Laboratory Procedure Manual

Analyte: **Folate Vitamers**

Matrix: **Serum**

Method: **Liquid Chromatography Tandem Mass Spectrometry**

Method No: 4012.01

Revised:

as performed by:

Nutritional Biomarkers Branch
Division of Laboratory Sciences
National Center for Environmental Health

contact:

James L. Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

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_G:

Variable Name	SAS Label
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)

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.

The primary circulating folate vitamer in serum is 5-methyltetrahydrofolate (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. 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-methenyl-tetrahydrofolate (5,10-methenylTHF), and an oxidation product of 5-methylTHF called MeFox (pyrazino-s-triazine derivative of 4-α-hydroxy-5-methyltetrahydrofolate) are measured by isotope-dilution high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [1-3]. The assay is performed by combining specimen (275 µL serum or whole blood hemolysate) with ammonium formate buffer and an internal standard mixture. Hemolysate samples have to be incubated for 4 h at 37°C to deconjugate folate polyglutamates prior to folate extraction [3]. Sample extraction and clean-up is performed by automated solid phase extraction (SPE) using 96-well phenyl SPE plates and takes ~5 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 calibration curve. The following analytes are quantified:

Compound	Abbreviation		
	Scientific literature (including this document)	Database serum analyte code	Database WB analyte code
5-Methyltetrahydrofolate	5-methylTHF	METS	METB
5-Formyltetrahydrofolate	5-formylTHF	FOTS	FOTB
Tetrahydrofolate	THF	THFS	THFB
5,10-Methenyltetrahydrofolate	5,10-methenylTHF	MYTS	MYTB
Pteroylglutamic acid	PGA	PGAS	PGAB
Pyrazino-s-triazine derivative of 4-α-hydroxy-5-methyltetrahydrofolate	MeFox	MFOS	MFOB
Total folate (sum of folate forms)	TFOL	FOL2	WBF2

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.

> *Formic acid and acetic acid: Handle with extreme care as these acids are caustic and toxic; avoid contact with skin and eyes.*
>
> *Organic solvents: Handle only in well-ventilated areas or as required under a fume hood.*
>
> *Ammonium hydroxide: Used to make ammonium formate buffer and produces strong fumes. Prepare only in chemical fume hood.*

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDSs) for all chemicals are readily available in the MSDS section as hard copies in the laboratory. MSDSs for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs.

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 via USB flash drive to the 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 "**4012_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 person. 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. The addition of 0.5% ascorbic acid to serum prior to storage improves folate stability. Specimens for red blood cell (RBC) folate analysis are frozen whole blood hemolysate (100 μL EDTA whole blood added to 1.0 mL of 1 g/dL ascorbic acid, corresponding to 1/11 dilution, frozen promptly, which keeps the folate in the reduced state).

C. A 750-μL serum specimen is preferable to allow for repeat analyses; a volume of 275 μL is required for analysis. A 1.1-mL whole blood hemolysate specimen is preferable to allow for repeat analysis; a volume of 275 μL is required for analysis.

D. Serum specimens may be collected with regular red-top Vacutainers. Whole blood is collected with lavender-top Vacutainers containing EDTA as an anticoagulant. A hematocrit measurement used for the RBC folate calculations is made at the time of collection. The appropriate amount of serum or whole blood hemolysate is dispensed into a Nalgene cryovial or other plastic screw-capped vial labeled with the participant's ID.

E. Specimens collected in the field should be kept cold and protected from light. After processing, specimens should be frozen and shipped on dry ice by overnight mail. Once received, they should be stored at ≤-20°C until analyzed. Folates are stable for only a few weeks if the specimen is frozen at -20°C. For long-term storage, specimens should always be frozen at -70°C. Multiple freeze-thaw cycles will cause folate degradation. Undiluted whole blood is particularly sensitive to folate degradation during thawing and exposure to room temperature.

F. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was collected. Previously published methods recommended 90-min incubation for complete deconjugation of folate polyglutamates to monoglutamates prior to the RBC folate analysis [6]. However, we have shown that whole blood hemolysates prepared with 1% ascorbic acid need to be incubated for 4 h 37°C to convert all folate polyglutamates to monoglutamates if HPLC-based methods are used [3]. No incubation is required for folate analysis by microbiologic assay. 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 analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood, serum or plasma should be transferred into a sterile Nalge cryovial labeled with the participant's ID.

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 using deionized water with a resistance of at least 15 MΩ/cm and filter water before use, using 0.45 µm nylon filters.

(1) 1% Ammonium formate buffer (Solvent #1: 1% formic acid, 0.5% ascorbic acid, pH 3.2)
In a 1 L reagent bottle add 980 mL deionized water and 10 mL concentrated formic acid. Titrate this mixture with ammonium hydroxide (30%) to adjust pH to 3.2. Transfer the buffer in to a 1 L measuring glass cylinder and make up the final volume to 1 L with deionized water. Transfer back into 1L reagent bottle, 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 only.

Note: 10x concentrated ammonium formate buffer can be prepared as above (use concentrated formic acid 100 mL instead of 10 mL). 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 SPE cartridges or 96-well plates
1% Ammonium formate (1% formic acid, pH 3.2) conditioning buffer for SPE cartridges is prepared as Solvent #1 above without the addition of ascorbic acid.
Acetronitrile (1 mL is used to condition cartridges or sorbent on 96-well plates).
Methanol (1 mL is used to condition cartridges or sorbent on 96-well 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 measuring glass cylinder. Transfer into 1L reagent bottle check pH & degas under vacuum for 3-5 min. This buffer can be stored at room temperature for maximum one week. Add ascorbic acid powder to a final concentration of 0.1% (0.1 g/100 mL) just before use.

(4) Sample elution buffer (Solvent #3: 49% deionized water, 40% methanol, 10% acetonitrile, 1% acetic acid, 0.5% ascorbic acid)
Using measuring glass cylinders (0.5L and 100mL) add 490 mL of deionized water, 400 mL of methanol, and 100 mL of acetonitrile into a 1L reagent bottle degas under vacuum for 3-5 min. At the time of use add 10 mL of acetic acid (1% final concentration) and ascorbic acid powder to a final concentration of 0.5% (0.5 g/100 mL).

(5) Reservoir solvent for Gilson 215 automated SPE system (40% methanol)
Using a 1L measuring glass cylinder add 600 ml of deionized water and 400 mL of methanol into a 1L reagent bottle degas under vacuum for 3-5 min. This solvent can be stored at room temperature and replaced as needed. This solvent is used to purge the solvent lines of 215 Gilson system before, during and after sample clean-up.

(6) L-Ascorbic acid (solid)
0.1g or 0.5 g portions of ascorbic acid powder are weighed into microcentrifuge vials on a calibrated balance as needed for daily use.

(7) HPLC mobile phase (49.5% deionized water, 40% methanol, 10% acetonitrile, 0.5% acetic acid)
Using measuring glass cylinders (0.5L and 100mL) add 495 mL of deionized water, 400 mL of methanol, and 100 mL of acetonitrile into a 1L reagent bottle degas under vacuum for 3-5 min. At the time of use add 5 mL of acetic acid (0.5% final concentration).

(8) L-Ascorbic acid solution (1%)
In a 50 mL volumetric flask, add 0.5 g ascorbic acid and 40 mL deionized water and mix well to dissolve. Make up final volume to 50 mL. Filter using 0.45 µm 10 mL syringe filter (Millipore) into two 50 mL falcon plastic vials (25 mL in each vial). Degas each vial under a stream of nitrogen for 5 min just before use.

(9) Hemolysate buffer (1% ascorbic acid, pH 2.7)
In a 100 mL volumetric flask, add 1.0 g of ascorbic acid to ~90 mL deionized water and mix well (pH 2.7) & make final volume up to 100 mL cap tightly until needed. This buffer should be made fresh always before use. To prepare hemolysates add 100 uL of WB into 1.0 mL of this buffer, vortex mix well before storage at appropriate temperature.

(10) PPG dilution solvent for mass spectrometer calibration
Dissolve 15.4 milligrams 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.

B. Standards Preparation

(1) Individual stock and intermediate solutions

The concentrations of individual folate stock solutions are calculated using molar absorptivity. Information on absorption maxima, absorption coefficients, and formulas to calculate the concentration for each folate stock solution are provided in **Appendix 1.**

(a) Stock solution I (~200 µg/mL):

PGA : Prepare a stock solution by dissolving ~5 mg of the respective compound in degassed 20 mM phosphate buffer (pH 7.2) in a 25-mL volumetric flask. Vortex briefly and add a few drops (10-20 uL) of 30% ammonium hydroxide to help dissolve the salt. Make volume up to 25 mL. A small aliquot (1 mL) of this stock solution is taken in a microcentrifuge vial to determine the concentration by UV spectrophotometry. Prepare a 1/20 dilution of the above aliquot with phosphate buffer and record absorbance at 282 nm and 345 nm for PGA gainst phosphate buffer as a blank on a UV/VIS spectrophotometer using scan analysis. The $^{13}C_5$-labeled compounds are used as internal standards and are prepared the same way.

MeFox: Prepare a stock solution by dissolving ~5 mg of the respective compound in degassed 0.1N NaOH (12.4) in a 25-mL volumetric flask. Vortex mix briefly to help dissolve the salt and make volume up to 25 mL. A small aliquot (1 mL) of this stock solution is taken in a microcentrifuge vial to determine the concentration by UV spectrophotometry. Prepare a 1/20 dilution of the above aliquot with 0.1N NaOH and record absorbance at 280 nm against 0.1N NaOH as a blank on a UV/VIS spectrophotometer using scan analysis. The $^{13}C_5$-labeled compounds are used as internal standards and are prepared the same way.

5-MethylTHF, 5-FormylTHF and THF: These reduced folates are treated the same way. Prepare a stock solution by dissolving ~5 mg of a reduced folate in degassed 20 mM phosphate buffer (pH 7.2) containing 0.1% cysteine in a 25-mL volumetric flask. Vortex briefly and make volume up to 25 mL. A small aliquot (1 mL) of this stock solution is placed in a microcentrifuge vial to determine the concentration by UV spectrophotometry. Add to the remaining stock solution ascorbic acid powder to a final concentration of ~1% (0.25 g). Prepare a 1/20 dilution of the 1-mL aliquot with phosphate buffer and record the absorbance at the following wavelengths against phosphate buffer as a blank on a UV/VIS spectrophotometer using scan analysis: 5-methylTHF 290 nm and 245 nm; 5-formylTHF 285 nm; THF 298 nm. For 5-methylTHF, the ratio of absorbance at 290/245 nm is also monitored to ensure that no oxidation took place. This ratio should exceed 3.3. The $^{13}C_5$-labeled compounds are used as internal standards and are prepared the same way.

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. Prepare a stock solution by dissolving ~5 mg 5,10-methenylTHF in 1 M HCl in a 25-mL volumetric flask. Vortex briefly and keep the flask in a beaker with warm water to help dissolve the salt. Make the volume up to 25 mL. A small aliquot (1 mL) of this stock solution is placed in a microcentrifuge vial to determine the concentration by UV spectrophotometry. Add to the remaining stock solution ascorbic acid powder to a final concentration of 1% (0.25 g). Prepare a 1/20 dilution of the 1-mL aliquot with 1 M HCl and record the absorbance at 288 nm and 348 nm against 1 M HCl as a blank on a UV/VIS spectrophotometer using scan analysis. The $^{13}C_5$-labeled compounds are used as internal standards and are prepared the same way.

(b) Stock solution II (100 µg/mL): Based on the actual concentration of stock solution I, the solution is diluted in a 25-mL volumetric flask to yield a 100 µg/mL stock solution II using the diluent specified in the table below. Aliquots of this stock solution II (1.2 mL) are stored at -70°C in 2-mL labeled cryovials.

(c) Stock solution III (20 µmol/L): Thaw one vial of stock solution II approximately once in two months and dilute in a 10-mL volumetric flask approximately 1/10 (depending on the MW) to yield a 20 µmol/L stock solution III using the diluent specified in the table below. Aliquot 500-µL portions into 25 microcentrifuge tubes and store at -70°C. This stock solution III is used as a working standard for daily folate analysis.

Compound	Diluent for stock solution II	Diluent for stock solution III
5-MethylTHF	Degassed 1% ascorbic acid	Degassed 0.5% ascorbic acid
5-FormylTHF	Degassed 1% ascorbic acid	Degassed 0.5% ascorbic acid
THF	Degassed 1% ascorbic acid	Degassed 1% ascorbic acid
5,10-MethenylTHF	Degassed 0.5 M HCl containing 1% ascorbic acid	Degassed 0.5 M HCl containing 0.5% ascorbic acid
PGA	Degassed deionized water	Degassed 0.1% ascorbic acid
MeFox	Degassed deionized water	Degassed 0.1% ascorbic acid

> **Note:**
> Fresh individual stock solutions I are prepared approximately every 2 years. Individual stock solutions II are prepared at the same time as stock solutions I. Individual stock solutions III are prepared approximately every 2 months. Analyte and internal standard stock solutions are always prepared together. Our in-house long-term storage stability data has shown that the analyte and internal standard stock solutions II (1% ascorbic acid) and III (0.1% ascorbic acid) are stable for at least 9 years when stored at -70°C.

(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 stock III standards solutions.

(a) Calibrator mix (Mix A):

Contains a mixture of each calibrator prepared in 0.1% ascorbic acid as shown below.

	5-MethylTHF	PGA	5-FormylTHF	MeFox	THF	5,10-MethenylTHF
Stock III (µL)	100	50	50	50	50	50
Ascorbic acid (µL)				650		
Concentration in mix A (µmol/L)	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 0.1% ascorbic acid as shown below.

	$^{13}C_5$-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
Stock III (µL)	100	25	25	25	25	25
Ascorbic acid (mL)				9.775		
Concentration in mix B (nmol/L)	200	50	50	50	50	50

(3) Calibration standards:

Prepare mixed calibrators S1-S5 for the calibration curve in 1.5-mL microcentrifuge tubes: add 50 µL mix A to 950 µL of 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, 5,10-methenylTHF, and THF). Prepare calibrators S4-S1 from calibrator S5 by using the amounts specified in the table below.

Calibrator level	Calibrator mix	Solvent #1	Concentration (nmol/L): 5-methylTHF/PGA/5-formylTHF/MeFox/5,10-methenylTHF/THF
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); for RBC TFOL target values are 300, 600, and 1,000 nmol/L, respectively. The low QC aims to be close to the deficiency cutoff value of 7 (serum) and 305 (RBC) nmol/L. The high QC aims to be in the top third of the population distribution (75th percentile is ~50 [serum] and ~1,400 [RBC] nmol/L). Because we have mandatory food fortification with folic acid in the US, it is difficult to find donors with low serum and RBC folate levels and the prevalence of 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 BioRad protein diluent or physiologic sodium chloride solution is considered. It is advisable to including a few blood donors with MTHFR T/T genotype to obtain whole blood that has endogenous levels of THF and 5,10-methenylTHF. Not all folate forms have to be low in the "low" pool, medium in the "medium" pool, and high in the "high" pool, just as long as there is sufficient distinction between the pools.

The serum is pooled and pools are filtered through gauze before being dispensed to remove fibrin. Serum (usually 800 µL) is aliquoted into 2.0-mL Nalge cryovials, capped, and frozen. The QC pools are stored at -70°C and are stable for at least 3 years. More recently, we started adding 0.5% ascorbic acid to the serum pools to ensure even better long-term folate stability.

To generate whole blood QC pools, fresh EDTA whole blood (~40 mL) is collected from blood donors. The Vacutainers are rocked for 5-10 min at room temperature and the whole blood is diluted with 1% ascorbic acid to achieve a 1:11 dilution. Whole blood hemolysate (usually 800 µL) is aliquoted into 2.0-mL Nalge cryovials, capped, and frozen. The QC pools are stored at -70°C and are stable for at least 3 years.

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

D. Other Materials

(1) Automated sample dilution on Hamilton liquid handler
 (a) 96-well sample collection plate (Whatman)
 (b) 96-well collection plate seals (Whatman)
 (c) 1 mL and 0.2 mL plastic pipet tips (Hamilton)
 (d) 300 mL plastic reusable reagent and water troughs (Hamilton)

(2) Automated solid phase extraction (SPE) on Gilson 215
 (a) 96-well Versaplate base that uses 1 mL adjustable cartridges - (Varian, Harbor City, CA/Agilent, Lake Forest, CA)
 (b) 1 mL cartridges for Versaplate -100 mg phenyl sorbent (Varian, Harbor City, CA/Agilent, Lake Forest, CA)
 (c) 96-well BondElut SPE block- 100 mg phenyl sorbent (Varian, Harbor City, CA/Agilent, Lake Forest, CA)
 (d) Captiva 96-well filter plates (0.45 µm PVDF embedded into the well [Varian, Harbor City, CA/Agilent Lake Forest, CA) for efficient automated filtration with vacuum manifold (IST Vacmaster-VCU)

(3) Manual SPE
 (a) Phenyl solid phase extraction cartridges 100 mg bed, 1 mL capacity (Varian, harbor City,CA)
 (b) Disposable teflon valve liners (Supelco, Bellefonte, PA)
 (c) 12-port manual vacuum manifold (Supelco)
 (d) 12x75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY)
 (e) 5.75" disposable glass Pasteur pipettes (Kimble, Toledo, OH)

(4) Other Items
 (a) C-8(2) analytical HPLC column, 150x3.2 mm, 5 µm (Phenomenex, Torrance, CA)
 (b) 0.5 µm stainless frits A-102X (Chromtech, Apple Valley, MN)
 (c) PEEK tubing 0.005 and 0.007 ID (Supelco)
 (d) HPLC Solvent glass inlet filters, purge frits, gold seal and outlet caps (Agilent, Lake forest, CA)
 (e) Blue tips (100-1000 µL) for Eppendorf pipette (Brinkmann)
 (f) White tips (1000 µL) for Eppendorf pipette (Brinkmann)
 (g) Yellow tips (10-100 µL) for Eppendorf pipettes (Brinkmann)
 (h) Yellow tips (0.5-10 µL) for Eppendorf pipette (Brinkmann)
 (i) Combitip plus (500 µL) for Eppendorf repeater pipette (Brinkmann)
 (j) Positive displacement pipette tips (50 µL , 100 µL, 1000 µL) for Gilson pipette (Gilson)
 (k) 30 mm Nunc 1-mL 96-well HPLC collection plate for 96-well autosampler (Fischer Scientific)
 (l) Nunc plastic seals for 30 mm 1 mL 96-well plates (Fischer Scientific)
 (m) 12x32-mm glass autosampler vials and 200 µL glass inserts (Kimble)
 (n) HPLC solvent filter degasser, model FG-256 (Lazar Research Laboratories, Inc., Los Angeles, CA)
 (o) 0.45 µm PVDF filters (Millipore, Bedford, MA)
 (p) 0.45 µm water filtration units 500 mL capacity (Nalgene)
 (q) 2.0 mL polypropylene cryovials (Nalgene Company, Rochester, NY)
 (r) 1.0 mL disposable syringes (Hamilton)
 (s) Syringe filters (Millipore)

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

(5) Folate Standards

(a) PGA (Pteroylglutamic acid, free acid or Na_2-salt), 5-methylTHF([6S]-5CH-3-H_4PteGlu, Ca- or Na2-salt), 5-formylTHF([6S]-5CHO- H_4PteGlu, Ca-or Na_2-salt), MeFox ([6S](pyrazino-s-triazine derivative), 5,10-methenylTHF([6S]-5-10-CH-H_4PteGlu-Cl x HCl salt), and THF([6S]- H_4PteGlu, free acid, Ca-or-Na_2-salt) (Merck & Cie [formerly Eprova]), Im Laternenacker 5, Schaffhausen 8200 [CH] Switzerland, http://www.merck.ch/en/company/merck_in_switzerland/merck_cie_schaffhouse/merck_cie_schaffhausen.html)

(b) $^{13}C_5$-PGA, $^{13}C_5$-5-methylTHF, $^{13}C_5$-5-formylTHF, $^{13}C_5$-MeFox $^{13}C_5$-5,10-methenylTHF, and $^{13}C_5$-THF (Merck & Cie)

(6) 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%-spectrum (Fisher Scientific Co)
(g) Hydrochloric acid- 36.5-38% (JT Baker)
(h) Water, 15 MΩ/cm, HPLC grade (Millipore)
(i) Methanol, acetonitrile HPLC certified solvent (Burdick & Jackson Laboratories, Muskegan
(j) Nitrogen ultra pure (>99.99 % purity) (Air Products, Atlanta, GA)

E. Instrumentation

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) HP1100 HPLC system (Agilent, Santa Claire, CA)

Models G1329A standard and G1367A WPALS 96-well plate thermostatted autosamplers, G1316A column heater, G1312A binary pump and G1322A in-line mobile phase degasser Models G1321A

(2) HP1200 HPLC system (Agilent, Santa Claire, CA)

Models G1367B HIP 96-well plate thermostatted autosamplers, ALS thermostat G1330B, G1316A thermostated column heater, G1312A binary pump and G1379 B in-line mobile phase degasser

(3) Sciex API 5500 triple quadrupole mass spectrometer with turboionspray (TIS) as ion source in ESI mode, with Analyst 1.5.2 Windows Microsoft software (AB Sciex, Foster City, CA) is used for serum folate analysis.

(4)   Sciex API 4000 QTRAP triple quadrupole mass spectrometer with turbo ionspray (TIS) as ion source in ESI mode, with Analyst 1.4.2 Windows Microsoft software (AB Sciex, Foster City, CA) is used for WB folate analysis.

(5)   Peak Scientific nitrogen generator (Model Table-31N) that is connected to the in house gas supply and compresses it to supply curtain, exhaust, and source gases to the mass spectrometer instrument (API 5500's) in addition to nitrogen gas for the collision cell (Peak Scientific Instruments, Chicago, IL)

(6)   Parker Balston Analytical Gas generator-LC-MS/MS Gas Generator Source 5000 (5001NTNA) for API 4000 Qtrap.

(7)   Gilson 215 automated solid phase extraction system (Gilson, Inc. 3000 W. Beltline Hwy, Middleton WI) (2 instruments to allow parallel processing of 2 runs/day)

(8)   Hamilton Liquid Handler for sample preparation and dilution (Hamilton, Reno, NV)

(9)   Tecan Freedom EVO 100 automated liquid handler to perform sample transfer for filtration on Captiva 96 well-plate filters (Tecan Genesis)

(10)   Harvard syringe pump (Harvard apparatus, Inc, Holliston, MA)

(11)   Eppendorf repeater pipettor (Brinkmann Instruments, Inc)

(12)   Eppendorf pipettes 10 µL, 100 µL, 200 µL, & 1000 µL (Reference & Research; Eppendorf)

(13)   Positive air displacement pipettes (Pipetman)50 µL, 100 µL & 1000 µL (Gilson Inc, Middleton, WI)

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

(15)   Galaxy Mini table top microcentrifuge (VWR Scientific Products)

(16)   Daigger Vortex Genie 2 mixer (VWR)

(17)   Magnetic stirrer (Baxter Scientific Products)

(18)   pH meter (Beckman- 360, pH/temp/mv meter)

(19)   Mettler, Toledo Analytical Balance Model AG104 (Mettler Instrument Corp., Hightstown, NJ

(20)   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. Aqueous calibrators are carried through the entire sample processing procedure. Calculation of folate concentrations in QCs and unknown patient samples is based on results obtained from a daily 5-point calibration curve (S1-S5). A blank sample (S0, containing internal standard mix) is included as a zero point. Area ratios of analyte to internal standard from single analysis of each calibrator are calculated and a linear regression equation (no weighting) is generated. At the end of each run, the calibration curve is re-injection to assess potential calibrator drift. The calculated calibrator concentrations should be within ± 15% of the calculated calibrator concentrations from the first injection.

This method is linear from 0-220 nmol/L for each folate form. The calibration range is from 0-100 nmol/L for 5-methylTHF and from 1-50 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.

Calibration verification is conducted at least twice a year using international reference materials. For details, see **4012_SOP Calibration and Calibration Verification FOL LCMSMS**.

In 2005, the National Institute of Standards and Technology (NIST) has released a new three levels standard reference material 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 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. The NIBSC also provides a WHO certified reference material (lyophilized, one level) for whole blood folate (95/528, established 1996). The folate concentration in this material has been determined by consensus value assignment, but is mainly representative for the microbiologic assay.

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.

Method figures of merit are presented in the **Appendix 3**.

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, still considered an accurate "reference point" for total folate. For serum samples, there is excellent correlation between the two assays **[4]**. The microbiologic assay gives results that are within ± 10% of the LC-MS/MS results. For whole blood samples, there is good correlation and agreement between the two assays as long as folate polyglutamates have been properly deconjugated to monoglutamates, however overall the microbiologic assay gives results that are 10-25% higher than LC-MS/MS results **[5]**.

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 the service engineer from Applied Biosystems. If necessary, the analyst can recalibrate 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 and third 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 **4012_SOP Tuning and mass calibration of mass spectrometer** for additional details.

(2) Hamilton Microlab Starlet liquid handler

Twice a year a Hamilton service engineer performs a preventative maintenance including volume verification at 10 µL and 1000 µL.

A volume verification of the various steps of the method can also be performed gravimetrically (e.g., using online gravimetric kit, Hamilton) by the user. Imprecision should be commensurate or exceed that obtained using manual pipettes.

(3) Tecan liquid handler

The Tecan liquid handler is used for non-critical solvent transfer. Once per year a Tecan service engineer performs a preventative maintenance including volume verification at 10 µL and 10.0 mL.

A volume verification of the various steps of the method can also be performed either gravimetrically (e.g., using pre-weighed sample vessels) or photometrically (e.g., using a microplate reader and a suitable chromophore) by the user. Imprecision should be commensurate or exceed that obtained using manual pipettes.

(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 by weight, 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 pre-calibrated solutions of pH 4.0 and pH 7.0.

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, 75 patient samples, and second set of QCs, for a total of 88 samples. Three levels of serum or whole blood hemolysate QCs are analyzed in duplicate in each run as bench quality control materials. The assay can be run in the 96-well plate format (routine runs) or in a single cartridge format (R & D). In preparation for SPE, samples can either be pipetted by a liquid handler (routine runs) or manually (R&D or trouble shooting). SPE can either be carried out using an automated Gilson SPE instrument (routine runs) or manually using a vacuum manifold (R&D or trouble shooting).

A. Preliminaries

(1) Thaw frozen serum or whole blood hemolysate specimens (QCs and unknown patient samples), folate stock standard III and internal standard III solutions; 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 1% 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 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 (automated pipetting for routine runs) or label 1.5-mL microcentrifuge tubes for the reagent blank, blank (S0), calibrators S1-S5, controls, and patient samples (manual pipetting for repeat and occasional R&D runs).

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

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

(9) Vortex all thawed specimens thoroughly prior to pipetting.

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 applied to automated SPE sample extraction and clean-up.

(2) For a detailed step-by-step description, see **4012_SOP Automated Sample Pipetting using Hamilton Microlab Starlet**.

(a) Tip racks have to be checked and restocked.

(b) Reagent troughs have to be filled and put in place.

(c) Calibrators, QC samples, and unknown patient samples have to be put in place.

(d) Internal standard mixture has to be put in place.

(e) A 96-well collection plate (destination plate) has to be put in place.

(f) The pipetting program is executed and dispenses according to the scheme shown below for a final volume of 1.1 mL.

Well	Type of sample	Solvent #1	Calibrator mix	Internal standard mix	Water	QC, or patient specimen
1	Reagent blank (double blank)	825 µL	---	---	275 µL	---
2	Calibrator S0 (blank)	770 µL	---	55 µL mix B	275 µL	---
3	Calibrator S1	495 µL	275 µL S1	55 µL mix B	275 µL	---
4	Calibrator S2	495 µL	275 µL S2	55 µL mix B	275 µL	---
5	Calibrator S3	495 µL	275 µL S3	55 µL mix B	275 µL	---
6	Calibrator S4	495 µL	275 µL S4	55 µL mix B	275 µL	---
7	Calibrator S5	495 µL	275 µL S5	55 µL mix B	275 µL	---
8	Low QC – Set 1	770 µL	---	55 µL mix B	---	275 µL
9	Medium QC – Set 1	770 µL	---	55 µL mix B	---	275 µL
10	High QC – Set 1	770 µL	---	55 µL mix B	---	275 µL
11-93	Patient samples	770 µL	---	55 µL mix B	---	275 µL
94	Low QC – Set 2	770 µL	---	55 µL mix B	---	275 µL
95	Medium QC – Set 2	770 µL	---	55 µL mix B	---	275 µL
96	High QC – Set 2	770 µL	---	55 µL mix B	---	275 µL

(g) After the pipetting is completed, the destination plate is removed, covered with the 96-well plastic seal, and incubated at 4°C for 20 min (serum only) prior to SPE for the internal standards to equilibrate with the endogenous folates.

(h) The WB folate method on the Hamilton is split into two sub-methods: The WB sample aliquots (275 µL hemolysate + 55 µL internal standard mix) are prepared on day 1. The calibration curve (275 µL calibrator + 55 µL internal standard mix) and reagent blank are also prepared on day 1 in the 96 well-plate. The sample plate is incubated at 37°C for 4 h to deconjugate the folate polyglutamates. It is then stored at-20°C until the next morning. On day 2 the samples in the 96-well plate are allowed to thaw at room temperature (45 min) and then diluted with buffer and water in reagent blank, blank and calibrators, and only diluted with buffer in patient specimens according to the standard protocol prior to SPE on the Gilson.

C. Manual Sample Pipetting to Prepare for SPE (occasional R&D runs)

(1) Arrange labeled vials in pre-marked racks.

(2) To construct a 5-point calibration curve, follow the pipetting scheme shown in the above table. A blank that contains only the internal standard mix is included as S0. A reagent blank (double blank) is also included in each run.

(3) QC and patient samples (vial 8 and beyond): add 770 µL solvent #1, 275 µL specimen, and 55 µL of internal standard mix (mix B) for a final volume of 1.1 mL.

(4) Vortex all tubes and incubate at 4°C for 20-30 min for the internal standards to equilibrate with the endogenous folates.

D. Automated Solid Phase Extraction (routine runs)

(1) The Gilson 215 SPE is used for automated SPE. The instrument processes 8 wells simultaneously (~25 min/row). All SPE steps, such as cartridge conditioning, sample loading, cartridge washing and sample elution are performed automatically. It takes ~2.5 h to extract 6 rows of samples (48 samples), which includes blanks, calibrators, 2 sets of QC, and 35 unknown patient specimens.

(2) For a detailed step-by-step description, see **4012_SOP Automated SPE using Gilson 215**.

    (a) The instrument is prepared by first priming the pump at a flow rate of 6 mL/min, for a total of 15 mL solvent from the reservoir. An automated prime/ purge method is set up in the instrument method files which is started 15 min before the actual SPE of samples is performed.

    (b) Next, the sample rack and collection rack are loaded on the instrument.

    (c) Finally, the SPE method is run.

- 96-well block (routine) conditioning is carried out in three steps; 1.0 mL methanol from solvent bottle 1; 1.0 mL acetonitrile from solvent bottle2 ; and 1 mL of conditioning buffer from solvent bottle 3.
- Sample loading is carried out in one step; 1 mL from tray at plate front-1 rack.
- 96-well block washing is carried out in two steps; 1.5 mL each with wash solvent from bottle # 4.
- Sample elution is carried out in one step; 1 mL Elution solvent from solvent bottle 5.

E. Manual Solid Phase Extraction (occasional R&D runs)

1) Place BondElut Phenyl cartridges on vacuum manifold using disposable Teflon valve liners (work in chemical fume hood).

2) In the rack inside the chamber place 15-mL glass or plastic tubes to collect the waste wash.

3) Condition 1: 1x1 mL of methanol and 1x1mL of acetonitrile using plastic disposable pipette.

4) Condition 2: 1x1 mL of solvent #2 (ammonium formate buffer, pH 3.2) using disposable plastic pipettes.

5) Apply 1.0 mL of sample (aqueous, plasma or serum) prepared as described in Section 8a-b on the conditioned cartridge and allow to pass through at a flow rate of ~1mL/min under vacuum.

6) Do not allow excessive air drying of the column bed during this step.

7) Wash with 3x1 mL of solvent #2 wash buffer. Do not allow the column to dry during this step.

8) Pipette 1.0 mL of solvent #3 (elution buffer) onto the cartridges. Incubate for 1 min at room temperature.

9) Lift the lid of the vacuum manifold that has the filled cartridges and place it on a clean rack.

10) Replace the 15-mL glass or plastic tubes containing the waste washes from the vacuum chamber with clean plastic tubes.

11) Place empty labeled 1.5-mL microcentrifuge vials on top of the new 15-mL tubes and put the lid back on.

12) Put the vacuum back on and collect eluate at a rate of 1 mL/min.

F. Automated Sample Filtration for LC-MS/MS Analysis (routine runs)

(1) After SPE is completed, using a transfer method on the Tecan liquid handler transfer 600 µL of the extracted sample into a fresh Captiva filter plate (Varian) that is placed on top of a fresh autosampler collection plate (31 mm Nunc plate) .

(2) The samples inside the Captiva filter plate are then filtered through into the HPLC collection plate using a vacuum manifold (IST) at 5 mm pressure within 5 min.

(3) The autosampler collection plates are sealed with the preslit seal and arranged on the HP1100 or HP1200 96-well model autosampler for LC-MS/MS analysis.

G. Manual Sample Filtration for LC-MS/MS Analysis (occasional R&D runs)

(1) Using a 1-mL disposable syringes, aspirate SPE eluate from the collection plate.

(2) Fit in the disposable 0.45 µm filters to these syringes before dispensing.

(3) Dispense through the filter 250-300 µL of the eluate directly into 250-µL glass inserts contained in pre-labeled autosampler vials.

(4) Cap the vials and vortex briefly to release trapped air bubbles.

(5) Arrange the vials in the correct sequence on the autosampler tray for injection according to the Batch file.

H. 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 and whole blood.

(2) For a detailed step-by-step description, see **4012_SOP LC-MS/MS Instrument Preparation.**

(a) Prior to every 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 ~ 5min at a flow rate of 5 mL/min. The column is primed in this solvent for ~20 min at a flow rate of 500 uL/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 uL/min. Pressure is recorded. HPLC system is ready for analysis.

(d) The tandem mass spectrometer is prepared.

- Cleaning of orifice plate
- Cleaning of curtain plate
- 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 two sample solvent injections are run 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 current run

(g) The sample plate is loaded into the autosampler and the batch is submitted for analysis

(h) The HPLC rinse method is loaded. It runs isocratically (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 separate by the HPLC rinse method.

(i) The HPLC shutdown method is loaded. It runs isocratically (90:10 methanol: water) for 1 min in MRM mode after the rinse method.

I. 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 **4012_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, 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 via a USB flash drive 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):

- 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 areas 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 the data printed for review and documentation.

J. Exporting a run

The procedure how a run is exported to the LIMS database is described in section 3.

K. 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 species have to be added up. If a vitamer result is less than the LOD, the LOD divided by the square root of the 2 is used for summation.

For whole blood samples we obtain direct results for each folate species as nmol/L lysate. These results have to be multiplied by 11 to obtain results as nmol/L whole blood. Then, the individual results from each folate vitamer are summed up to obtain a whole blood TFOL result. If a vitamer result is less than LOD, the LOD divided by square root of the 2 is used for summation. This whole blood TFOL can now be converted to a RBC folate result by using the hematocrit and the serum TFOL of the sample:

$$RBC\ folate = \frac{(Whole\ blood\ lysate\ TFOL \times 11) - Serum\ TFOL \times (1 - Hematocrit)/100}{Hematocrit/100}$$

RBC folate results have to be calculated because clinical cutoffs are defined based on RBC folate levels. If the hematocrit value is not available for a patient or QC sample, assume a hematocrit of 40% for calculation or alternatively the RBC folate result is not reportable (study dependent). If the serum folate value is not available, assume a value of 18 nmol/L or alternatively the RBC folate result is not reportable (study dependent).

When the serum value is low compared to the RBC value (i.e., non-fortified population), the formula can be simplified to:

$$RBC\ folate = \frac{Whole\ blood\ TFOL}{Hematocrit/100}$$

L. 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 with methanol dabbed lint-free Kimwipes. Preventative maintenance is performed by service engineers on all major equipment (MS/MS, HPLC, Gilson, Tecan, Hamilton) at least once a year.

M. Special Method Notes

Since the majority of folate in red blood cells occurs in the polyglutamate form, and those polyglutamates need to be deconjugated to monoglutames so that they can be measured by LC-MS/MS, the whole blood samples should be lysed with 1% ascorbic acid, pH 2.7 (1:11 dilution) and incubation at 37°C/4h before analysis. Alternatively, samples can be lysed in 1% ascorbic acid, pH 4.0, and incubated at 37°C/3 h before analysis for complete polyglutamate deconjugation.
To capture least stable tetrahydrofolate (THF) in WB samples (typical for T/T genotype), WB samples (275 µL) are incubated at 37°/4h with internal standard (55 µL) on day-1 and diluted with sample buffer (Sample solvent #1) on day-2 prior to SPE procedure. The calibration curve is also prepared on day-1 (275 µL calibrators + 55 µL IS mix) incubated 37C/4h and next day diluted with respective volumes of sample buffer.

9. Reportable Range of Results

This method is linear from the LOD (see section 7) to 100 nmol/L for MET, and 50 nmol/L for FOT, PGA, THF, and MYT. Samples with MET results <7 nmo/L (3 ng/mL) for serum folate and <127 nmol/L for whole blood (equivalent to 317 nmol/L [140 ng/mL] RBC folate) 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.

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. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

B. Bench Quality Controls

Bench QC specimens are prepared from three serum pools and three whole blood pools, which represent low, medium and high levels of MET, FOT, PGA, MYT, and THF. These QC samples are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

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

1) If all three QC run means are within 2Sm limits and individual results are within 2Si limits, accept the run

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

    a. 1_{3s}: Any of the three QC results are outside the 3s limit

    b. 2_{2s}: Two of the three 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 six QC individual results is outside a 2 Si limit – reject run if:

    a. Outlier – One individual result is beyond the characterization mean $\pm$ 4 Si 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

Si = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).
Sm = Standard deviation of the run means (the limits are shown on the chart).
Sw = Within-run standard deviation (the limits are not shown on the chart).

The QC results are checked after each run using of a multi-rule quality control program **[8]**. 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 quarterly. 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. A hardcopy of the QC results from each run is also maintained by the analyst.

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

A. Check to make sure that the hardware is functioning properly. Make sure the mass spec calibrations are ok. Run PPG's in Q1 and Q3 scan mode to verify the instrument calibration.

B. Run folate standards in Q1 scan mode to see if molecular ion is detected.

C. Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator.

D. Check the autosampler for proper sample injections.

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

F. Check the calibrations of the pipettes.

G. 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.

H. 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 imprecision are intermittently inaccurate micro-pipettors or pipetting errors.

B. Stock standards, internal standards and specimens should be vortex-mixed thoroughly before pipetting.

C. Handling stocks and internal standards in step-wise sequential manner will minimize the chances of cross-contamination.

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

E. Also change of gloves after preparations of stock and working standards and internal standards are recommended to avoid any contamination.

F. All solvents should be degassed before use, solid-phase extraction reservoir solvent in particular, as without doing so the air gets trapped in the solvent lines which results in incomplete sample extraction.

G. Buffers should be made fresh daily and pH should be checked.

H. Ascorbic acid powder (0.5 g/100 mL) should be added to sample solvents #1, #3 and 0.1% to the sample solvent #2 only before use.

I. Acetic acid (1 mL/100 mL) should be added to sample solvent #3 and 0.5% to the mobile phase only before use.

J. Hemolyzed serum samples may give falsely elevated values.

K. The entire sample preparation and calibration 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. Frozen WB samples should be handled carefully, allow only 15-40 min thaw time (depends on sample volume in the vial) at room temperature prior to hemolysate preparations.

N. Exposure to strong sunlight for more than eight hours may cause 10-20% serum folate degradation.

O. Nitrogen gas cylinder for 215 Gilson SPE system should be carefully monitored for gas. The pressure for out flow should be always 40 psi, and the gas pressure to the instrument at the regulator should be adjusted at 5 psi. Change the cylinder before the gauge reads 500 psi.

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

Q. Only 250 µL of the filtered samples should be added to the insert and vial should be closed properly and vortex mix briefly before analysis on LC/MS/MS (manual filtration).

13. Reference Ranges (Normal Values)

Clinical reference ranges reported for serum folate are 11-36 nmol/L with the microbiologic assay and 7-36 with the chemiluminescence assay **[9]**. Clinical reference ranges reported for RBC folate are 317-1422 nmol/L with the CPB radioassay **[9]**.

The newest post-fortification reference ranges for the U.S. population generated with the microbiologic assay for NHANES 2005-2010 are shown below **[10]**. 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 **[10]**.

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

RBC folate: 505-2,490 nmol/L (2.5th -97.5th percentile; n = 23,528

New reference ranges for individual folate vitamers based on LC/MS/MS will have to be determined with NHANES 2011-2012 samples.

Serum folate levels <7 nmol/L (3 ng/mL) are usually indicative of inadequate folate intake. High PGA values in serum are caused by supplementation or consumption of significant quantities of fortified food. RBC folate values are more indicative of body stores, whereas serum levels reflect only recent dietary intake. RBC folate levels <317 nmol/L (140 ng/mL) are usually indicative of inadequate folate stores.

14. Critical Call Results ("Panic Values")

Any NHANES samples with serum folate levels <7 nmol/L (3 ng/mL) or RBC folate levels <317 nmol/L (140 ng/mL) are considered to require follow-up. Since survey data are transmitted several times 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.

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 ASCII text file or Excel file, generally through electronic mail or via ftp site.

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

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

The 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 attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing 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
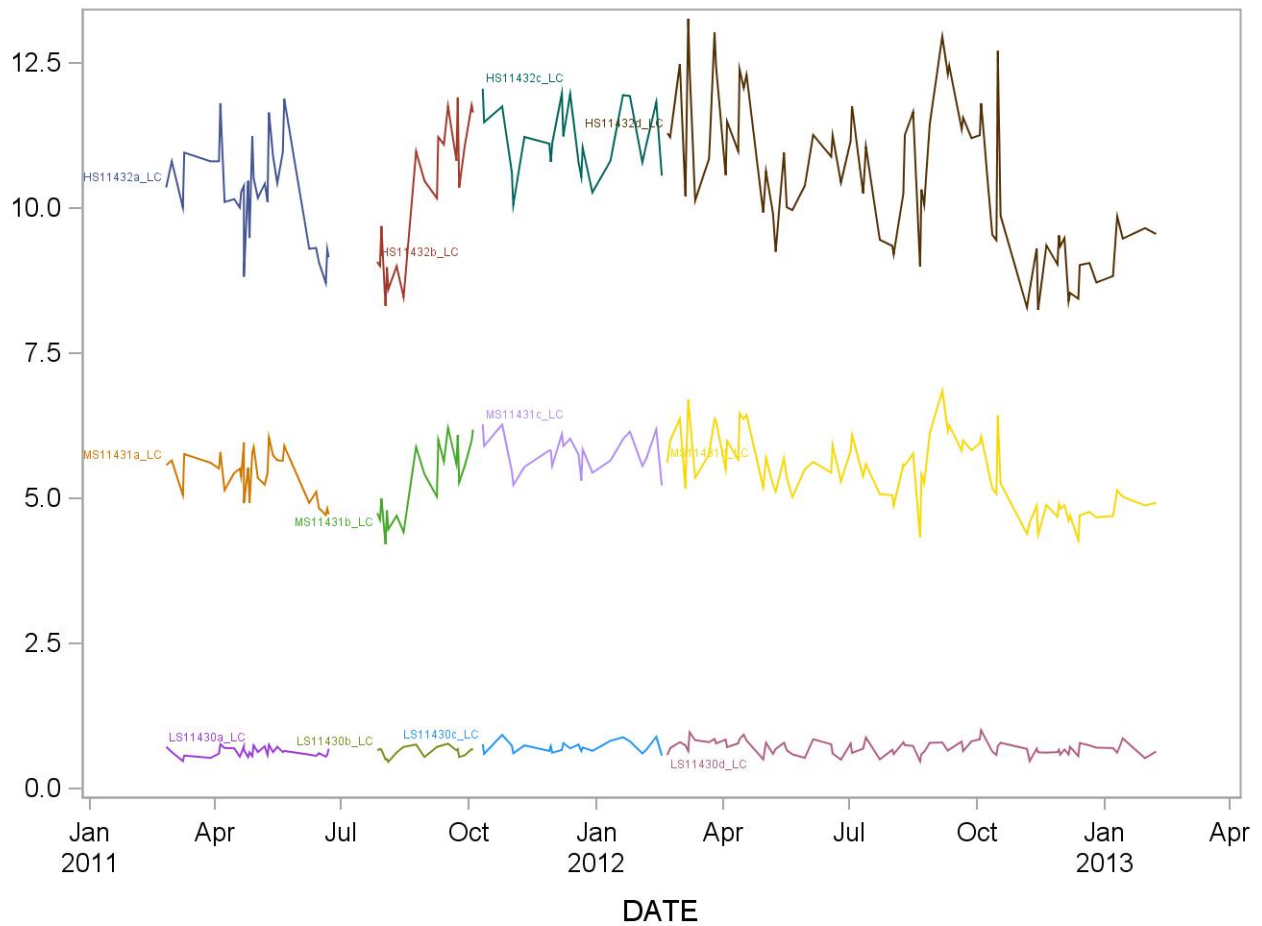
See following pages.

2011-2012 Summary Statistics and QC Chart for 5-Methyl-tetrahydrofolate (nmol/L)

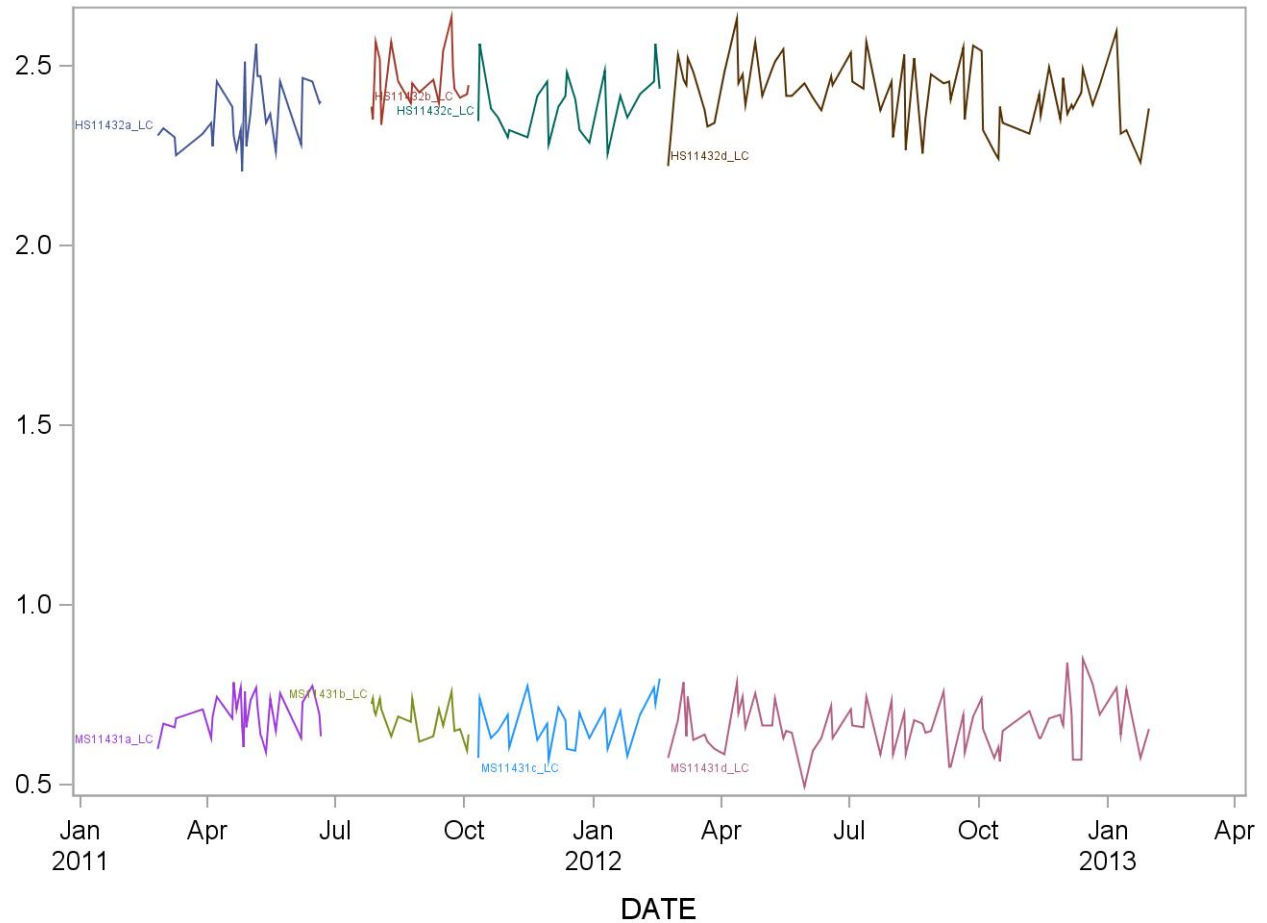| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11432a_LC	31	25FEB11	07JUL11	48.0800	0.9709	2.0
LS11430a_LC	31	25FEB11	07JUL11	18.2853	0.4795	2.6
MS11431a_LC	31	25FEB11	07JUL11	33.5987	0.6968	2.1
HS11432b_LC	21	27JUL11	04OCT11	48.1964	1.2123	2.5
LS11430b_LC	21	27JUL11	04OCT11	18.2876	0.3378	1.8
MS11431b_LC	21	27JUL11	04OCT11	33.3821	0.7828	2.3
HS11432c_LC	22	11OCT11	17FEB12	48.5414	1.1066	2.3
LS11430c_LC	22	11OCT11	17FEB12	18.3516	0.3412	1.9
MS11431c_LC	22	11OCT11	17FEB12	33.4677	0.7402	2.2
HS11432d_LC	77	21FEB12	07FEB13	48.3499	1.3467	2.8
LS11430d_LC	77	21FEB12	07FEB13	18.1925	0.4909	2.7
MS11431d_LC	77	21FEB12	07FEB13	33.4708	0.7629	2.3
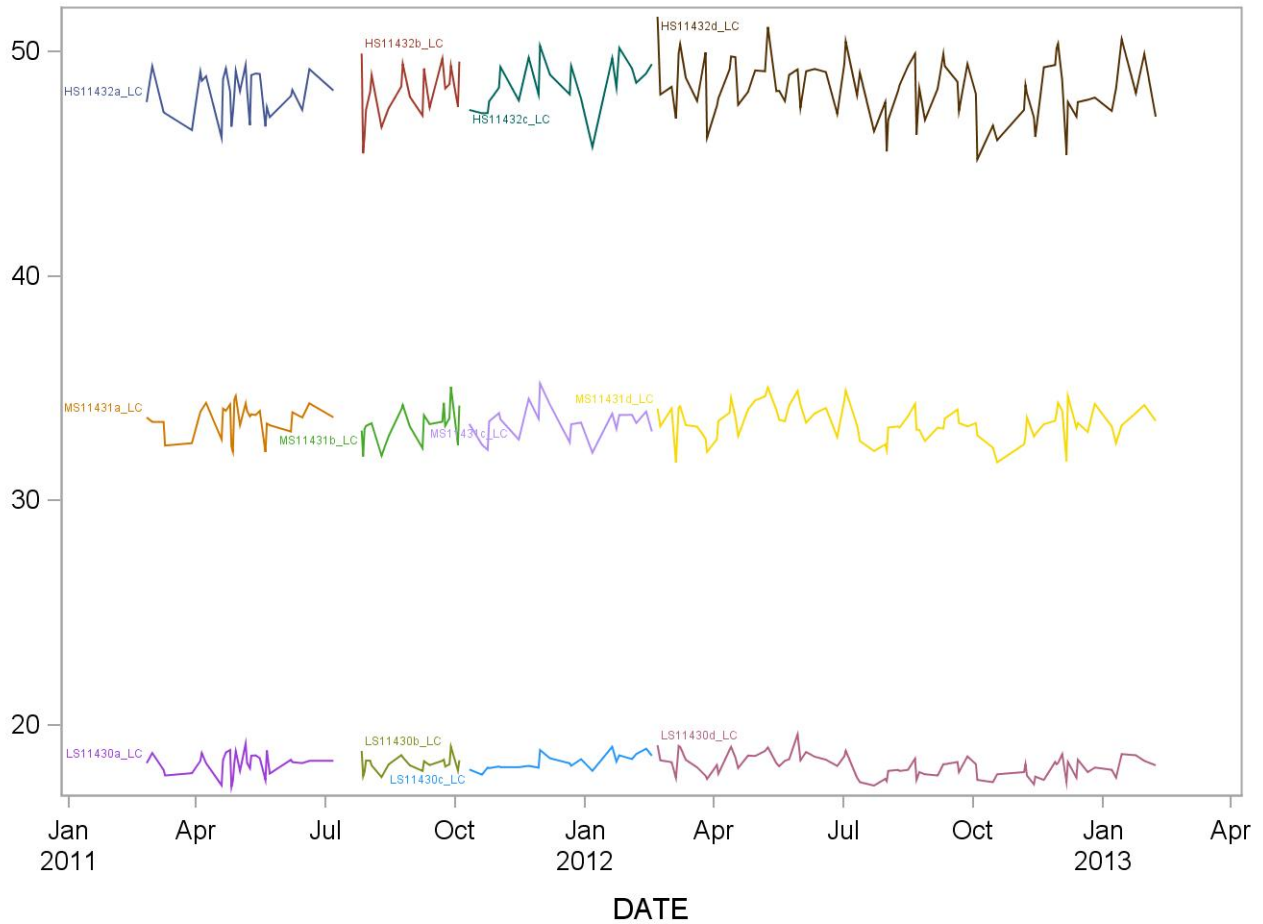
2011-2012 Summary Statistics and QC Chart for Folic acid (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11432a_LC	31	25FEB11	22JUN11	10.2639	0.8369	8.2
LS11430a_LC	31	25FEB11	22JUN11	0.6329	0.0757	12.0
MS11431a_LC	31	25FEB11	22JUN11	5.4040	0.3938	7.3
HS11432b_LC	20	27JUL11	04OCT11	10.2140	1.2255	12.0
LS11430b_LC	20	27JUL11	04OCT11	0.6460	0.0930	14.4
MS11431b_LC	20	27JUL11	04OCT11	5.2893	0.6494	12.3
HS11432c_LC	23	11OCT11	17FEB12	11.1678	0.5969	5.3
LS11430c_LC	23	11OCT11	17FEB12	0.7204	0.1004	13.9
MS11431c_LC	23	11OCT11	17FEB12	5.7693	0.3224	5.6
HS11432d_LC	74	21FEB12	07FEB13	10.4734	1.3136	12.5
LS11430d_LC	74	21FEB12	07FEB13	0.7048	0.1183	16.8
MS11431d_LC	74	21FEB12	07FEB13	5.4467	0.6314	11.6

2011-2012 Summary Statistics and QC Chart for 5-Formyl-tetrahydrofolate (nmol/L)

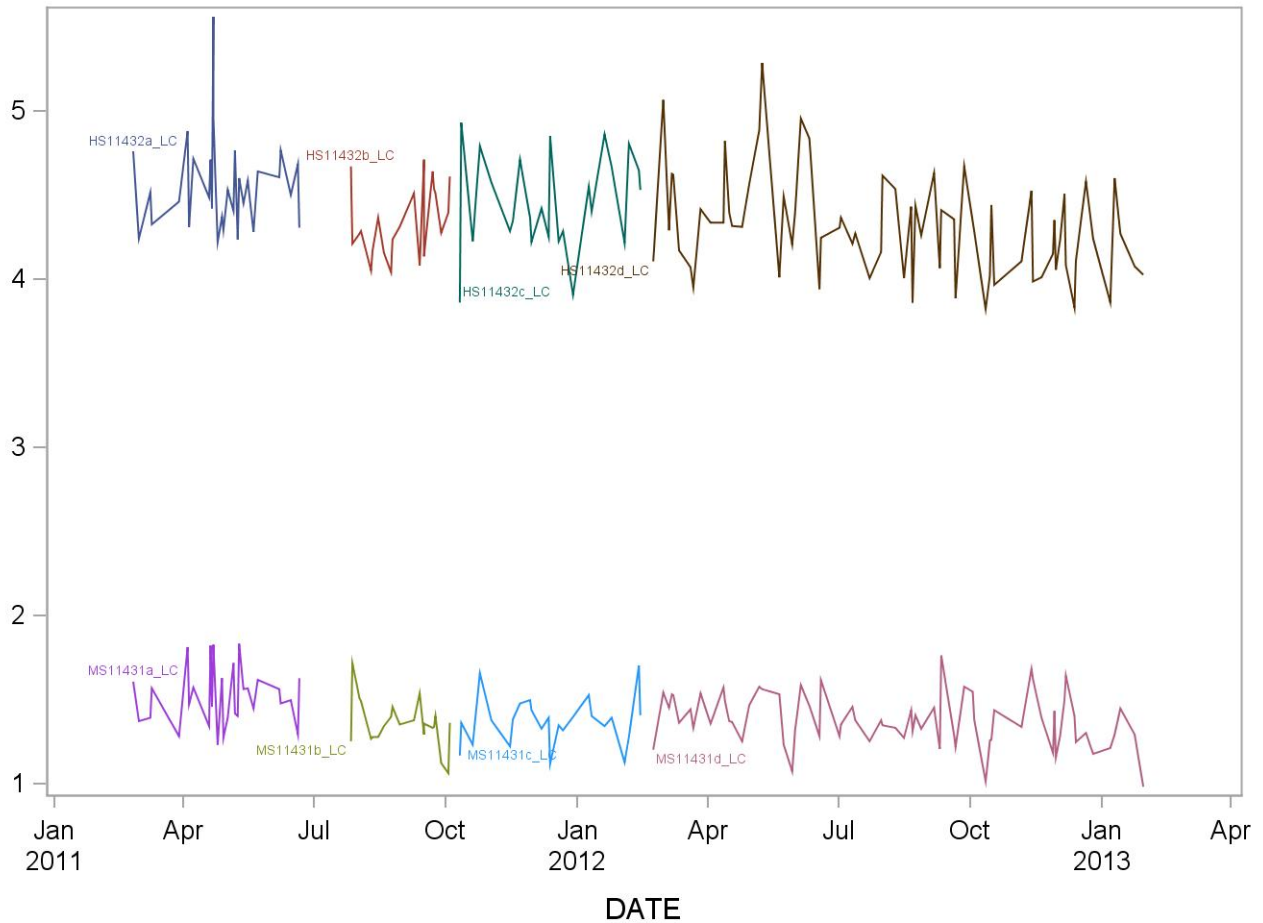| Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11432a_LC	30	25FEB11	21JUN11	2.3633	0.0883	3.7
MS11431a_LC	30	25FEB11	21JUN11	0.6907	0.0572	8.3
HS11432b_LC	20	27JUL11	04OCT11	2.4563	0.0764	3.1
MS11431b_LC	20	27JUL11	04OCT11	0.6843	0.0460	6.7
HS11432c_LC	24	11OCT11	17FEB12	2.3908	0.0835	3.5
MS11431c_LC	24	11OCT11	17FEB12	0.6681	0.0672	10.1
HS11432d_LC	70	23FEB12	30JAN13	2.4215	0.0926	3.8
MS11431d_LC	70	23FEB12	30JAN13	0.6647	0.0722	10.9

2011-2012 Summary Statistics and QC Chart for Tetrahydrofolate (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11432a_LC	30	25FEB11	21JUN11	4.5522	0.2770	6.1
MS11431a_LC	30	25FEB11	21JUN11	1.5273	0.1782	11.7
HS11432b_LC	21	27JUL11	04OCT11	4.3407	0.2119	4.9
MS11431b_LC	21	27JUL11	04OCT11	1.3560	0.1408	10.4
HS11432c_LC	24	11OCT11	14FEB12	4.4546	0.2915	6.5
MS11431c_LC	24	11OCT11	14FEB12	1.3681	0.1435	10.5
HS11432d_LC	70	23FEB12	30JAN13	4.3081	0.3047	7.1
MS11431d_LC	70	23FEB12	30JAN13	1.3774	0.1551	11.3

2011-2012 Summary Statistics and QC Chart for 5,10-Methenyl-tetrahydrofolate (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11432a_LC	30	25FEB11	21JUN11	4.3527	0.1588	3.6
MS11431a_LC	30	25FEB11	21JUN11	1.4278	0.1249	8.8
HS11432b_LC	20	27JUL11	04OCT11	4.4315	0.1354	3.1
MS11431b_LC	20	27JUL11	04OCT11	1.4440	0.0709	4.9
HS11432c_LC	24	11OCT11	14FEB12	4.3858	0.1904	4.3
MS11431c_LC	24	11OCT11	14FEB12	1.4246	0.1063	7.5
HS11432d_LC	70	23FEB12	30JAN13	4.4814	0.1823	4.1
MS11431d_LC	70	23FEB12	30JAN13	1.4722	0.0819	5.6
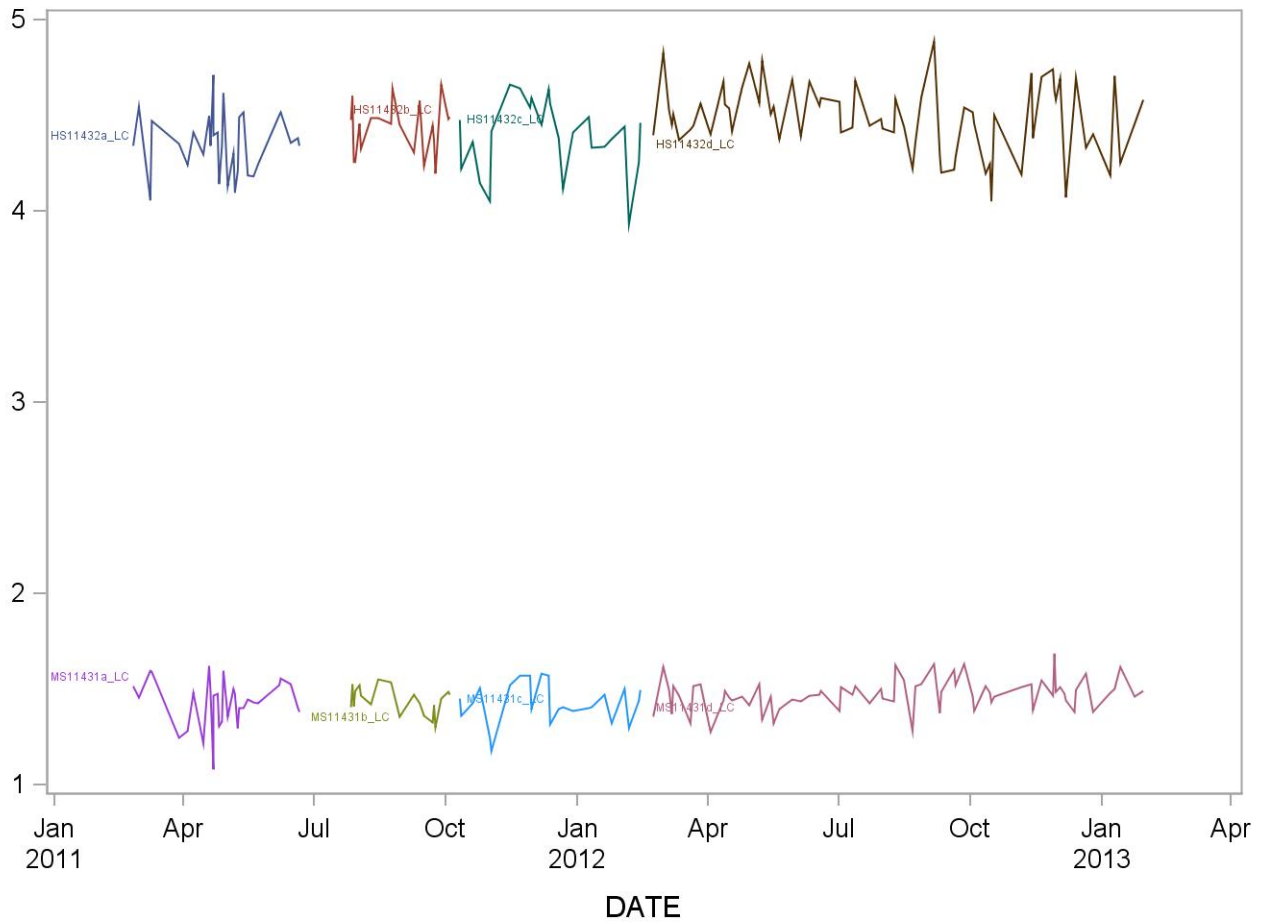
2011-2012 Summary Statistics and QC Chart for Mefox oxidation product (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS11432a_LC	30	25FEB11	21JUN11	2.9905	0.1057	3.5
LS11430a_LC	30	25FEB11	21JUN11	1.4503	0.0758	5.2
MS11431a_LC	30	25FEB11	21JUN11	1.5675	0.0805	5.1
HS11432b_LC	20	27JUL11	04OCT11	3.0208	0.1365	4.5
LS11430b_LC	20	27JUL11	04OCT11	1.4770	0.0822	5.6
MS11431b_LC	20	27JUL11	04OCT11	1.5833	0.0807	5.1
HS11432c_LC	24	11OCT11	14FEB12	2.8100	0.1119	4.0
LS11430c_LC	24	11OCT11	14FEB12	1.3623	0.0731	5.4
MS11431c_LC	24	11OCT11	14FEB12	1.4446	0.0711	4.9
HS11432d_LC	70	23FEB12	07FEB13	2.8684	0.1387	4.8
LS11430d_LC	70	23FEB12	07FEB13	1.4133	0.0874	6.2
MS11431d_LC	70	23FEB12	07FEB13	1.5049	0.0850	5.7
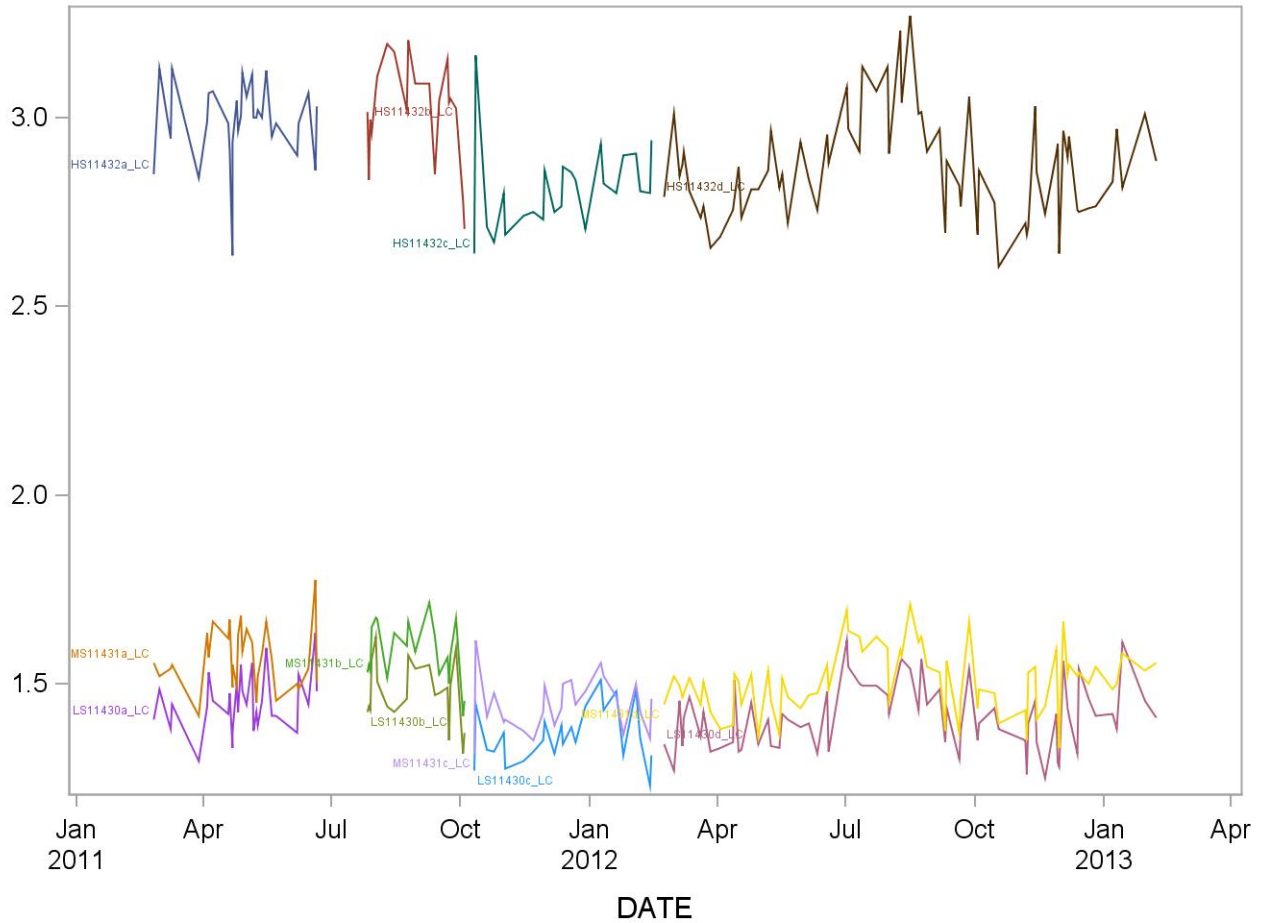
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ACKNOWLEDGMENTS

Appendix 1 - Formulas to calculate the concentration of folate stock solutions based on molar absorptivity

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

Or

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

€ max = molar extinction coefficient

Conc. = concentration

Example - Folic acid (PGA)

Abs	0.661
Dilution	10
€ max	27600
MW	441.4

Concentration (ppm or µg/mL) = 0.661 x 10 x 1000 x 441.4/27600   = **105.7**
Concentration (µmol/L)   = 0.661 x 10 x 1000 x1000 /27600   = **239.5**

The table below shows the molecular weights and conversion factors for different folate vitamers

	5-MethylTHF	5-FormylTHF	PGA	THF	5,10-MethenylTHF	MeFox
Folate, MW	459.46	473.44	441.4	445.43	455.45	473.4
Conversion factor (ng/mL to nmol/L)	2.176	2.112	2.266	2.245	2.196	2.112
13C5-Folate MW	464.46	478.44	446.4	450.43	460.45	478.44
Conversion factor (ng/mL to nmol/L)	2.153	2.09	2.24	2.22	2.172	2.09

Appendix 2 - Approximate target concentrations (nmol/L) for the various folate vitamers in QC pools

Matrix	Level	5-Methyl-THF	PGA	MeFox	5-Formyl-THF	THF	5,10-Methenyl-THF	TFOL
Serum	Low QC	5*	1	1	1	1	1	7*
	Medium QC	15	2.5	2.5	2.5	2.5	2.5	25
	High QC	30	5	5	5	5	5	50
WB hemolysate	Low QC	5*	1	1	1	1	1	11[&]
	Medium QC	15	2.5	2.5	2.5	2.5	2.5	25
	High QC	30	5	5	5	5	5	50

*As low as possible

[&] A WB hemolysate TFOL concentration of 11 nmol/L corresponds to a WB TFOL concentration of 122 nmol/L and a RBC TFOL concentration of 305 nmol/L or 140 ng/mL (based on a Hct of 40% and a molar conversion factor of 2.176 [derived from 5-methylTHF]).

Appendix 3 - Method Figures of Merit

Accuracy

Results of in-house SPE efficiency experiments independent of the IS showed a mean (± SD) recovery of 75% ± 3% for MET, 72% ± 4% for PGA, and 77% ± 6% for FOT, if these standards were added to serum at different levels (0, 5, 50 ng/mL MET, and 0, 2.5 and 25 ng/mL PGA & FOT) [1]. Results of in-house recovery studies based on area ratios (analyte/IS) of the spiked serum were complete for all three analytes (90% ± 10%) [1].

The SPE efficiency (independent of the IS) for folate extraction from whole blood was 77% ± 3% for MET, 80% ± 4% for FOT, 81% ± 6% for PGA, 72% ± 12% for MYT, and 46% ± 8% for THF [2]. Recoveries of spiked folate species in whole blood based on area ratios (analyte/IS) were complete for all analytes: 92% ± 2% for MET, 93% ± 1% for FOT, 90% ± 4% for PGA, 100% ± 12% for MYT, and 101% ± 20% for THF [2].

Precision

Representative information on method precision for serum and whole blood folate is shown below

	Serum folate nmol/L (Inter-assay CV [n = 122 days])					
	5-MethylTHF	**PGA**	**MeFox**	**5-FormylTHF**	**THF**	**5,10-MethenylTHF**
Low QC	18.36 2.5%	0.68 17%	1.44 6.7%	-	-	-
Medium QC	33.5 2.7%	5.45 12%	1.54 6.4%	0.67 9.5%	1.41 13.1%	1.43 7.7%
High QC	48.2 2.7%	10.52 11%	2.93 5.1%	2.38 3.9%	4.4 6.9%	4.39 4.4%
	Whole blood hemolysate folate nmol/L (Inter-assay CV [n = 13 days])					
	5-MethylTHF	**PGAS**	**MeFox**	**5-FormylTHF**	**THFS**	**5,10-MethenylTHF**
Low QC	9.1 3.0%	-	2.3 11%	-	-	-
Medium QC	15.4 4.2%	0.27 60%	3 11%	0.52 22%	4.1 13%	1.91 10%
High QC	22.6 3.5%	0.45 31%	4 7.7%	0.91 16%	1.71 25%	3.12 6.8%

Limits of detection

Determination of the limit of detection (LOD) was conducted by serially diluting a "low" serum and whole blood hemolysate QC pool with 0.1% ascorbic acid and by estimating the SD at a concentration of zero (σ_0) by extrapolating repeat analyte measurements (n = 9) made near the detection limit in these dilutions (LOD defined as 3 σ_0). The calculated method LOD values and corresponding method LOQ values (representing a signal to noise of 10) are shown in the table below. Because RBC folate is a calculated value, no LOD applies.

	API 5500	
Analytes	**LOD (nmol/L)**	**LOQ (nmol/L)**
5-MethylTHF	0.31	10.
Folic Acid	0.14	0.47
MeFox	0.34	1.13
5-FormylTHF	0.30	1.00
5,10-MethenylTHF	0.34	1.13
THF	0.37	1.23

Appendix 4 – Ruggedness Testing

This method has also undergone a series of in-house **ruggedness testing** experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. 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.

Folate is an important nutrient involved in one carbon cellular metabolism. Serum and whole blood folate 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 or whole blood samples.

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

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

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

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

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

Table 1: Ruggedness testing 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	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

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

Table 2: Ruggedness testing for whole blood folate vitamers by LC-MS/MS

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

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