 | 0.5104 | 1.18 | | |
| Between Run | 3.0420 | 0.3380 | 0.3369 | 1.37 | | | Between Run | 8.5205 | 0.9467 | 0.5858 | 1.36 | | |
| Total | 4.15 | | 0.4738 | 1.93 | | | Total | 11.13 | | 0.7769 | 1.80 | | |

| Analyte: | MeFox | | | | | | | | | | | | |
|------------------------------|-------------|---------------|---------|-----------------|-------|----------|------------------------------|-------------|---------------|---------|-----------------|-------|----------|
| Quality material 1 (LS16430) | | | | | | | Quality material 2 (HS16432) | | | | | | |
| 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 | 4.77 | 4.76 | 4.77 | 0.000 | 0.000 | 45.4105 | 1 | 2.02 | 2.09 | 2.06 | 0.001 | 0.001 | 8.4461 |
| 2 | 5.03 | 5.05 | 5.04 | 0.000 | 0.000 | 50.8032 | 2 | 2.34 | 2.15 | 2.25 | 0.009 | 0.009 | 10.0801 |
| 3 | 4.85 | 4.68 | 4.77 | 0.007 | 0.007 | 45.4105 | 3 | 2.09 | 2.02 | 2.06 | 0.001 | 0.001 | 8.4461 |
| 4 | 4.71 | 4.73 | 4.72 | 0.000 | 0.000 | 44.5568 | 4 | 2.00 | 2.12 | 2.06 | 0.004 | 0.004 | 8.4872 |
| 5 | 4.82 | 4.50 | 4.66 | 0.026 | 0.026 | 43.4312 | 5 | 2.10 | 2.13 | 2.12 | 0.000 | 0.000 | 8.9465 |
| 6 | 5.14 | 5.08 | 5.11 | 0.001 | 0.001 | 52.2242 | 6 | 2.33 | 2.01 | 2.17 | 0.026 | 0.026 | 9.4178 |
| 7 | 4.72 | 4.69 | 4.71 | 0.000 | 0.000 | 44.2741 | 7 | 2.06 | 2.02 | 2.04 | 0.000 | 0.000 | 8.3232 |
| 8 | 4.60 | 4.56 | 4.58 | 0.000 | 0.000 | 41.9528 | 8 | 2.05 | 2.08 | 2.07 | 0.000 | 0.000 | 8.5285 |
| 9 | 5.14 | 4.94 | 5.04 | 0.010 | 0.010 | 50.8032 | 9 | 2.17 | 2.18 | 2.18 | 0.000 | 0.000 | 9.4613 |
| 10 | 4.88 | 4.74 | 4.81 | 0.005 | 0.005 | 46.2722 | 10 | 2.11 | 2.14 | 2.13 | 0.000 | 0.000 | 9.0313 |
| Grand sum | 96.39 | Grand mean | 4.82 | | | | Grand sum | 42.21 | Grand mean | 2.11 | | | |
| | | Std Dev | 0.18 | | | | | | Std Dev | 0.07 | | | |
| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | | | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | |
| Within Run | 0.0989 | 0.0099 | 0.0995 | 2.06 | | | Within Run | 0.0836 | 0.0084 | 0.0914 | 4.33 | | |
| Between Run | 0.5869 | 0.0652 | 0.1663 | 3.45 | | | Between Run | 0.0835 | 0.0093 | 0.0215 | 1.02 | | |
| Total | 0.69 | | 0.1938 | 4.02 | | | Total | 0.17 | | 0.0939 | 4.45 | | |

| Analyte: Folic acid | | | | | | | | | | | | | |
|------------------------------|--------------------|----------------------|----------------|------------------------|--------|----------|------------------------------|--------------------|----------------------|----------------|------------------------|-------|----------|
| Quality material 1 (LS16430) | | | | | | | Quality material 2 (HS16432) | | | | | | |
| 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 | 0.62 | 0.62 | 0.62 | 0.0000 | 0.0000 | 0.769 | 1 | 9.79 | 9.49 | 9.64 | 0.022 | 0.023 | 185.86 |
| 2 | 0.63 | 0.67 | 0.65 | 0.0004 | 0.0004 | 0.845 | 2 | 9.83 | 10 | 9.92 | 0.007 | 0.007 | 196.61 |
| 3 | 0.63 | 0.7 | 0.67 | 0.0012 | 0.0012 | 0.884 | 3 | 10.3 | 9.54 | 9.92 | 0.144 | 0.144 | 196.81 |
| 4 | 0.62 | 0.63 | 0.63 | 0.0000 | 0.0000 | 0.781 | 4 | 10.2 | 9.91 | 10.06 | 0.021 | 0.021 | 202.21 |
| 5 | 0.64 | 0.61 | 0.63 | 0.0002 | 0.0002 | 0.781 | 5 | 10.1 | 9.92 | 10.01 | 0.008 | 0.008 | 200.40 |
| 6 | 0.64 | 0.61 | 0.63 | 0.0002 | 0.0002 | 0.781 | 6 | 9.63 | 9.79 | 9.71 | 0.006 | 0.006 | 188.57 |
| 7 | 0.61 | 0.65 | 0.63 | 0.0004 | 0.0004 | 0.794 | 7 | 9.83 | 10.4 | 10.12 | 0.081 | 0.081 | 204.63 |
| 8 | 0.61 | 0.61 | 0.61 | 0.0000 | 0.0000 | 0.744 | 8 | 9.66 | 9.64 | 9.65 | 0.000 | 0.000 | 186.25 |
| 9 | 0.60 | 0.64 | 0.62 | 0.0004 | 0.0004 | 0.769 | 9 | 10.2 | 10.5 | 10.35 | 0.023 | 0.023 | 214.25 |
| 10 | 0.61 | 0.57 | 0.59 | 0.0004 | 0.0004 | 0.696 | 10 | 9.70 | 9.95 | 9.83 | 0.016 | 0.016 | 193.06 |
| Grand sum | 12.52 | Grand mean | 0.63 | | | | Grand sum | 198.38 | Grand mean | 9.92 | | | |
| | | Std Dev | 0.02 | | | | | | Std Dev | 0.22 | | | |
| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | | | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | |
| Within Run | 0.0066 | 0.0007 | 0.0257 | 4.10 | | | Within Run | 0.6582 | 0.0658 | 0.2566 | 2.59 | | |
| Between Run | 0.0075 | 0.0008 | 0.0092 | 1.48 | | | Between Run | 0.9074 | 0.1008 | 0.1323 | 1.33 | | |
| Total | 0.01 | | 0.0273 | 4.36 | | | Total | 1.57 | | 0.2887 | 2.91 | | |

| Analyte: Tetrahydrofolate | | | | | | | | | | | | | |
|------------------------------|--------------------|----------------------|----------------|------------------------|-------|----------|------------------------------|--------------------|----------------------|----------------|------------------------|-------|----------|
| Quality material 1 (MS16431) | | | | | | | Quality material 2 (HS16432) | | | | | | |
| 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 | 2.64 | 2.21 | 2.43 | 0.046 | 0.046 | 11.761 | 1 | 4.59 | 4.25 | 4.42 | 0.029 | 0.029 | 39 |
| 2 | 2.53 | 2.52 | 2.53 | 0.000 | 0.000 | 12.751 | 2 | 4.85 | 4.54 | 4.70 | 0.024 | 0.024 | 44 |
| 3 | 2.09 | 2.55 | 2.32 | 0.053 | 0.053 | 10.765 | 3 | 4.32 | 4.46 | 4.39 | 0.005 | 0.005 | 39 |
| 4 | 2.47 | 2.53 | 2.50 | 0.001 | 0.001 | 12.500 | 4 | 4.89 | 4.65 | 4.77 | 0.014 | 0.014 | 46 |
| 5 | 2.73 | 2.66 | 2.70 | 0.001 | 0.001 | 14.526 | 5 | 4.73 | 4.97 | 4.85 | 0.014 | 0.014 | 47 |
| 6 | 2.66 | 2.70 | 2.68 | 0.000 | 0.000 | 14.365 | 6 | 5.02 | 4.78 | 4.90 | 0.014 | 0.014 | 48 |
| 7 | 2.49 | 2.62 | 2.56 | 0.004 | 0.004 | 13.056 | 7 | 4.96 | 4.88 | 4.92 | 0.002 | 0.002 | 48 |
| 8 | 2.57 | 2.40 | 2.49 | 0.007 | 0.007 | 12.350 | 8 | 4.74 | 4.68 | 4.71 | 0.001 | 0.001 | 44 |
| 9 | 2.75 | 2.89 | 2.82 | 0.005 | 0.005 | 15.905 | 9 | 4.78 | 4.87 | 4.83 | 0.002 | 0.002 | 47 |
| 10 | 2.78 | 2.47 | 2.63 | 0.024 | 0.024 | 13.781 | 10 | 4.90 | 4.74 | 4.82 | 0.006 | 0.006 | 46 |
| Grand sum | 51.26 | Grand mean | 2.563 | | | | Grand sum | 94.6 | Grand mean | 4.73 | | | |
| | | Std Dev | 0.15 | | | | | | Std Dev | 0.19 | | | |
| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | | | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | |
| Within Run | 0.2841 | 0.0284 | 0.1686 | 6.58 | | | Within Run | 0.2239 | 0.0224 | 0.1496 | 3.16 | | |
| Between Run | 0.3813 | 0.0424 | 0.0835 | 3.26 | | | Between Run | 0.6229 | 0.0692 | 0.1530 | 3.23 | | |
| Total | 0.67 | | 0.1881 | 7.34 | | | Total | 0.85 | | 0.2140 | 4.52 | | |

| Analyte: 5-FormylTHF | | | | | | | | | | | | | |
|------------------------------|--------------------|----------------------|----------------|------------------------|--------|----------|------------------------------|--------------------|----------------------|----------------|------------------------|-------|----------|
| Quality material 1 (MS16431) | | | | | | | Quality material 2 (HS16432) | | | | | | |
| 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 | 2.59 | 2.6 | 2.60 | 0.0000 | 0.0000 | 13.4681 | 1 | 5.18 | 5.33 | 5.26 | 0.006 | 0.006 | 55.230 |
| 2 | 2.69 | 2.75 | 2.72 | 0.0009 | 0.0009 | 14.7968 | 2 | 5.47 | 5.48 | 5.48 | 0.000 | 0.000 | 59.951 |
| 3 | 2.71 | 2.64 | 2.68 | 0.0012 | 0.0012 | 14.3113 | 3 | 5.33 | 5.35 | 5.34 | 0.000 | 0.000 | 57.031 |
| 4 | 2.67 | 2.80 | 2.74 | 0.0042 | 0.0042 | 14.9605 | 4 | 5.71 | 5.42 | 5.57 | 0.021 | 0.021 | 61.938 |
| 5 | 2.80 | 2.64 | 2.72 | 0.0064 | 0.0064 | 14.7968 | 5 | 5.52 | 5.39 | 5.46 | 0.004 | 0.004 | 59.514 |
| 6 | 2.78 | 2.67 | 2.73 | 0.0030 | 0.0030 | 14.8513 | 6 | 5.63 | 5.45 | 5.54 | 0.008 | 0.008 | 61.383 |
| 7 | 2.73 | 2.66 | 2.70 | 0.0012 | 0.0012 | 14.5261 | 7 | 5.34 | 5.39 | 5.37 | 0.001 | 0.001 | 57.566 |
| 8 | 2.70 | 2.69 | 2.70 | 0.0000 | 0.0000 | 14.5261 | 8 | 5.25 | 5.30 | 5.28 | 0.001 | 0.001 | 55.651 |
| 9 | 2.94 | 2.85 | 2.90 | 0.0020 | 0.0020 | 16.7621 | 9 | 5.48 | 5.63 | 5.56 | 0.006 | 0.006 | 61.716 |
| 10 | 2.77 | 2.86 | 2.82 | 0.0020 | 0.0020 | 15.8485 | 10 | 5.41 | 5.16 | 5.29 | 0.016 | 0.016 | 55.862 |
| Grand sum | 54.54 | Grand mean | 2.73 | | | | Grand sum | 108.2 | Grand mean | 5.41 | | | |
| | | Std Dev | 0.08 | | | | | | Std Dev | 0.12 | | | |
| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | | | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | |
| Within Run | 0.0422 | 0.0042 | 0.0650 | 2.38 | | | Within Run | 0.1232 | 0.0123 | 0.1110 | 2.05 | | |
| Between Run | 0.1166 | 0.0130 | 0.0661 | 2.42 | | | Between Run | 0.2660 | 0.0296 | 0.0928 | 1.72 | | |
| Total | 0.16 | | 0.0927 | 3.40 | | | Total | 0.39 | | 0.1447 | 2.67 | | |

| Analyte: 5,10-MethenylTHF | | | | | | | | | | | | | |
|------------------------------|--------------------|----------------------|----------------|------------------------|--------|----------|------------------------------|--------------------|----------------------|----------------|------------------------|-------|----------|
| Quality material 1 (MS16431) | | | | | | | Quality material 2 (HS16432) | | | | | | |
| 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 | 2.54 | 2.67 | 2.61 | 0.0042 | 0.0042 | 13.5721 | 1 | 5.17 | 4.88 | 5.03 | 0.021 | 0.021 | 50.501 |
| 2 | 2.74 | 2.64 | 2.69 | 0.0025 | 0.0025 | 14.4722 | 2 | 5.20 | 5.55 | 5.38 | 0.031 | 0.031 | 57.781 |
| 3 | 2.37 | 2.38 | 2.38 | 0.0000 | 0.0000 | 11.2813 | 3 | 4.61 | 4.47 | 4.54 | 0.005 | 0.005 | 41.223 |
| 4 | 2.93 | 2.20 | 2.57 | 0.1332 | 0.1332 | 13.1585 | 4 | 5.54 | 4.89 | 5.22 | 0.106 | 0.106 | 54.392 |
| 5 | 2.63 | 2.70 | 2.67 | 0.0012 | 0.0012 | 14.2045 | 5 | 5.26 | 5.00 | 5.13 | 0.017 | 0.017 | 52.634 |
| 6 | 2.73 | 2.60 | 2.67 | 0.0042 | 0.0042 | 14.2045 | 6 | 5.15 | 5.09 | 5.12 | 0.001 | 0.001 | 52.429 |
| 7 | 2.74 | 2.76 | 2.75 | 0.0001 | 0.0001 | 15.1250 | 7 | 5.24 | 5.48 | 5.36 | 0.014 | 0.014 | 57.459 |
| 8 | 2.71 | 2.69 | 2.70 | 0.0001 | 0.0001 | 14.5800 | 8 | 5.36 | 5.42 | 5.39 | 0.001 | 0.001 | 58.104 |
| 9 | 3.00 | 2.67 | 2.84 | 0.0272 | 0.0272 | 16.0745 | 9 | 5.45 | 5.40 | 5.43 | 0.001 | 0.001 | 58.861 |
| 10 | 2.74 | 2.79 | 2.77 | 0.0006 | 0.0006 | 15.2905 | 10 | 5.14 | 5.27 | 5.21 | 0.004 | 0.004 | 54.184 |
| Grand sum | 53.23 | Grand mean | 2.66 | | | | Grand sum | 103.6 | Grand mean | 5.18 | | | |
| | | Std Dev | 0.13 | | | | | | Std Dev | 0.26 | | | |
| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | | | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) | | |
| Within Run | 0.3470 | 0.0347 | 0.1863 | 7.00 | | | Within Run | 0.4003 | 0.0400 | 0.2001 | 3.86 | | |
| Between Run | 0.2911 | 0.0323 | 0.0000 | 0.00 | | | Between Run | 1.2322 | 0.1369 | 0.2201 | 4.25 | | |
| Total | 0.64 | | 0.1863 | 7.00 | | | Total | 1.63 | | 0.2974 | 5.74 | | |

| | | | | | | | |
|--|--|--|--|--|--|--|--|
| Stability - fill in yellow shaded cells | | | | | | | |
| The initial measurement can be from the same day for all stability experiments. | | | | | | | |
| Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions | | | | | | | |
| Describe condition: QC material thawed 3 times (2 hrs at room temperature) and re-frozen at -70°C (3 freeze-thaw cycles) | | | | | | | |
| Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typicall at room temperature) | | | | | | | |
| Describe condition: QC material (not yet processed) stored at room temperature for 3 hrs, then processed for analysis | | | | | | | |
| Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler | | | | | | | |
| Describe condition: (1): Processed samples were kept in the autosampler overnight prior to analysis next day | | | | | | | |
| (2): Processed samples were kept at -20°C for 3 days (over the weekend) prior to analysis | | | | | | | |
| Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis | | | | | | | |
| Describe condition: QC material stored at -70°C for 1.5 years | | | | | | | |
| All stability sample results should be within ±15% of nominal concentration | | | | | | | |
| Method name: Folate Forms by LC-MS/MS | | | | | | | |
| Method #: 4013.04 | | | | | | | |
| Matrix: Serum | | | | | | | |
| Units: nmol/L | | | | | | | |
| Run date: 2017-08-31 (HZ:6500) | | | | | | | |

| | | | | | | | |
|---------------------------------------|---------------------|--------------------------|---------------------|--------------------------------|--------------------------------|---------------------|---------------------|
| Analyte: | 5-MethylTHF | | | | | | |
| Quality material 1 (MS16431) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 25.10 | 25.00 | 25.30 | 25.50 | 25.00 | 22.98 | 25.10 |
| Replicate 2 | 25.10 | 25.20 | 25.70 | 25.40 | 24.90 | 23.21 | 25.10 |
| Replicate 3 | 25.50 | 25.10 | 24.80 | 25.00 | 24.90 | 23.89 | 25.50 |
| Mean | 25.23 | 25.10 | 25.27 | 25.30 | 24.93 | 23.36 | 25.23 |
| % difference from initial measurement | -- | -0.53 | 0.13 | 0.26 | -1.19 | -- | 8.02 |
| Quality material 2 (HS16432) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 44.50 | 43.70 | 44.20 | 44.00 | 44.20 | 41.11 | 44.50 |
| Replicate 2 | 44.10 | 43.80 | 43.40 | 43.60 | 44.30 | 41.58 | 44.10 |
| Replicate 3 | 44.70 | 44.50 | 44.10 | 43.40 | 43.20 | 40.98 | 44.70 |
| Mean | 44.43 | 44.00 | 43.90 | 43.67 | 43.90 | 41.22 | 44.43 |
| % difference from initial measurement | -- | -0.98 | -1.20 | -1.73 | -1.20 | -- | 7.79 |

| Analyte: | | MeFox | | | | | |
|---------------------------------------|---------------------|--------------------------|---------------------|---------------------------------|----------------------------------|---------------------|---------------------|
| Quality material 1 (MS16431) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1)* | Processed sample stability (2)** | Initial measurement | Long-term stability |
| Replicate 1 | 1.36 | 1.35 | 1.46 | 1.39 | 1.46 | 1.21 | 1.36 |
| Replicate 2 | 1.34 | 1.32 | 1.41 | 1.42 | 1.37 | 1.23 | 1.34 |
| Replicate 3 | 1.33 | 1.35 | 1.31 | 1.34 | 1.38 | 1.25 | 1.33 |
| Mean | 1.34 | 1.34 | 1.39 | 1.38 | 1.40 | 1.23 | 1.34 |
| % difference from initial measurement | -- | -0.25 | 3.72 | 2.98 | 4.47 | -- | 9.4 |
| Quality material 2 (HS16432) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 2.12 | 2.07 | 2.09 | 2.05 | 2.10 | 1.88 | 2.12 |
| Replicate 2 | 1.98 | 2.01 | 1.99 | 2.24 | 2.17 | 1.90 | 1.98 |
| Replicate 3 | 2.15 | 2.02 | 2.17 | 1.94 | 2.20 | 1.87 | 2.15 |
| Mean | 2.08 | 2.03 | 2.08 | 2.08 | 2.16 | 1.88 | 2.08 |
| % difference from initial measurement | -- | -2.40 | 0.00 | -0.32 | 3.52 | -- | 10.6 |
| Analyte: | | Folic acid | | | | | |
| Quality material 1 (MS16431) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1)* | Processed sample stability (2)** | Initial measurement | Long-term stability |
| Replicate 1 | 5.75 | 5.62 | 5.68 | 5.73 | 5.74 | 5.26 | 5.75 |
| Replicate 2 | 5.58 | 5.67 | 5.76 | 5.67 | 5.68 | 5.24 | 5.58 |
| Replicate 3 | 5.69 | 5.58 | 5.6 | 5.77 | 5.82 | 5.31 | 5.69 |
| Mean | 5.67 | 5.62 | 5.68 | 5.72 | 5.75 | 5.27 | 5.67 |
| % difference from initial measurement | -- | -0.88 | 0.12 | 0.88 | 1.29 | -- | 7.6 |
| Quality material 2 (HS16432) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 10.30 | 10.10 | 10.30 | 10.40 | 10.20 | 9.23 | 10.30 |
| Replicate 2 | 10.50 | 10.10 | 10.00 | 10.20 | 10.30 | 9.23 | 10.50 |
| Replicate 3 | 10.70 | 10.20 | 10.60 | 10.40 | 10.30 | 9.41 | 10.70 |
| Mean | 10.50 | 10.13 | 10.30 | 10.33 | 10.27 | 9.29 | 10.50 |
| % difference from initial measurement | -- | -3.49 | -1.90 | -1.59 | -2.22 | -- | 13.0 |

| Analyte: | | Tetrahydrofolate | | | | | |
|---------------------------------------|---------------------|--------------------------|---------------------|--------------------------------|--------------------------------|---------------------|---------------------|
| Quality material 1 (MS16431) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 2.69 | 2.41 | 2.70 | 2.93 | 2.49 | 2.60 | 2.69 |
| Replicate 2 | 2.40 | 2.52 | 2.54 | 2.83 | 2.48 | 2.46 | 2.40 |
| Replicate 3 | 2.61 | 2.37 | 2.29 | 2.60 | 2.55 | 2.71 | 2.61 |
| Mean | 2.57 | 2.43 | 2.51 | 2.79 | 2.51 | 2.59 | 2.57 |
| % difference from initial measurement | -- | -5.19 | -2.21 | 8.57 | -2.34 | -- | -0.9 |
| Quality material 2 (HS16432) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 4.65 | 4.74 | 4.77 | 4.88 | 4.81 | 4.56 | 4.65 |
| Replicate 2 | 5.01 | 4.64 | 4.45 | 4.75 | 4.62 | 4.54 | 5.01 |
| Replicate 3 | 4.83 | 4.47 | 4.74 | 4.89 | 4.86 | 4.63 | 4.83 |
| Mean | 4.83 | 4.62 | 4.65 | 4.84 | 4.76 | 4.58 | 4.83 |
| % difference from initial measurement | -- | -4.42 | -3.66 | 0.21 | -1.38 | -- | 5.5 |
| Analyte: | | 5-FormylTHF | | | | | |
| Quality material 1 (MS16431) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 2.83 | 2.79 | 2.86 | 2.68 | 2.82 | 2.54 | 2.83 |
| Replicate 2 | 2.73 | 2.81 | 2.84 | 2.77 | 2.76 | 2.65 | 2.73 |
| Replicate 3 | 2.72 | 2.67 | 2.8 | 2.79 | 2.81 | 2.54 | 2.72 |
| Mean | 2.76 | 2.76 | 2.83 | 2.75 | 2.80 | 2.58 | 2.76 |
| % difference from initial measurement | -- | -0.12 | 2.66 | -0.48 | 1.33 | -- | 7.2 |
| Quality material 2 (HS16432) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 5.40 | 5.44 | 5.76 | 5.38 | 5.57 | 5.13 | 5.40 |
| Replicate 2 | 5.39 | 5.55 | 5.40 | 5.61 | 5.58 | 5.20 | 5.39 |
| Replicate 3 | 5.56 | 5.55 | 5.54 | 5.54 | 5.55 | 5.18 | 5.56 |
| Mean | 5.45 | 5.51 | 5.57 | 5.51 | 5.57 | 5.17 | 5.45 |
| % difference from initial measurement | -- | 1.16 | 2.14 | 1.10 | 2.14 | -- | 5.4 |

| Analyte: 5,10-MethenylTHF | | | | | | | |
|---------------------------------------|---------------------|--------------------------|---------------------|---------------------------------|----------------------------------|---------------------|---------------------|
| Quality material 1 (MS16431) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1)* | Processed sample stability (2)** | Initial measurement | Long-term stability |
| Replicate 1 | 2.54 | 2.55 | 2.68 | 2.88 | 2.83 | 2.38 | 2.54 |
| Replicate 2 | 2.77 | 2.46 | 2.79 | 2.75 | 2.70 | 2.38 | 2.77 |
| Replicate 3 | 2.82 | 2.60 | 2.67 | 2.86 | 2.93 | 2.32 | 2.82 |
| Mean | 2.71 | 2.54 | 2.71 | 2.83 | 2.82 | 2.36 | 2.71 |
| % difference from initial measurement | -- | -6.40 | 0.12 | 4.43 | 4.06 | -- | 14.7 |
| Quality material 2 (HS16432) | | | | | | | |
| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability (1) | Processed sample stability (2) | Initial measurement | Long-term stability |
| Replicate 1 | 5.38 | 5.27 | 5.37 | 5.36 | 5.55 | 4.63 | 5.38 |
| Replicate 2 | 5.28 | 5.21 | 4.92 | 5.31 | 5.66 | 4.72 | 5.28 |
| Replicate 3 | 5.29 | 5.42 | 5.21 | 5.49 | 5.42 | 4.61 | 5.29 |
| Mean | 5.32 | 5.30 | 5.17 | 5.39 | 5.54 | 4.65 | 5.32 |
| % difference from initial measurement | -- | -0.31 | -2.82 | 1.32 | 4.26 | -- | 14.2 |

| LOD, specificity and fit for intended use - fill in yellow shaded cells | | | | |
|--|---|--------------------------|---|--|
| Analytes | Mass Spectrometer Instrument (2017-01-18) | 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 |
| Method name: | Folate Forms by LC-MS/MS | | | |
| Method #: | 4013.04 | | | |
| Matrix: | Serum | | | |
| Units: | nmol/L | | | |
| 5-MethylTHF | Hazel (6500) | 0.13 | yes | yes |
| Folic acid | Hazel (6500) | 0.14 | yes | yes |
| 5-FormylTHF | Hazel (6500) | 0.20 | yes | yes |
| Tetrahydrofolate | Hazel (6500) | 0.25 | yes | yes |
| 5, 10-MethenylTHF | Hazel (6500) | 0.20 | yes | yes |
| MeFox | Hazel (6500) | 0.10 | 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:

$$\text{Conc. (ppm or } \mu\text{g / mL)} = [\text{Absorbance (cm}^{-1}) \times \text{dilution} \times 1000 \times \text{MW (g mol}^{-1})] / \epsilon_{\text{max}} (\text{L mol}^{-1} \text{cm}^{-1})$$

$$\text{Conc. (}\mu\text{mol / L)} = [\text{Absorbance (cm}^{-1}) \times \text{dilution} \times 1000 \times 1000] / \epsilon_{\text{max}} (\text{L mol}^{-1} \text{cm}^{-1})$$

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 $\mu\text{g/mL}$) = $0.661 \times 10 \times 1000 \times 441.4 / 27600 = 105.7$

Concentration ($\mu\text{mol/L}$) = $0.661 \times 10 \times 1000 \times 1000 / 27600 = 239.5$

(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 |
| ¹³ C5-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 | 10* | 1 | 1 | 1 | 1 | 1 | 15* |
| Medium | 20 | 2.5 | 5 | 2.5 | 2.5 | 2.5 | 30 |
| High | 50 | 5 | 10 | 5 | 5 | 5 | 70 |

*As low as possible

Appendix D: Ruggedness Testing

A previous version of this method (method 4001) 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 method basics did not change by going to method 4013, there is no need to repeat the ruggedness testing with this method.

Folate is an important nutrient involved in one carbon cellular metabolism. Serum 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 species.

A. Variations in sample preparation

- (1) Principle: The buffers used for sample preparation, solid-phase extraction and analyte elution use formic acid, ammonium hydroxide (to adjust pH), ascorbic acid (as antioxidant), and acetic acid (acid modifier). The changes in buffer pH, concentration of formic acid, ascorbic acid, and acetic acid are critical for analyte and/or sample matrix recovery during sample preparation and solid-phase extraction and will affect analyte sensitivity and potentially affect the results.
- (2) Proposal: To vary and test the sample preparation and solid-phase extraction conditions.
 - a) pH of ammonium formate buffer (Sample solvent #1)
 - b) Formic acid concentration in ammonium formate buffer (Sample solvent #1)
 - c) Ascorbic acid concentration in ammonium formate buffer (Sample solvent #1)
 - d) Ammonium formate concentration in intermediate wash step during which matrix compounds are eluted but analytes are retained (SPE wash buffer)
 - e) Ascorbic acid concentration in SPE elution buffer (Sample solvent # 3)
 - f) Acetic acid concentration in SPE elution buffer (Sample solvent # 3)
- (3) Findings:
 - a) Varying the pH of the ammonium formate sample preparation buffer does not appear to affect folate species results in serum samples.
 - b) Varying the formic acid concentration in the ammonium formate sample preparation buffer does not appear to affect folate species results in serum samples.
 - c) Varying the ascorbic acid concentration in the ammonium formate sample preparation buffer does not appear to affect folate species results in serum samples.
 - d) Varying the ammonium formate concentration in the SPE wash buffer does not appear to affect the folate species results in serum samples.
 - e) Varying the ascorbic acid concentration in the SPE elution buffer does not appear to affect folate species results in serum samples.

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

Table: Ruggedness testing results for serum 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 | MET: 22.3 FOT: 1.8 PGA: 1.8 | 3.0 | MET: 22.5 FOT: 2.1 PGA: 1.8 | 3.4 | MET: 22.2 FOT: 1.8 PGA: 1.9 |
| 2. Formic acid concentration in ammonium formate buffer (sample solvent #1) | 1% | MET: 22.7 FOT: 2.1 PGA: 2.0 | 0.8% | MET: 21.6 FOT: 2.1 PGA: 1.9 | 1.2% | MET: 21.5 FOT: 2.1 PGA: 1.9 |
| 3. Ascorbic acid in ammonium formate buffer (sample solvent #1) | 0.5% | MET: 22.4 FOT: 1.6 PGA: 1.9 | 0.3% | MET: 22.3 FOT: 1.6 PGA: 2.1 | 0.7% | MET: 23.1 FOT: 1.6 PGA: 2.2 |
| 4. Ammonium formate concentration in SPE wash buffer | 0.05% | MET: 22.2 FOT: 2.1 PGA: 2.0 | 0.04% | MET: 22.0 FOT: 2.3 PGA: 2.0 | 0.06% | MET: 21.7 FOT: 2.0 PGA: 2.0 |
| 5. Ascorbic acid concentration in SPE elution buffer | 0.5% | MET: 22.3 FOT: 1.3 PGA: 1.8 | 0.3% | MET: 22.5 FOT: 1.4 PGA: 1.9 | 0.7% | MET: 21.7 FOT: 1.5 PGA: 1.7 |
| 6. Acetic acid concentration in SPE elution buffer | 1% | MET: 22.0 FOT: 2.0 PGA: 1.9 | 0.8% | MET: 21.5 FOT: 2.0 PGA: 1.9 | 1.2% | MET: 21.6 FOT: 1.8 PGA: 2.0 |

^a Results are shown for the medium QC sample.

Abbreviations for folate vitamers in serum: MET (5-methylTHF); PGA (Folic acid); FOT (5-formylTHF), THF (tetrahydrofolate); MYT (5,10-methenylTHF).

Appendix E: 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 \rightarrow m/z 313.2) | 2.37 | 90 | 28 | 13 | 10 |
| ¹³ C ₅ -5-MethylTHF (m/z 465.2 \rightarrow m/z 313.2) | 2.37 | 90 | 28 | 13 | 10 |
| PGA (m/z 442.2 \rightarrow m/z 295.2) | 3.17 | 70 | 18 | 13 | 10 |
| ¹³ C ₅ -PGA (m/z 447.2 \rightarrow m/z 295.2) | 3.17 | 70 | 19 | 13 | 10 |
| MeFox (m/z 474.4 \rightarrow m/z 284.2) | 3.22 | 90 | 48 | 13 | 10 |
| ¹³ C ₅ -MeFox (m/z 479.4 \rightarrow m/z 284.4) | 3.22 | 90 | 48 | 13 | 10 |
| 5-FormylTHF (m/z 474.4 \rightarrow m/z 299.2) | 3.16 | 80 | 43 | 13 | 10 |
| ¹³ C ₅ -5-FormylTHF (m/z 479 \rightarrow m/z 299.2) | 3.16 | 80 | 43 | 13 | 10 |
| THF (m/z 446.2 \rightarrow m/z 299.2) | 2.33 | 85 | 25 | 13 | 10 |
| ¹³ C ₅ THF (m/z 451.1 \rightarrow m/z 299.1) | 2.33 | 85 | 25 | 13 | 10 |
| 5,10-MethenylTHF (m/z 456.1 \rightarrow m/z 412.2) | 2.30 | 155 | 41 | 13 | 10 |
| ¹³ C ₅ -5,10-MethenylTHF (m/z 461.1 \rightarrow 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, Entrance 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 °C; 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.