



## Laboratory Procedure Manual

*Analyte:* **Folate Vitamers**

*Matrix:* **Serum**

*Method:* **Liquid Chromatography Tandem Mass Spectrometry**

*Method No:* 4013.03

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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\_I:

File Name	Variable Name	SAS Label
FOLFMS_I	LBDFOTSI	Serum total folate (nmol/L)
	LBDFOT	Serum total folate (ng/mL)
	LBXSF1SI	5-Methyl-tetrahydrofolate (nmol/L)
	LBXSF2SI	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)

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## 1. Summary of Test Principle and Clinical Relevance

### A. Clinical relevance

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

Bailey *et al.* published in 2015 an indepth review of folate biology and biomarkers [1]. The primary circulating folate vitamer in serum is 5-methyltetrahydrofolate (5-methylTHF), while the actual bioactive form of folate is tetrahydrofolate (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. Red blood cells (RBCs) contain mainly 5-methylTHF polyglutamates as a storage form. In people with the 5,10-methylene-tetrahydrofolate reductase (MTHFR) C677T polymorphism mutation, a portion of the 5-methylTHF polyglutamates is replaced by formyl-folates. The measurement of folate forms circulating in serum and forms present in RBCs 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. Serum folate is an indicator of short-term status, while red blood cell (RBC) folate is an indicator of long-term status.

### B. Test principle

Five folate forms, 5-methylTHF, PGA, THF, 5-formyltetrahydrofolate (5-formylTHF), 5,10-methenyltetrahydrofolate (5,10-methenylTHF), and one oxidation product of 5-methylTHF called MeFox (pyrazino-s-triazine derivative of 4- $\alpha$ -hydroxy-5-methyltetrahydrofolate) 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 6 min using isocratic mobile phase conditions and measured by LC-MS/MS. Quantitation is based on peak area ratios interpolated against a five-point aqueous linear calibration curve using  $1/x^2$  weighting. The following analytes are quantified (Table I):

**Table I: Analytes and abbreviations**

Compound	Abbreviation	
	Scientific literature (including this document)	Database analyte code
5-Methyltetrahydrofolate	5-methylTHF	METS
5-Formyltetrahydrofolate	5-formylTHF	FOTS
Tetrahydrofolate	THF	THFS
5,10-Methenyltetrahydrofolate	5,10-methenylTHF	MYTS
Pteroylglutamic acid	PGA	PGAS
Pyrazino-s-triazine derivative of 4- $\alpha$ -hydroxy-5-methyltetrahydrofolate	MeFox	MFOS
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 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>.

## 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 “**4013.03\_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- $\mu$ L serum specimen is required to allow for repeat analysis and for automated sample pipetting; a volume of 150  $\mu$ 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  $-20^{\circ}\text{C}$ . 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-filters) deionized water with a resistance of at least 18 MΩ/cm. Use Class A volumetric glassware where a volumetric flask is specified. Though each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare larger or smaller quantities if desired.

- (1) 1% Ammonium formate buffer (Solvent #1: 1% formic acid, 0.5% ascorbic acid, 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 a week. Add 0.5% ascorbic acid (0.5 g/100 mL) at the time of use.

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, degas under vacuum, and add ascorbic acid powder to a final concentration of 0.5% (0.5 g/100 mL).

- (2) Conditioning solvents for 96-well SPE plates  
1% Ammonium formate (1% formic acid, pH 3.2) conditioning buffer for SPE is prepared as Solvent #1 above without the addition of ascorbic acid (1.3 mL of this reagent is used to condition the sorbent on 96-well SPE plates).  
Acetonitrile: 0.5 mL is used to condition sorbent on 96-well SPE plates.  
Methanol: 0.5 mL is used to condition sorbent on 96-well SPE plates.
- (3) 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 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.
- (4) 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).
- (5) L-Ascorbic acid (solid)  
0.1 g or 0.5 g portions of ascorbic acid powder are weighed into microcentrifuge vials on a calibrated balance as needed for daily use.
- (6) 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).



- (7) L-Ascorbic acid solution (1%)  
In a 50-mL falcon tube, add 0.5 g ascorbic acid and 40 mL deionized water and mix well to dissolve. Make up to final volume. Divide into two 50-mL falcon plastic tubes (25 mL in each tube). Degas each tube under a stream of nitrogen for a few minutes and filter using 0.45 µm 10-mL sterile syringe filter (Millipore) just prior to use.
- (8) 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/pipette).
- (9) 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 1. 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&F], degassed and filtered; [D], degassed

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

**5-MethylTHF, 5-FormylTHF and THF:** These reduced folates are treated the same way. The <sup>13</sup>C<sub>5</sub>-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 1**). 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  $\mu\text{mol/L}$  intermediate stock solution (see below under b).
4. Aliquot the remainder of the primary stock solution into cryovials that are stored at  $-70^{\circ}\text{C}$  and used approximately every two months to generate a fresh intermediate stock solution. The primary stock solution is stable for at least 2 years.

**5,10-MethenylTHF:** Although 5,10-methenylTHF is also a reduced folate, it is treated differently because it is only stable at acidic pH. At neutral pH it is in equilibrium with 5-formylTHF and at alkaline pH it converts to 10-formylTHF. The  $^{13}\text{C}_5$ -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 ( $\pm 0.1$  mg) of the pure solid compound in 1 M HCl, targeting a final concentration of  $\sim 100$   $\mu\text{g/mL}$  (e.g., 5 mg in 50 mL). Vortex briefly and keep the flask for  $\sim 10$  min in a beaker with warm water ( $\sim 70^{\circ}\text{C}$ ) to help dissolve the contents. Vortex a few times in between. After contents are completely dissolved, keep the volumetric flask at ambient temperature for  $\sim 30$  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 1**).
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 that are stored at  $-70^{\circ}\text{C}$  and used approximately every two months to generate a fresh intermediate stock solution. The primary stock solution is stable for at least 2 years.

**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 ( $\pm 0.1$  mg) of the pure solid compound in degassed 20 mM phosphate buffer (pH 7.2), targeting a final concentration of  $\sim 50$   $\mu\text{g/mL}$  (e.g., 5 mg in 100 mL). Vortex briefly to help dissolve the contents. Keep the volumetric flask in a luke-warm water bath ( $\sim 30^{\circ}\text{C}$ ) for  $\sim 20$  min to ensure complete solubility. Keep the volumetric flask at ambient temperature for  $\sim 20$  min to let it cool down. Make up to final volume.

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

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 x? mL/vial) that are stored at -70°C and used approximately every two months to generate a fresh intermediate stock solution. The primary stock solution is stable for at least 2 years.

**MeFox:** The <sup>13</sup>C<sub>5</sub>-labeled compound is used as internal standard and is prepared in the same way as described below.

1. Prepare a primary stock solution in a volumetric flask by dissolving an accurately known mass (±0.1 mg) of the pure solid compound in degassed 0.1 N NaOH (pH ~13), targeting a final concentration of ~100 µg/mL (e.g., 5 mg in 50 mL). Vortex briefly to help dissolve the contents and make up to final volume.

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

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

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

- (b) Intermediate stock solution II (20 µmol/L): Prepare a 20 µmol/L intermediate stock solution for each folate separately by diluting a portion of the primary stock solution in the diluent specified in Table II (typically 10 mL volume). Aliquot the intermediate stock solution into microcentrifuge 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 13C<sub>5</sub>-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 & filtered (0.45 µm sterile Millipore filters) 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 the intermediate stock II solutions.

(a) Calibrator mix (Mix A):

Contains a mixture of each standard prepared in solvent #1 as shown in **Table III**.

**Table III.** Information for calibrator mix A

<b>Mix A</b>	<b>5-MethylTHF</b>	<b>PGA</b>	<b>5-FormylTHF</b>	<b>MeFox</b>	<b>THF</b>	<b>5,10-MethenylTHF</b>
Intermediate stock II (μL)	100	50	50	50	50	50
1% Ammonium formate 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):

Contains a mixture of each internal standard prepared in solvent #1 as shown in **Table IV**.

**Table IV.** Information for internal standard mix B

<b>Mix B</b>	<sup>13</sup> C <sub>5</sub> - <b>5-MethylTHF</b>	<sup>13</sup> C <sub>5</sub> - <b>PGA</b>	<sup>13</sup> C <sub>5</sub> - <b>5-FormylTHF</b>	<sup>13</sup> C <sub>5</sub> - <b>MeFox</b>	<sup>13</sup> C <sub>5</sub> - <b>THF</b>	<sup>13</sup> C <sub>5</sub> - <b>5,10-MethenylTHF</b>
Intermediate stock II (μL)	80	20	20	20	20	20
1% Ammonium formate 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, 5-formylTHF, MeFox, THF, 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 (75<sup>th</sup> 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 2**, 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  $\mu$ 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  $\mu$ m (Phenomenex)
  - (b) 0.5  $\mu$ 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  $\mu$ L) for Eppendorf pipette (Brinkmann)
  - (f) Pipette tips (1000  $\mu$ L & 10 mL) for Eppendorf pipette (Brinkmann)
  - (g) Pipette tips (10-100  $\mu$ L) for Eppendorf pipettes (Brinkmann)
  - (h) Pipette tips (0.5-10  $\mu$ L) for Eppendorf pipette (Brinkmann)
  - (i) Combitip plus (500  $\mu$ L) for Eppendorf repeater pipette (Brinkmann)
  - (j) Positive displacement pipette tips (50  $\mu$ L, 100  $\mu$ L, 1000  $\mu$ 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  $\mu$ m PVDF filters (Millipore)
  - (o) 0.45  $\mu$ m 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 (Millipore)
  - (s) Various glass beakers, volumetric flasks (class A), graduated glass cylinders, and bottles
- (4) Folate Standards
  - (a) PGA (Pteroylglutamic acid, free acid or Na<sub>2</sub>-salt), 5-methylTHF ([6S]-5CH<sub>3</sub>-H<sub>4</sub>PteGlu, Ca-or Na<sub>2</sub>-salt), 5-formylTHF ([6S]-5CHO-H<sub>4</sub>PteGlu, Ca-or Na<sub>2</sub>-salt), MeFox ([6S](pyrazino-s-triazine derivative), THF ([6S]-H<sub>4</sub>PteGlu, free acid, Ca-or-Na<sub>2</sub>-salt) and 5,10-methenylTHF ([6S]-5,10-CH=H<sub>4</sub>PteGlu-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](http://www.merck.ch/en/company/merck_in_switzerland/merck_cie_schaffhouse/merck_cie_schaffhausen.html)
  - (b) <sup>13</sup>C<sub>5</sub>-PGA, <sup>13</sup>C<sub>5</sub>-5-methylTHF, <sup>13</sup>C<sub>5</sub>-5-formylTHF, <sup>13</sup>C<sub>5</sub>-MeFox, <sup>13</sup>C<sub>5</sub>-THF and <sup>13</sup>C<sub>5</sub>-5,10-methenylTHF (Merck & Cie)
- (5) Chemicals and Solvents
  - (a) Ammonium hydroxide (28–30% as NH<sub>3</sub>, 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 (Millipore)
- (i) Methanol, acetonitrile HPLC certified solvent (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) HP1200 HPLC systems (Agilent)  
Models G1367B HIP 96-well plate thermostatted autosamplers, ALS thermostat G1330B, G1316A thermostated 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 µL, 100 µL, 200 µL, 1000 µL, and 10 mL (Brinkmann Instruments Inc.)
- (9) Positive air displacement pipettes (Pipetman) 50 µL, 100 µL, and 1000 µL (Gilson Inc.)
- (10) Digiflex CX (ICN Biomedicals, Inc. Diagnostics Division)
- (11) Galaxy mini table top microcentrifuge (VWR Scientific Products)
- (12) Daigger Vortex Genie 2 mixer (VWR)
- (13) Magnetic stirrer (Baxter Scientific Products)
- (14) pH meter (Corning Pinnacle 530 or Accumet XL150 (pH/MV; Fisher Scientific)
- (15) Analytical Balance Model AG104 (Mettler Instrument Corp.)
- (16) Cary 3E UV/visible spectrophotometer (Varian)

## 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 ±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 “**4013.03\_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 two external proficiency testing programs twice a year: the UK NEQAS Haematinics survey, and the CAP Ligand survey. Details can be found in the proficiency testing form. For general information on the handling, analysis, review, and reporting of proficiency testing materials see “**NBB\_SOP Proficiency Testing Procedure**”.

Method figures of merit are presented in **Appendix 3** and have 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 4**.

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. 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 "4013.03\_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 µL and 1000 µL. Additionally, pipetting accuracy may be checked by using a yellow dye test.

Daily and weekly maintenance of the system is executed through the instrument operated software that checks for deck cleaning, tip waste, and 8 channel tightness. For details see, "4013.03\_SOP Automated Sample Pipetting, Maintenance, and Verification".

### **(3) Caliper-Zephyr SPE instrument**

A 96-probe automated SPE instrument is used for sample extraction and cleanup. Once per year a Perkin-Elmer service engineer performs preventive maintenance including arm adjustment and volume verification (10 µL to 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 “**4013.03\_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 $\mu$ L	150 $\mu$ L	---
2	Calibrator S0 (blank)	60 $\mu$ L mix B	---	340 $\mu$ L	150 $\mu$ L	---
3	Calibrator S1	60 $\mu$ L mix B	150 $\mu$ L S1	190 $\mu$ L	150 $\mu$ L	---
4	Calibrator S2	60 $\mu$ L mix B	150 $\mu$ L S2	190 $\mu$ L	150 $\mu$ L	---
5	Calibrator S3	60 $\mu$ L mix B	150 $\mu$ L S3	190 $\mu$ L	150 $\mu$ L	---
6	Calibrator S4	60 $\mu$ L mix B	150 $\mu$ L S4	190 $\mu$ L	150 $\mu$ L	---
7	Calibrator S5	60 $\mu$ L mix B	150 $\mu$ L S5	190 $\mu$ L	150 $\mu$ L	---
8	Low QC – Set 1	60 $\mu$ L mix B	---	340 $\mu$ L	---	150 $\mu$ L
9	Medium QC – Set 1	60 $\mu$ L mix B	---	340 $\mu$ L	---	150 $\mu$ L
10	High QC – Set 1	60 $\mu$ L mix B	---	340 $\mu$ L	---	150 $\mu$ L
11-93	Patient samples	60 $\mu$ L mix B	---	340 $\mu$ L	---	150 $\mu$ L
94	Low QC – Set 2	60 $\mu$ L mix B	---	340 $\mu$ L	---	150 $\mu$ L
95	Medium QC – Set 2	60 $\mu$ L mix B	---	340 $\mu$ L	---	150 $\mu$ L
96	High QC – Set 2	60 $\mu$ L mix B	---	340 $\mu$ L	---	150 $\mu$ L

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

- (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  $\mu$ L solvent #1, 60  $\mu$ L of internal standard mix (mix B) and 150  $\mu$ L 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 **“4013.03\_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 **“4013.03\_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  $\geq 15$  min at a flow rate of 250  $\mu\text{L}/\text{min}$ . 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 5**:
  - 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 “**4013.03\_SOP Processing and Reporting 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.
  - Print the results for each analyte as a PDF to allow future review and documentation (routine procedure) or print hardcopies (exception).
  - To process the results on another PC, copy the data file and batch file to the network.
  - Import the results file into the LIMS database for further data review.
- (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  $R^2$  is automatically generated (linear, not forced through zero, no weighting) based on area ratios.
  - The concentrations of QCs and unknowns are automatically calculated using the slope and intercept information.
  - The file is saved and maintained for documentation.
  - For studies where data is not imported into the database, we directly transfer the results from the Analyst result table into the Excel sheet for final summary and results interpretation since our results are from a weighted calibration curve ( $1/x^2$ weighted).

## H. Exporting a run

The procedure to export a run to a LIMS database is described in section 3.

## 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 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 total folate may or may not include MeFox, depending on the study and investigator request. It is still of scientific debate whether MeFox is only generated *in vitro* or may already be present *in vivo* [10]. Including MeFox into the total folate may slightly overestimate folate status, while excluding it may slightly underestimate status, however the difference between the two approaches is rather small (~5%) [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

This method is linear from the LOD (see section 7 and **Appendix 3**) 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  $2S_m$  limits and individual results are within  $2S_i$  limits, accept the run
- 2) If 1 of the 3 QC run means is outside a  $2S_m$  limit – reject run if:
  - a.  $1_{3s}$ : Any of the 3 QC results are outside the 3s limit
  - b.  $2_{2s}$ : Two of the 3 QC results in the run are outside the 2s limit (same side of mean)
  - c.  $10_x$ : Ten sequential QC results (across pools and across runs) are on the same side of the mean.
- 3) If one of the 6 QC individual results is outside a  $2S_i$  limit – reject run if:
  - a. Outlier – One individual result is beyond the characterization mean  $\pm 4S_i$  or
  - b.  $R_{4s}$ : Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean

*$S_i$  = 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.

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

- 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 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 (2.5<sup>th</sup>–97.5<sup>th</sup> percentile;  $n = 23,528$ )

In our hands, the LC/MS/MS method and the microbiologic assay give relatively comparable results. 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 [14]. While these values are of interest to researchers, they cannot be interpreted clinically. Serum total folate levels <7 nmol/L (3 ng/mL) represent a negative folate balance and are usually indicative of inadequate folate intake [15].

#### **14.Critical Call Results (“Panic Values”)**

Any NHANES samples with serum total folate 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 may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

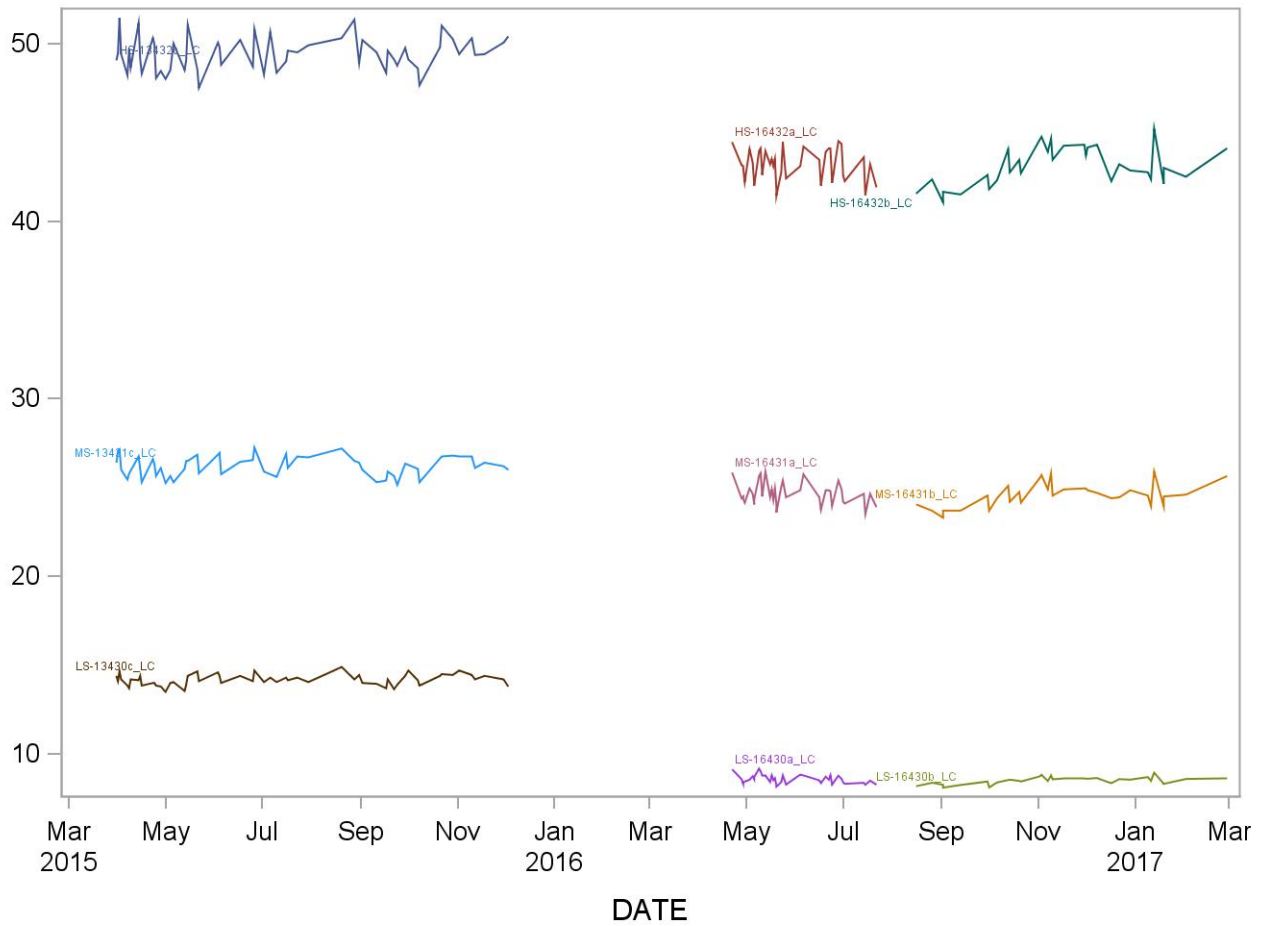
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70°C. The specimen ID is read off of the vial by a barcode reader 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 to note down the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

## **19. Summary Statistics and QC graphs**

Please see following pages

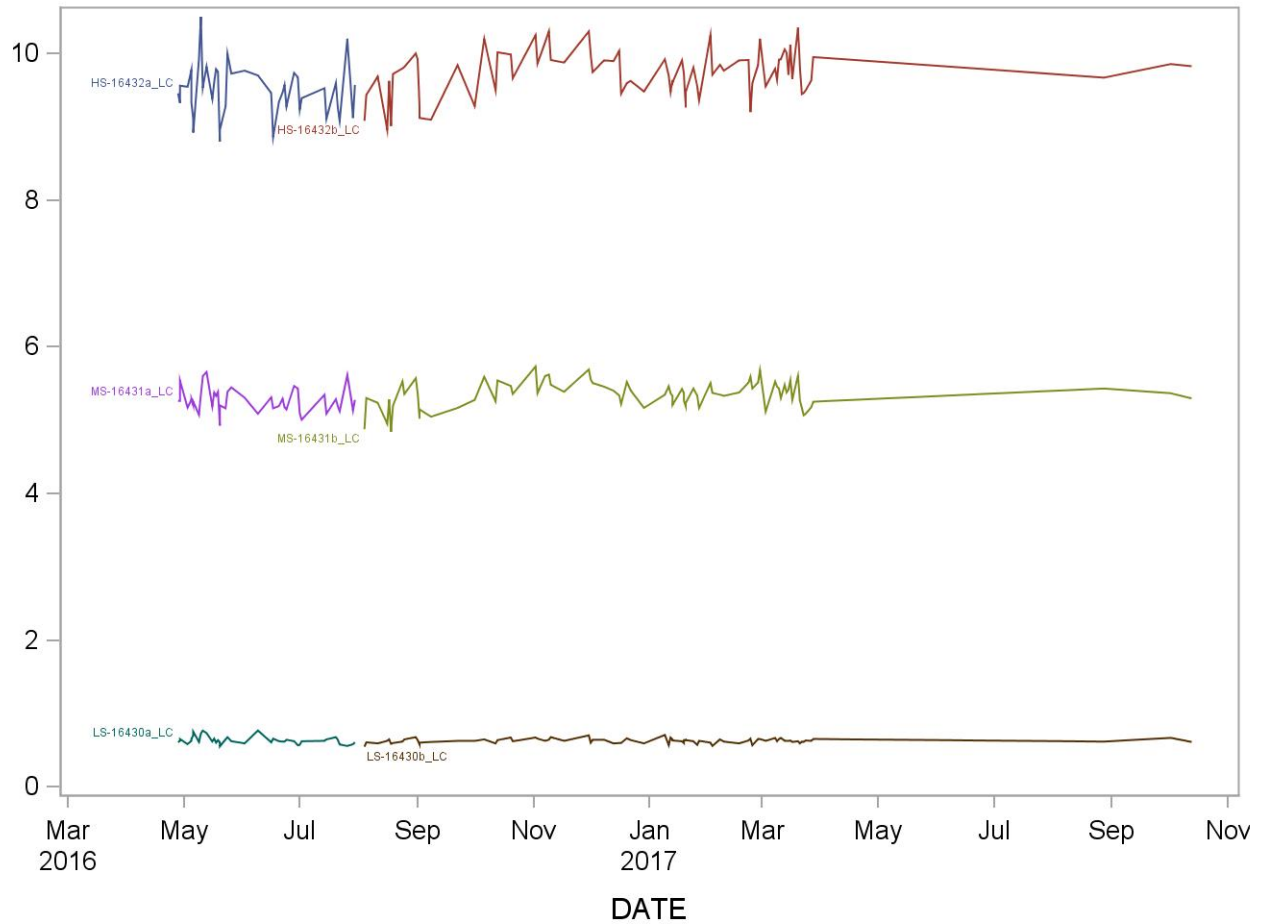
### Summary Statistics and QC Chart for 5-Methyl-tetrahydrofolate (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS-13432c_LC	57	31MAR15	03DEC15	49.4228	0.9536	1.9
LS-13430c_LC	57	31MAR15	03DEC15	14.1728	0.3130	2.2
MS-13431c_LC	55	31MAR15	03DEC15	26.2145	0.5677	2.2
HS-16432a_LC	38	22APR16	22JUL16	43.1250	0.9164	2.1
LS-16430a_LC	38	22APR16	22JUL16	8.5775	0.2520	2.9
MS-16431a_LC	38	22APR16	22JUL16	24.6513	0.6287	2.6
HS-16432b_LC	32	16AUG16	28FEB17	43.1281	1.1036	2.6
LS-16430b_LC	32	16AUG16	28FEB17	8.5072	0.2010	2.4
MS-16431b_LC	32	16AUG16	28FEB17	24.5922	0.6656	2.7



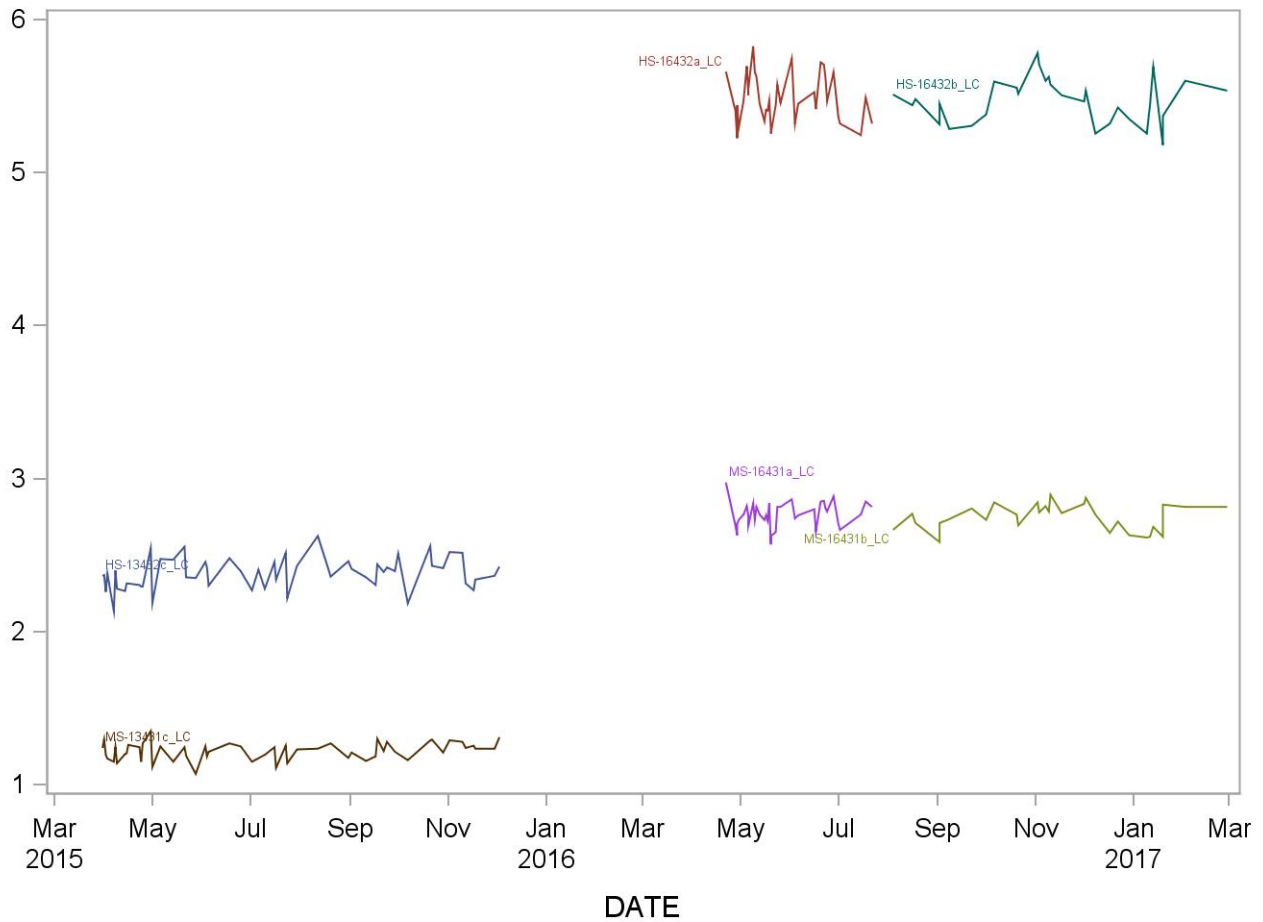
### Summary Statistics and QC Chart for Folic acid (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS-16432a_LC	41	28APR16	30JUL16	9.4879	0.3546	3.7
LS-16430a_LC	41	28APR16	30JUL16	0.6340	0.0536	8.5
MS-16431a_LC	41	28APR16	30JUL16	5.2683	0.1629	3.1
HS-16432b_LC	80	04AUG16	13OCT17	9.7323	0.3096	3.2
LS-16430b_LC	80	04AUG16	13OCT17	0.6246	0.0313	5.0
MS-16431b_LC	80	04AUG16	13OCT17	5.3519	0.1814	3.4



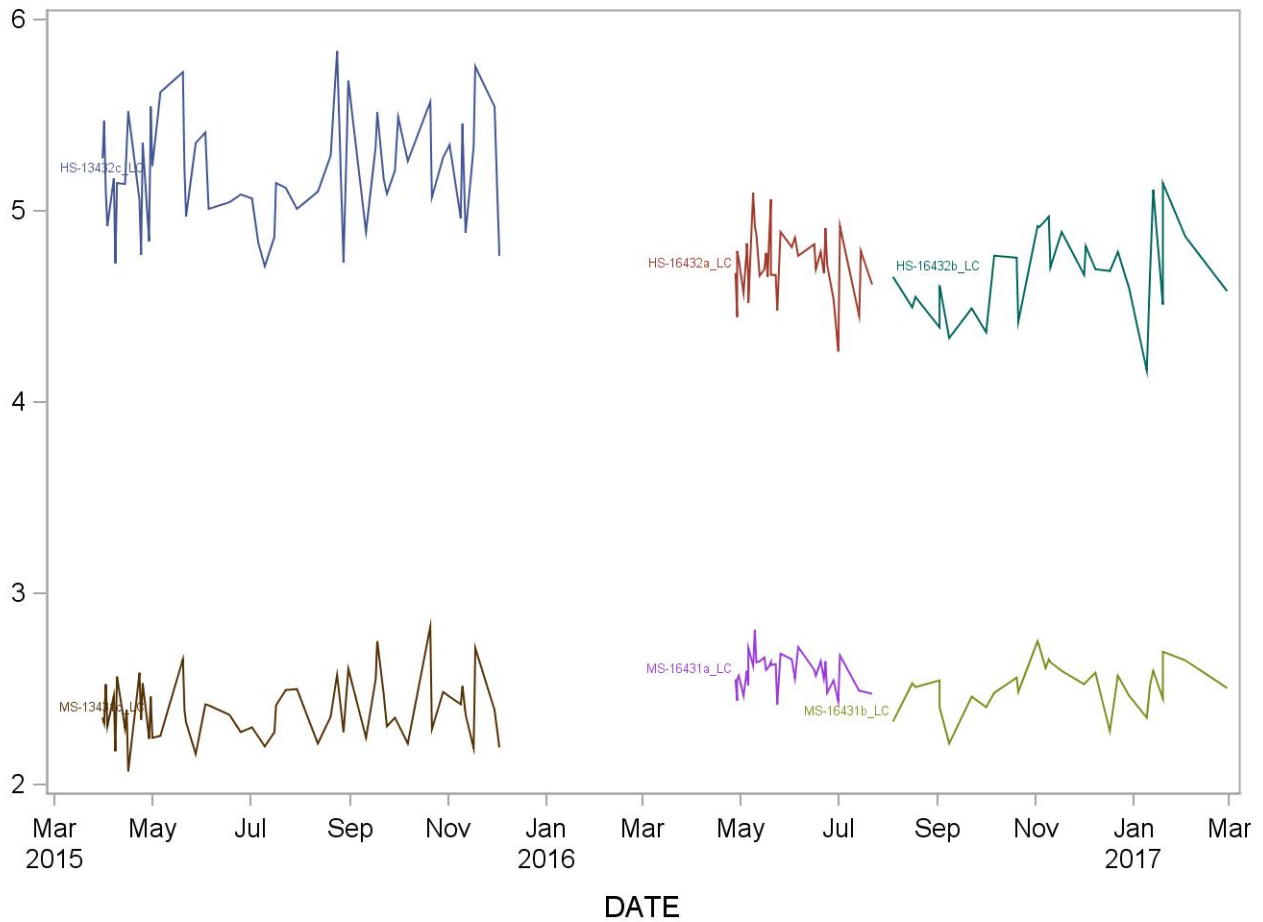
### Summary Statistics and QC Chart for 5-Formyl-tetrahydrofolate (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS-13432c_LC	55	31MAR15	03DEC15	2.3798	0.1027	4.3
MS-13431c_LC	53	31MAR15	03DEC15	1.2203	0.0578	4.7
HS-16432a_LC	37	22APR16	22JUL16	5.4808	0.1561	2.8
MS-16431a_LC	37	22APR16	22JUL16	2.7634	0.0839	3.0
HS-16432b_LC	30	04AUG16	28FEB17	5.4682	0.1469	2.7
MS-16431b_LC	30	04AUG16	28FEB17	2.7462	0.0854	3.1



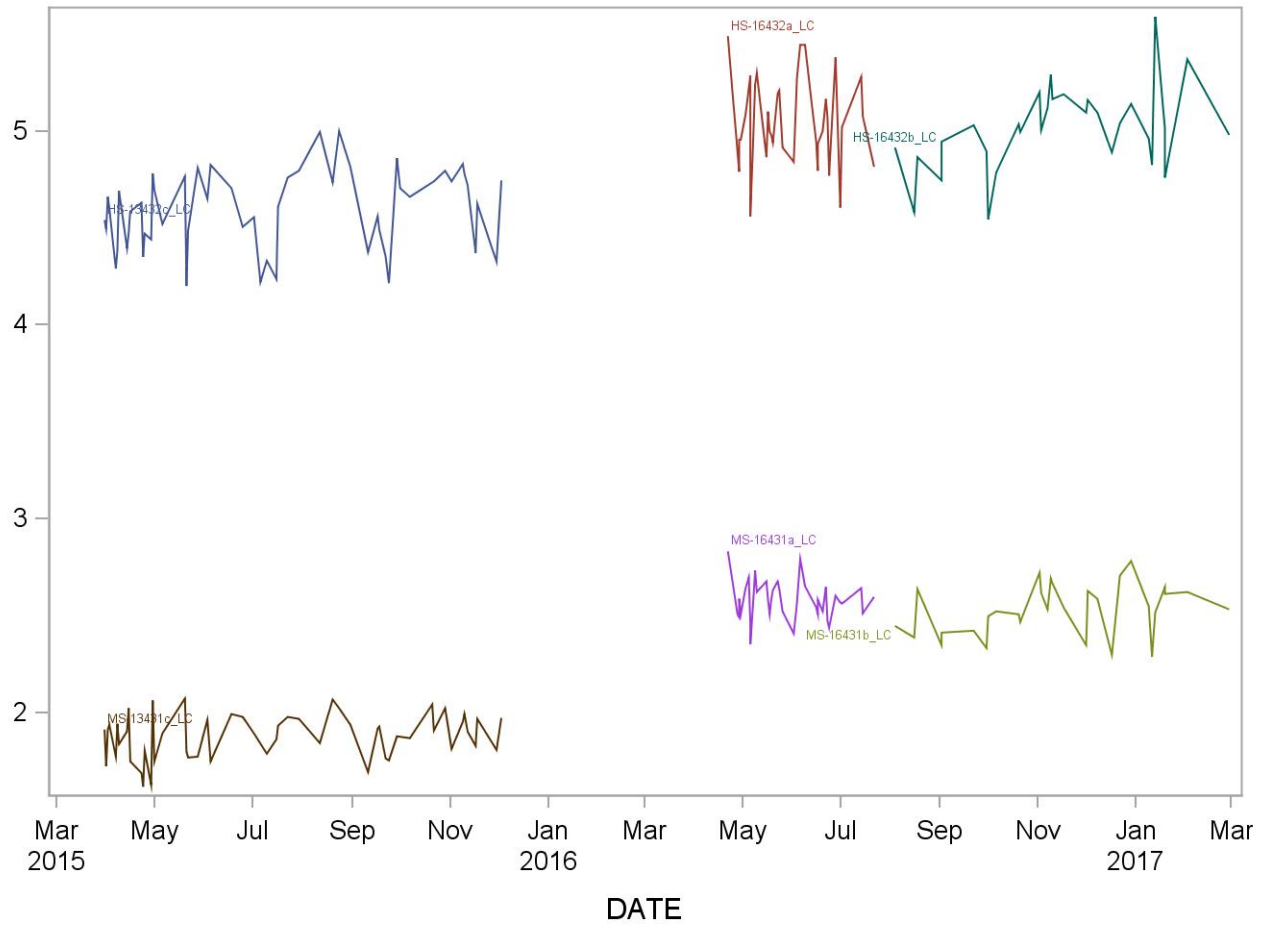
### Summary Statistics and QC Chart for Tetrahydrofolate (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS-13432c_LC	56	31MAR15	03DEC15	5.2024	0.2836	5.5
MS-13431c_LC	54	31MAR15	03DEC15	2.3902	0.1589	6.6
HS-16432a_LC	37	28APR16	22JUL16	4.7280	0.1722	3.6
MS-16431a_LC	37	28APR16	22JUL16	2.5873	0.0875	3.4
HS-16432b_LC	30	04AUG16	28FEB17	4.6822	0.2324	5.0
MS-16431b_LC	30	04AUG16	28FEB17	2.5208	0.1260	5.0



### Summary Statistics and QC Chart for 5,10-Methenyl-tetrahydrofolate (nmol/L)

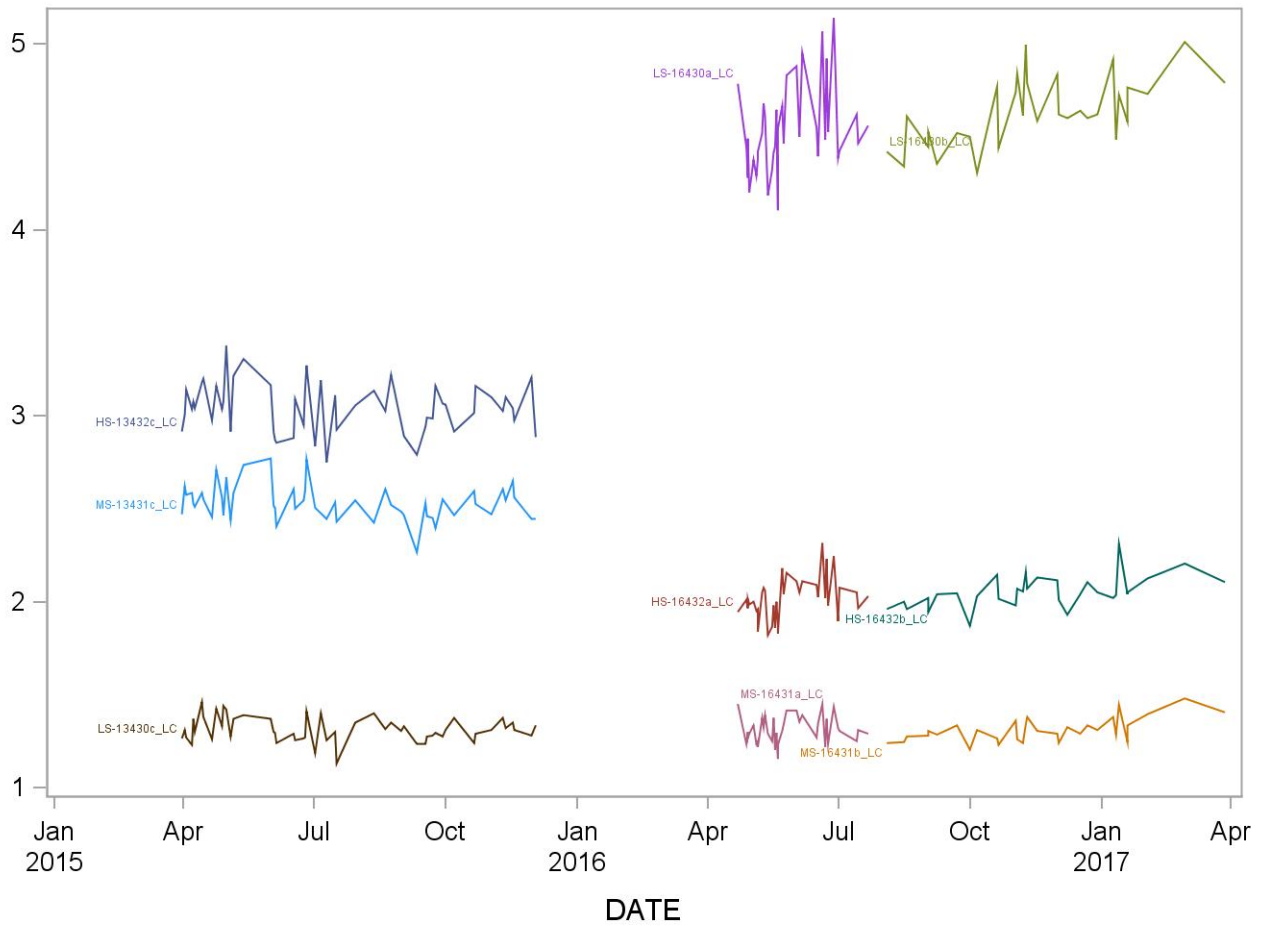
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS-13432c_LC	55	31MAR15	03DEC15	4.5930	0.2016	4.4
MS-13431c_LC	53	31MAR15	03DEC15	1.8755	0.1143	6.1
HS-16432a_LC	36	22APR16	22JUL16	5.0508	0.2306	4.6
MS-16431a_LC	36	22APR16	22JUL16	2.5772	0.1016	3.9
HS-16432b_LC	30	04AUG16	28FEB17	5.0075	0.2188	4.4
MS-16431b_LC	30	04AUG16	28FEB17	2.5267	0.1334	5.3





### Summary Statistics and QC Chart for Mefox oxidation product (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS-13432c_LC	53	31MAR15	03DEC15	3.0431	0.1343	4.4
LS-13430c_LC	53	31MAR15	03DEC15	1.3132	0.0659	5.0
MS-13431c_LC	51	31MAR15	03DEC15	2.5325	0.0965	3.8
HS-16432a_LC	37	22APR16	22JUL16	2.0174	0.1154	5.7
LS-16430a_LC	37	22APR16	22JUL16	4.5397	0.2386	5.3
MS-16431a_LC	37	22APR16	22JUL16	1.3105	0.0740	5.6
HS-16432b_LC	31	04AUG16	28MAR17	2.0527	0.0861	4.2
LS-16430b_LC	31	04AUG16	28MAR17	4.6356	0.1835	4.0
MS-16431b_LC	31	04AUG16	28MAR17	1.3103	0.0653	5.0



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## **ACKNOWLEDGMENTS**

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

## Appendix 1 - 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
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

### References for UV-spectrophotometric determination of folate concentration:

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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.

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

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

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

with  $\epsilon \text{ max}$  = molar extinction coefficient and conc = concentration

Example calculation for folic acid (PGA):

Absorbance	Dilution	$\epsilon \text{ max}$	MW
0.661	10	27600	441.4

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

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

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

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

**Appendix 2 - Approximate QC pool target concentrations (nmol/L) for the various folate vitamers and the 5-methylTHF oxidation product (MeFox)**

<b>QC level</b>	<b>5-MethylTHF</b>	<b>5-FormylTHF</b>	<b>PGA</b>	<b>THF</b>	<b>5,10-MethenylTHF</b>	<b>MeFox</b>	<b>TFOL</b>
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 3 - Method Figures of Merit**

### **Accuracy**

Results of in-house recovery studies based on area ratios (analyte/IS) of spiked serum showed complete (100% ± 10%) recovery for all folate analytes [2]:

- 5-methylTHF: 99.4% ± 17%
- 5-formylTHF: 92% ± 10%
- PGA: 100% ± 8.8%
- THF: 99.5% ± 6.8%
- 5,10-methenylTHF: 108% ± 14%
- MeFox: 100% ± 7.9%.

In-house SPE efficiency experiments (independent of the IS) showed the following mean ± SD recovery [2]:

- 5-methylTHF: 87% ± 9%
- 5-formylTHF: 94% ± 8.5%
- PGA: 89.5% ± 10%
- THF: 78% ± 6.3%
- 5,10-methenylTHF: 88% ± 10.5%
- MeFox: 85% ± 9.7%.

In these experiments, standards were added to serum at different levels (1.0, 2.0, 4.0, 20 and 100 nmol/L for 5-methylTHF, and 0.5, 1.0, 2.0, 10 and 50 nmol/L for other folate forms).

### **Precision**

Representative information on method precision for serum folate showing the mean concentration (nmol/L) and the inter-assay CV (%) from 21 runs performed with the 96-probe SPE method on the AB 6500

QC level	Estimate	5-MethylTHF	PGA	5-FormylTHF	THF	5,10-MethenylTHF	MeFox
Low	Conc.	19.9	0.64				1.54
	CV	3.5%	6.5%				4.4%
Medium	Conc.	36.6	5.52	0.63	1.32	1.60	1.65
	CV	3.9%	8.8%	6.5%	8.4%	6.3%	4.8%
High	Conc.	52.9	10.6	2.41	4.39	4.95	3.15
	CV	3.4%	9.6%	5.3%	5.6%	5.0%	5.3%

### **Limit of detection**

Determination of the limit of detection (LOD) was conducted with the 96-probe SPE method on the AB 6500 by serially diluting a serum QC pool with 0.1% ascorbic acid and by estimating the SD at a concentration of zero ( $\sigma_0$ ) by extrapolating repeat analyte measurements made near the detection limit in these dilutions (LOD defined as  $3\sigma_0$ ). The calculated method LOD values and corresponding method LOQ values (representing a signal to noise of 10) are shown in the table below.

Analyte	LOD (nmol/L)	LOQ (nmol/L)
5-MethylTHF	0.13	0.43
PGA	0.14	0.46
5-FormylTHF	0.20	0.66
THF	0.25	0.83
5,10-MethenylTHF	0.20	0.66
MeFox	0.10	0.33

Note: Determination of LODs in matrix (4% albumin) gave similar values as in non-matrix (aqueous) [2].

## **Appendix 4 – 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 21 of the 2008 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.

### **Variations in sample preparation**

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



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

**Table: Ruggedness testing for serum folate vitamers by LC-MS/MS**

Factor	Method specifics	Results <sup>a</sup> (nmol/L)	Lower level	Results <sup>a</sup> (nmol/L)	Higher level	Results <sup>a</sup> (nmol/L)
1. pH of ammonium formate buffer (sample solvent #1)	3.2	METS: 22.3 FOTS: 1.8 PGAS: 1.8	3.0	METS: 22.5 FOTS: 2.1 PGAS: 1.8	3.4	METS: 22.2 FOTS: 1.8 PGAS: 1.9
2. Formic acid concentration in ammonium formate buffer (sample solvent #1)	1%	METS: 22.7 FOTS: 2.1 PGAS: 2.0	0.8%	METS: 21.6 FOTS: 2.1 PGAS: 1.9	1.2%	METS: 21.5 FOTS: 2.1 PGAS: 1.9
3. Ascorbic acid in ammonium formate buffer (sample solvent #1)	0.5%	METS: 22.4 FOTS: 1.6 PGAS: 1.9	0.3%	METS: 22.3 FOTS: 1.6 PGAS: 2.1	0.7%	METS: 23.1 FOTS: 1.6 PGAS: 2.2
4. Ammonium formate concentration in SPE wash buffer	0.05%	METS: 22.2 FOTS: 2.1 PGAS: 2.0	0.04%	METS: 22.0 FOTS: 2.3 PGAS: 2.0	0.06%	METS: 21.7 FOTS: 2.0 PGAS: 2.0
5. Ascorbic acid concentration in SPE elution buffer	0.5%	METS: 22.3 FOTS: 1.3 PGAS: 1.8	0.3%	METS: 22.5 FOTS: 1.4 PGAS: 1.9	0.7%	METS: 21.7 FOTS: 1.5 PGAS: 1.7
6. Acetic acid concentration in SPE elution buffer	1%	METS: 22.0 FOTS: 2.0 PGAS: 1.9	0.8%	METS: 21.5 FOTS: 2.0 PGAS: 1.9	1.2%	METS: 21.6 FOTS: 1.8 PGAS: 2.0

<sup>a</sup> Results are shown for the medium QC sample.

Abbreviations for folate vitamers in serum: METS (5-methylTHF); PGAS (Folic acid); FOTS (5-formylTHF), THFS (tetrahydrofolate); MYT (5,10-methenylTHF)

## Appendix 5 - 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
$^{13}\text{C}_5$ -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
$^{13}\text{C}_5$ -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
$^{13}\text{C}_5$ -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
$^{13}\text{C}_5$ -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
$^{13}\text{C}_5$ 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
$^{13}\text{C}_5$ -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.