Laboratory Procedure Manual

Analyte: **Folate Species**

Matrix: **Serum/Whole Blood**

Method: **Liquid Chromatography Tandem Mass Spectrometry**

Method No: 4001.02

Revised:

as performed by:

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

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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:

Data file name	Variable name	SAS Label
FOLFMS_E	LBXSF2	Folic acid, serum (nmol/L)
	LBXSF1	5-Methyl-tetrahydrofolic acid, (ser) (nmol/L)

1. Summary of Test Principle and Clinical Relevance

a. Clinical relevance

Folate is required in cellular C1-metabolism and hematopoiesis, and 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, and to increase plasma homocysteine levels, a risk factor for cardiovascular disease, in the general population. 5-Methyltetrahydrofolic acid (MET) is the major folate vitamer circulating in serum. If intake of folic acid (PGA) from fortified food or supplements is >200 µg per meal, unmetabolized PGA may appear in serum. Red cells contain mainly MET polyglutamates as a storage form. It has been shown that in people with mutation of the MTHFR enzyme, a portion of the MET polyglutamates is replaced by formyl-folates. It is thus desirable to determine these main folate vitamers in serum and whole blood rather than measuring total folate (TFOL).

b. Test principle

Five folate species, 5-methytetrahydrofolic acid (MET), folic acid (PGA), 5-formyl-tetrahydrofolic acid (FOT), tetrahydrofolic acid (THF), and 5,10 methenyl-tetrahydrofolic acid (MYT) are measured by isotope-dilution high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) (1, 2, 3). The sum of these folate species represents TFOL. The assay is performed by combining serum (275 µL) with ammonium formate buffer (770 µL) and the internal standard mixture (55 µL). The samples are vortex mixed and incubated at 4°C for 20 min. Sample clean-up is performed using phenyl solid phase extraction (SPE) cartridges. Finally, samples are eluted with 1 mL elution buffer and analyzed by LC/MS/MS. Quantitation is by peak area ratio (analyte to internal standard) and is based on a six-point calibration curve in an aqueous medium. The sample extraction is fully automated and can handle a throughput of 48 to 96 samples. This LC/MS/MS method is highly specific and sensitive, and is a candidate reference method for the determination of serum and red cell folate species in various studies. We recently compared the LC/MS/MS method to the microbiological assay and the Bio-Rad radioassay for serum (4) and whole blood samples (5) to provide the link for future NHANES trend analyses.

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: Is 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. Mass spectrometry raw data files and respective batch files are collected and stored using Analyst Software of the Sciex API 4000 (Applied Biosystems) mass spectrometer workstation. The data files and batch files are exported via USB flash disk to the Q drive on the network (Folate Project directory under the Analyst 1.4.2 Software). The raw data (containing patient data as well as QC data) stored in the data file is processed into results using the Quantitation Wizard software and the results are saved in the Results folder under the main directory. The results generated are mostly based on auto-integration by the Analyst software, but do allow for manual peak selection and integration when necessary. The final data file results (including peak areas of both analyte and internal standards, analyte retention time, analyte and internal standard names, dilution factor, data file name, acquisition time etc) from the instrument results file are saved in the import data file folder on Q drive from where the these files are further exported into the Microsoft Access Database (FrontEnds) for statistical evaluation and QC check as described below.

<u>Step 1 – Analyst – Import data file into ACCESS:</u>

- Double click the FrontEnds icon on desktop, password entry required
- [Add Instrument data file Results to Database] (under Batch & X-Batch)
- [Import Instrument Data File] - Enter information (instrument, assay, date, time, analyst, study)
- [Import] – In “select data file” window, choose A: and import file number assigned. Check that sample ID’s are recognized.
- [Transfer]

<u>Step 2 – Analyst – Review run in ACCESS:</u>

- [Run Review] (under Batch & X-Batch) – Select assay
- [Show runs] – Cursor to desired run, enter sample set name and comments
- [QC Results] – Review QC results for transmission errors and whether they pass the 2S limits
- [Print Report] [Back]
- [Sample Results] – Review patient results to assure proper information transmission, enter appropriate comment codes on flagged samples
- [Set Final] results that are ready to be reported
- [Set Reviewed]
- [Print Report] [Back]

<u>Step 3 – Analyst – Send email and run folder to QA Officer:</u>

An e-mail is sent to the QA Officer including the following run information: Analysis date, Instrument, Study, Groups, File name, Batch ID, Run #, and QC Status. Noteworthy comments are included in the email. All printouts including raw data are submitted in a run folder to the QA Officer who reviews the Bench QC data via the ACCESS database as described below.

Step 4 – QA Officer – Review Bench QC via ACCESS:

- Double click the ACCESS icon on desktop, password entry required
- [Export QC to SAS] (under Batch & X-Batch) – Select Assay, Date range and Controls
- [Make QC Data Infile] – Save file to I:, appropriate subfolder for archival
- [Run SAS] – SAS will automatically open, [go], review each generated plot, print QC cover page and standard deviation plot, [Back]
- [Run Review] (under Batch & X-Batch) – Select assay
- [Show runs]
- [Sample Results]
- [Set Batch QC] – accept or reject
- [Set Reviewed]
- Forward email from Analyst to Second QA reviewer (for Blind QC review) specifying Bench QC status of the run.

Step 5 – Second QA Reviewer – Review Blind QC and other parameters in ACCESS:

- Double click the ACCESS icon on desktop, password entry required
- [Run Review] (under Batch & X-Batch) – Select assay, then desired run
- [Blind QC Results] – Review whether Blind QC results pass the 2S limits
- [Print Report] [Back]
- Check other parameters if applicable (i.e., background, calibration curve, repeat values, replicates, signal intensity)
- [Set RQC] – accept or reject
- Verify that appropriate comment codes have been applied and that final values have been set correctly
- [Set Ready] – Final results will be set ready to be exported
- [Set Reviewed]
- Forward email from QA Officer to Supervisor specifying Blind QC status of the run and other relevant comments.

Step 6 – Supervisor – Approval and Export of Results via FrontEnds:

- Double click the FrontEnds icon on desktop, password entry required
- [Run Review] (under Batch & X-Batch) – Select assay, then desired run
- Perform a final review
- [Set Reviewed]
- [Export/Report Results] (under Study Functions) – Select study, select analytes/panel, use selected panel
- [Generate Pre-Export Text File] – Review file on \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\To be transmitted

- [Generate Export Text File and Set Results Exported] – \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\To be transmitted
- FTP file to Westat
- Send Westat an email that file was transmitted
- Move transmittal file from \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\To be transmitted to \\cdc\project\CCEHIP_NCEH_DLS_NBB_OC\QA\Data handling\Transmitted\Appropriate Year Folder.

For NHANES, data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. 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 to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.

c. The instrument raw and results files in the Analyst software are typically backed up to the CDC network on a daily basis and periodically (at least monthly) backed up to a CD-ROM for long-term storage. All sample, QC, and calibration data (both raw and results) are stored on the network and are the responsibility of the analyst. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively.

d. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

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. RBC folate samples are prepared from 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 275-µL serum specimen is required for serum folate assays. A 275-µL whole blood lysate specimen is required for red cell folate assay.

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 red cell folate calculations is made at the time of collection. The appropriate amount of serum or whole blood/ascorbic acid solution 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 frozen and then shipped on dry ice by overnight mail. Once received, they should be stored at ≤-20°C until analyzed. Serum folate and red cell folate are stable for a few weeks if the specimen is frozen at -20°C before analysis. For long-term storage (more than a few weeks), specimens should always be frozen at -70°C. Ascorbic acid is typically not added to the serum specimen. Multiple freeze-thaw

cycles will cause degradation of the folate in serum and particularly whole blood folate is sensitive to extended freeze-thaw degradation.

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 assay for RBC folate (6). However, our recently published data has shown that incubation of whole blood lysates prepared with 1% ascorbic acid either at pH 2.7 or pH 4.0 needs 4 h or 3 h incubation at 37°C to convert all folate polyglutamates to monoglutames if HPLC based methods are considered for folate measurements (3). No incubation is required for analysis either by microbiologic assay or BioRad radioassay. Results from hemolyzed serum specimens should be interpreted with caution because they may have falsely elevated values. Folate specimens exposed to light for longer than 8 h may undergo 10-20% 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 Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA\DLS Policies and Procedures Manual). The protocol discusses collection and transport of specimens and the special equipment required. In general, plasma should be transported and stored at no more than -20°C. Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood 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 resistance to at least 15 megaOhm-cm and filter before use, using 0.45 µm nylon filters.

(1) Ammonium formate buffer (Solvent #1, 1% formic acid, 0.5% ascorbic acid, pH 3.2)
To 980 mL deionized water add 10 mL concentrated formic acid and titrate with 30% ammonium hydroxide to adjust pH to 3.2. Make up volume to 1 liter with deionized water. The buffer can be stored at room temperature for a week without adding ascorbic acid. 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 (except for using 100 instead of 10 mL of concentrated formic acid). This buffer can be stored at room temperature for about 6 months. At the time of use take 100 mL from 10x buffer and dilute to 1 liter with deionized water and add ascorbic acid powder to a final concentration of 0.5%.

(2) Conditioning solvents for SPE cartridges

(a) 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.
(b) Acetronitrile (1 mL is used to condition cartridges).
(c) Methanol (1 mL is used to condition cartridges).

(3) Wash buffer (Solvent #2, 0.05% ammonium formate, pH 3.4)
Dilute 50 mL of 1% ammonium formate buffer to 1 L with deionized water and store at room temperature for maximum one week after preparation. Add ascorbic acid powder to 0.1% final concentration.

(4) Sample elution buffer or sample solvent (Solvent #3, 40% methanol, 10% acetonitrile, 1% acetic acid, 0.5% ascorbic acid)
Mix 400 mL of methanol, 100 mL of acetonitrile, and 450 mL of deionized water in a 1-L graduated cylinder. Degas under vacuum for 5 min. Add 10 mL of acetic acid (1% final concentration) and make volume up to 1 L. At the time of use, add 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)
Mix 400 mL of methanol and 600 mL of deionized water in a 1-L graduated cylinder. Degas under vacuum for 5 min. This solvent is used to purge the solvent lines of the system as a system solvent during sample extraction.

(6) Ascorbic acid
Weigh 0.5 g portions of ascorbic acid powder into microcentrifuge vials and store them closed in a cardboard box at room temperature. When needed, add the contents of one vial to a 100-mL portion of solvent #1 or solvent #3. Ascorbic acid portions are pre-weighed on a weekly basis.

(7) HPLC mobile phase (40% methanol, 10% acetonitrile, 0.5% acetic acid)
Mix 400 mL of methanol, 100 mL of acetonitrile, and 450 mL of deionized water(filtered through 0.45 µm) in a 1-L graduated cylinder. Degas under vacuum for 5 min. Add 5 mL of acetic acid (0.5% final concentration) and make volume up to 1 L with filtered water.

(8) Ascorbic acid (1%)
Add 1.0 g L-ascorbic acid to 100 mL deionized water and mix well to dissolve. Filter using 0.45 µm filter and degas under a stream of nitrogen for 5 min.

(9) Lysate buffer (1% ascorbic acid, pH 4.0)
Add 1.0 g L-ascorbic acid to ~90 mL deionized water and mix well, adjust pH to 4.0 using 5 N NaOH. Make final volume up to 100 mL.

(10) PPG dilution solvent for mass spec calibration (stable at 4ºC for 6 months)
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 1/50 dilution for positive ion calibration (400 µL of PPG standard 2000 + 19.6 mL of PPG dilution solvent) for calibration of the API 4000.

b. Standards Preparation

(1) Individual PGA stock solutions

a. PGA stock solution I (~200 µg/mL): Prepare a stock solution by dissolving ~5 mg PGA in degassed 20 mM phosphate buffer (pH 7.2) in a 25-mL volumetric flask.

Vortex briefly and add a few drops of 1% acetic acid 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 against phosphate buffer as a blank on a UV/VIS spectrophotometer using scan analysis.

b. PGA stock solution II (100 µg/mL): Based on the actual concentration of stock solution I, the solution is diluted to yield 25 mL of a 100 µg/mL stock solution II. The dilution is done with degassed deionized water in a 25-mL volumetric flask. Aliquots of this stock solution II (1.2 mL) are stored at -70°C in 2-mL labeled cryovials.

c. PGA stock solution III (20 µmol/L): Thaw one vial of stock solution II approximately once a month and dilute it approximately 1/10 (depending on the MW) with 0.1% ascorbic acid in a 10-mL volumetric flask to yield a 20 µmol/L stock solution III. Aliquot 500-µL portions into 25 microcentrifuge vials and store at -70°C. This stock solution III is used as a working standard for folate analysis on a daily basis.

(2) <u>Individual MET, FOT, and THF stock solutions</u>

The three reduced folates: MET, FOT, and THF are treated the same way. Only MYT is treated differently because it is only stable at acidic pH.

a. Reduced folate stock solution I (~200 µg/mL): Prepare a stock solution by dissolving ~5 mg of a reduced folate in degassed 20 mM psphate 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 taken 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 absorbance at the following wavelengths against phosphate buffer as a blank on a UV/VIS spectrophotometer using scan analysis: MET 290 nm and 245 nm; FOT 285 nm; THF 298 nm. For MET, the ratio of absorbance at 290/245 nm is also monitored to ensure that no oxidation took place. This ratio should exceed 3.3.

b. Reduced folate stock solution II (100 µg/mL): Based on the actual concentration of stock solution I, the solution is diluted to yield 25 mL of a 100 µg/mL stock solution II. The dilution is done with degassed 1% ascorbic acid solution in a 25-mL volumetric flask. Aliquots of this stock solution II (1.2 mL) are stored at -70°C in 2-mL labeled cryovials.

c. Reduced folate stock solution III (20 µmol/L): Thaw one vial of stock solution II approximately once a month and dilute approximately 1/10 (depending on the MW) with degassed 0.1% ascorbic acid in a 10-mL volumetric flask to yield a 20 µmol/L stock solution III. Aliquot 500-µL portions into 25 microcentrifuge vials and store at -70°C. This stock solution III is used as a working standard for folate analysis on a daily basis.

(3) Individual MYT stock solutions

a. MYT stock solution I (~200 µg/mL): Prepare a stock solution by dissolving ~5 mg MYT in 1M HCl in a 25-mL volumetric flask. Vortex briefly and keep 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 taken 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%. Prepare a 1/20 dilution of the 1-mL aliquot with 1M HCl and record absorbance at 288 nm and 348 nm against 1M HCl as a blank on a UV/VIS spectrophotometer using scan analysis.

b. MYT stock solution II (100 µg/mL): Based on the actual concentration of stock solution I, the solution is diluted to yield 25 mL of a 100 µg/mL stock solution II. The dilution is done with degassed 0.5 M HCl containing 1% ascorbic acid in a 25-mL volumetric flask. Aliquots of this stock solution II (1.2 mL) are stored at -70°C in 2-mL labeled cryovials.

c. MYT stock solution III (20 µmol/L): Thaw one vial of stock solution II approximately once a month and dilute approximately 1/10 (depending on MW) with degassed 0.5 M HCl containing 0.1% ascorbic acid in a 10-mL volumetric flask to yield a 20 µmol/L stock solution III. Aliquot 500-µL portions into 25 microcentrifuge vials and store at -70°C. This stock solution III is used as a working standard for folate analysis on a daily basis.

(4) $^{13}C_5$-PGA (Internal standard): Stock solution I, II and III for PGA internal standard are prepared exactly as unlabeled PGA.

(5) $^{13}C_5$-MET, $^{13}C_5$-FOT, and $^{13}C_5$-THF (Internal standard): Stock solution I, II and III for these three reduced folate internal standards are prepared exactly as unlabeled reduced folates.

(6) $^{13}C_5$-MYT (Internal standard): Stock solution I, II and III for MYT internal standard are prepared exactly as unlabeled MYT.

Information on absorption maxima, absorption coefficients, and formulas to calculate the molar concentration for each folate stock solution is provided in **Addendum 1.**

Note:
Both labeled and unlabeled stock solution III are made fresh bimonthly from stock solution II for each folate analyte. The remainder of stock solution III is discarded after use.
Both labeled and unlabeled stock solutions II & III have been shown to be stable for at least 9 months at -70°C.

c. Preparation of Quality Control Materials

Three levels of serum and whole blood controls are analyzed in duplicate in each run as bench quality control materials. Approximate target values for serum TFOL are 7-10, 25, 50nmol/L; for RBC TFOL 300, 600, and 1000 nmol/L RBC. Since foods are fortified with folic acid in the US, it is difficult to find donors with low serum and RBC folate levels. In addition to TFOL, concentrations of individual folate species are considered. If specimens don't contain the approximate target values for the individual folate forms as shown in **Addendum 2**, manipulation through spiking with standard compounds or dilution with BioRad protein diluent or physiologic sodium chloride solution is considered. Including one or two donors with MTHFR T/T genotype is advisable to obtain whole blood that has endogenous levels of THF and MYT. It is acceptable to have one folate species at low concentration and the next species at a high or medium concentration in the same pool.

The serum is pooled and pools are filtered through gauze before being dispensed to remove fibrin. Serum (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.

To generate whole blood QC pools, fresh blood (~40 mL) is collected from volunteers in 7-mL 1.5% K3EDTA vacutainers. The vacutainers are rocked for 5-10 min at room temperature and the whole blood is diluted with 1% ascorbic acid, pH 4.0 to achieve a 1:11 dilution. Whole blood lysate (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.
Means plus range limits for all pools are established by analyzing duplicates for at least 20 consecutive runs.

d. Other Materials

a. Automated sample dilution on Hamilton Liquid Handler
- 96-well sample collection plate (Whatman)
- 96-well collection plate seals (Whatman)
- 1 mL and 0.2 mL plastic pipet tips (Hamilton)
- 300 mL plastic reusable reagent and water troughs (Hamilton)

b. Automated solid phase extraction (SPE) on Gilson 215
- 96-well Versaplate adjustable loose 1mL, 100 mg bed phenyl cartridges (Varian, Harbor City, CA)
- 96-well Versaplate with cartridges 1mL, 100 mg bed phenyl (Varian)
- 96-well BondElut SPE 100 mg bed phenyl block (Varian)
- 96-well format Versaplate base (Varian)
- Captiva 96-well filter plates (0.45 µm PVDF embedded into the well) for efficient automated filtration with vacuum manifold (Varian)

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

d. Other Items
- C-8(2) analytical HPLC column, 150 x 3.2 mm, 5 µm (Phenomenex, Torrance, CA)
- 0.5 µm stainless frits A-102X (Chromtech, Apple Valley, MN)
- PEEK tubing 0.005, and 0.007 ID (Supelco)
- HPLC Solvent glass inlet filters, purge frits, gold seal and outlet caps (Agilent)
- Blue tips (100-1000 µL) for Eppendorf pipette (Brinkmann)
- White tips (1000 µL) for Eppendorf pipette (Brinkmann)
- Yellow tips (10-100 µL) for Eppendorf pipettes (Brinkmann)
- Yellow tips (0.5-10 µL) for Eppendorf pipette (Brinkmann)
- Combitip plus (500 µL) for Eppendorf repeater pipette (Brinkmann)
- Positive displacement pipette tips (50 µL , 100 µL, 1000 µL) for Gilson pipette (Gilson)

- 30 mm Nunc 1-mL 96-well HPLC collection plate for 96-well autosampler (Fischer Scientific)
- Nunc plastic seals for 30 mm 1 mL 96-well plates (Fischer Scientific)
- 12x32-mm glass autosampler vials and 200 µL glass inserts (Kimble)
- HPLC solvent filter degasser, model FG-256 (Lazar Research Laboratories, Inc., Los Angeles, CA)
- 0.45 µm PVDF filters (Millipore, Bedford, MA)
- 0.45 µm water filtration units 500 mL capacity (Nalgene)
- 2.0 mL polypropylene cryovials (Nalgene Company, Rochester, NY)
- 1.0 mL disposable syringes (Hamilton)
- Syringe filters (Millipore)
- Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware

e. Folate Standards
- PGA, MET, FOT, MYT, and THF (Merck Eprova, AG, Im Laternenacker 5, Schaffhausen 8200 [CH] Switzerland, www.eprova.com)
- $^{13}C_5$-PGA, $^{13}C_5$-MET, $^{13}C_5$-FOT, $^{13}C_5$MYT, and $^{13}C_5$THF (Merck Eprova, AG)

f. Chemicals and Solvents
- Ammonium hydroxide (JT Baker Co)
- L-Cysteine (Sigma)
- Potassium phosphate dibasic and monobasic salts (Fisher Scientific Co)
- Formic acid reagent grade (Sigma)
- Acetic acid reagent grade (Sigma)
- L-ascorbic acid, ACS certified (Fisher Scientific Co)
- Methanol, acetonitrile HPLC grade (Burdick & Jackson Laboratories, Muskegan)
- Water, 15 megaOhm-cm, HPLC grade (Millipore)
- Nitrogen ultra pure (>99.99 % purity) (Air Products, Atlanta, GA)

e. Instrumentation

To provide adequate throughput for this method we have 2 LC-MS/MS instruments since November 2007. Not both instruments are necessarily used daily, but quite frequently.

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 fluorescence and G1315B photodiode array detectors
- HP Chemstation with Windows NT software (CPU Kit - with G2170AA, G2180AA)

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

Sciex API 4000 triple quadrupole mass spectrometer with turboionspray as ion source (TIS) in ESI mode, with Analyst 1.4.2 Windows Microsoft software (AB Sciex, Foster City, CA)

Sciex API 4000 QTRAP triple quadrupole mass spectrometer with turbo ionspray as ion source (TIS) in ESI mode, with Analyst 1.4.2 Windows Microsoft software (AB SCiex, Foster City, CA)

Peak Scientific nitrogen generator (Model No: 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 in addition to nitrogen gas for the collision cell (Peak Scientific Instruments,1300 West Belmont Ave, Chicago, IL)

Parker Balston Analytical Gas generator (5001 NTNA LC-MS/MS) Gas Generator Source 5000.

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)

Hamilton Liquid Handler for sample preparation and dilution (Hamilton)

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

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

Gilson Pipetman pipettor, 50 µL, 100 µL & 1000 µL size (Gilson Inc.)

Eppendorf repeater pipettor (Brinkmann Instruments, Inc)

Galaxy Mini table top microcentrifuge (VWR Scientific Products)

Daigger Vortex Genie 2 mixer (VWR)

Magnetic stirrer (Baxter Scientific Products)

pH meter (Beckman, 360)

Cary 3E UV/visible spectrophotometer (Varian)

f. Preparation of Infusion Solvents and Folate Analytes for Manual Tuning

Infusion solvent (50:50 Water: Methanol with 0.1% acetic acid)

- Add 20 mL of MEOH in a 50 mL conical tube
- Next add 19.960 mL of water to above conical tube
- Add 40 uL of acetic acid, mix well

Preparation of analytes:

- We need 2 µg/mL concentration of folates for infusion (Can use lower or higher conc. as well depending on the analyte response on mass spec).
- We have stock solution III as 20 µmol/L (~10 µg/mL).
- We have stock solution II 100 µg/mL.

Preparation from Stock solution III

$$\frac{2\ \mu g \times 1000\ \mu L}{10\ \mu g} = 200\ \mu L \text{ from stock solution III}$$

Add 800 µL Infusion solvent + 200 µL from stock III
Total volume = 1000 µL

- Preparation from stock solution II (100 µg/mL)

$\frac{2\ \mu g \times 2000\ \mu L}{100\ \mu g} =$ 40 µL from stock solution III

Add 1.960 mL Infusion solvent + 40 µL from stock II = 2000 µL

Manual Tuning and Quantitative Optimization

- Open manual tuning by double clicking on "manual tuning".
- Choose positive polarity.
- Deselect parameter range.
- Choose Q1 Scan & enter start 100 amu and stop 500 amu (based on mol. wt of analyte), enter time as 3.000 seconds.
- The duration will be calculated as 5 minutes in duration column.
- Start the syringe pump (Harverd) containing analyte at sufficient concentration in the syringe at 10 µL/min flow rate (ignore the warning that appears by pressing OK).
- Then click Start on Analyst and look for your molecular ion of interest in the profile that appears on explore window as TIC and MS/MS spectrum.
- In positive ion look for M+1, and for M -1 in negative ion (m/z).
- Intensify your ion of interest by changing parameters under source gas and compound tabs.
- Under source gas change following:
    - Curtain gas ~ 15
    - Gas 1 ~ 12
    - Gas 2 ~ 6-10
    - Ion spray voltage 4500-5000
    - Temperature 0 always in Q1 scan
- Under compound tab:
    - The DP range is 40-80 for folate analytes & is compound dependent (instrument dependent). Optimize in small increments or decrements to clear adjacent clusters from the molecular ion of interest. For API 5000 default DP is 150.
    - EP should be always left at 10 (default).
    - After you see the molecular ion in Q1 Scan, click on center width and enter your molecular ion mass. Select width as 5 amu, time 0.5 seconds go to advanced MS Tab and select fast profile and hit start.
        - In explore window containing TIC and MS/MS spectrum a sharp molecular ion peak M+1 is seen.
        - Now go on advanced tab and select MCA, and hit start again.
        - Observe for 10 or 50 MCA for molecular ion & stop. Right click on the profile and select user text/captions functions and write the analyte name/molecular ion m/z/analyte conc./date/composition of infusion solvent.

  - Click off the MCA and optimize and decluster the sensitivity of molecular ion by changing DP from low to high gradually.
  - DP will help reduce the background and your molecular ion will look as a dominant peak.
    - Save it here as Q1 Scan_Pos_Opt.
- Now select the multiple ion and optimize again the DP, and Gas 1, Curtain gas, Ion Spray voltage etc.
- Next select product ion (MS2 Scan) and enter mass range 50-500, time 3.000 seconds.
- Enter the exact mass for the precursor ion (product of use mol ion here) and click Start.
- The explore window containing the TIC and MS/MS spectrum, you should be able to see the molecular ion first as a dominant peak. Next the fragments (products) of molecular ion should be produced and see in the profile.
- While in product ion (MS2) go to edit ramp icon and ramp for DP, CE and CXP. Note that CE & CXP are already pre-selected and need to be optimized.
- For DP choose 20-300 voltage range, changes steps to 10.
- For CE choose Start - Stop voltage range between 5-100, change steps to 5
- For CXP use the default scan range.
- Record new optimum ramped values for DP, CE, and CXP in the MS method table.
- Click off the “Ramp Edit” icon and with the new updated method parameters perform MS2 scan again.
- Save again here as MS2_Scan_Pos_Opt.
- Next go to the MRM scan and enter your Q1 (molecular ion) and Q3 (MS2 mass) masses here. Set dwell time 300 ms per transition.
- If the parameters optimized in MS2 scan change by bringing MRM scan, then re-enter those values and select by clicking on “Edit Ramp” again in MRM mode for DP, CE, CXP optimizations.
- Observe optimum CE trace for transitions in the explore window. If the DP, CE, CXP parameters change in MRM in response to new optimization, update the method file again.
- Save the file as MRM_Pos_Opt.
- Now close the tune page [manual tune - can click on T] the instrument asks to save changes (say – NO).
- Go to the Quantitative Optimization tab and double click.
- Optimization page will open up and a small screen pops up:
  - Select infusion.
  - Select mass ion ± 2.0 amu.
  - Select MS/MS.
  - Go to criteria and select how many fragments you are interested to get form the precursor ion. (Enter 18 for water and 30 for threshold) or you can try default numbers as well.
  - Click Next.
- After you click Next another window screen opens up
  - Select positive ion mode here.
  - Select Q1 as unit resolution and Q3 as unit resolution.

- Click Next.
- In the next window screen enter the name of your compound, its molecular weight (exact), and charge. Example: FA is name of the analyte, its molecular weight is **441.4** (molecular ion is **442.4** in positive ion mode). The charge is always 1 if the molecule gains or loses one ion.

- Click finish and automatic optimization will start.
- At the end of the automatic optimization, in explore window you will get the profile of most intense precursor to fragment peaks (selected in the method earlier). This will appear as Q1/Q3 transition window. Note - Do not close this page prior to printing (will get lost).
- While on the same page right click on mouse and select user text enter comments about the compound (name/concentration etc.) and print as pane/window to save for documentation or save as a PDF in the respective folder.
- Quantitative optimization generates & automatically saves on main drive method, data and log files.
- In explorer go to the file/create separate folders for method, data, & log files and keep same date for all folders. For example: Give following name to the method, data & log folder and move all related files together: 06-10-08 (month/day/year).

Trouble shooting

- If signal is too weak stop the analysis, go to tune page detector tab and increase CEM up to 2200 (usually 1800-1900).
- If signal is too strong then stop the analysis, got to tune page detector tab and decrease the CEM to 1800 or 1900.
- Then start the analysis again.
- Or as an alternative you can dilute your analyte.
- If you have too many isotopic peaks or clusters around your mol ion more intense than the ion of interest then change ion mass ± 2.0 amu as ± 1 amu or even you can select as ± 0.5 amu.

LC/MS/MS method: acquisition method

- To establish the LC method with the MS/MS, select and activate LC/MS/MS from the hardware profile.
- Print the log file from the computer.
- Open acquisition method page from the Analyst software.
- Enter all method information from the log file (always cross check the values with manual tune method) for Q 1 and Q3 transitions.
- Go to Pump enter LC parameters.
- Go to the Autosampler and enter the parameters here.
- Go to column compartment and enter the parameters here.
- Save this method _Save as and give it a unique name. For example give Analyte/ Project name. Close the window.

Making Batches in the Analyst

- Go to open batch tab.
- Enter set name.
- Using drop down menu choose the acquisition method.
- Click on enter sample number-a window opens up.
- Enter sample id, data file name here.
- Right click on mouse and select autosampler features (sample Id, vial, plate, injection, comments etc).
- Choose relevant components here.
- Click on quantitation icon and enter quantitation method.
- Save the batch by selecting as save feature give it a proper name relevant to the project.

7. Calibration and Calibration Verification Procedures

Results of in-house SPE recovery studies 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). Results of in-house recovery studies based on area ratios (analyte/IS) of the spiked serum were complete for all three analytes (90% ± 10%).

The SPE efficiency (independent of the IS) for folate extraction from whole blood found was 77% ± 3% for MET, 80% ± 4% for FOT, 81% ± 6% for PGA, 72% ± 12% for MYT, and 46% ± 8% for THF. 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.

Limits of detection (LOD at S/N = 3) and quantitation (LOQ at S/N = 10) for serially diluted serum and whole blood lysate samples are shown below. Due to variabilities in hematocrit and serum folate concentration from sample to sample, we cannot report an exact LOD for RBC folate.

API 4000 Instruments

	LOD (nmol/L)	LOQ (nmol/L)
MET	0.5	1.5
FOT	0.1	0.3
PGA	0.3	0.9
MYT	0.3	0.9
THF	1.0	3.0

In-house studies showed that aqueous calibration gave the same results as calibration in serum. Daily calibrations are therefore performed in water.

a. Preparation of a daily 6-point calibration curve

At the beginning of each run, prepare two mixed calibrators (**Mix A & Mix B**) using the working standard solutions prepared as described in section 6 of this document. :

Standard Mix (Mix A): Contains mixture of standards (MET 2.0 µmol/L; PGA, FOT, THF, and MYT, all at 1.0 µmol/L) prepared in 0.1% ascorbic acid.

Internal standard Mix (Mix B): Contains mixture of internal standards ($^{13}C_5$-MET 200 nmol/L; $^{13}C_5$-PGA**,** $^{13}C_5$-FOT, $^{13}C_5$-MYT, and $^{13}C_5$-THF, all at 50 nmol/L) prepared in 0.1% ascorbic acid.

In a 1.5-mL microcentrifuge vial, add 50 µL from **Mix A** (mixed calibrators) to 950 µL of ammonium formate buffer (Solvent #1, pH 3.2, 0.5% ascorbic acid) to prepare the highest calibrator as-**S5** (100 nmol/L MET, and 50 nmol/L each of PGA, FOT, MYT, and THF).

From this calibrator **S5**, an aliquot of 200, 40, 20 and 10 µL is added to 1.5-mL microcentrifuge vials containing 800, 960, 980, and 990 µL of ammonium formate buffer (Solvent #1) to prepare calibrators **S4, S3, S2**, and **S1**. Calibrator **S0**- is a zero-calibrator that only contains the internal standard mixture. A reagent blank is included prior to the zero-calibrator.

To construct a 6-point calibration curve, an aliquot of 275 µL from calibrators 1 to 5 is added into the first row of a 96 well-plate starting from wells 2-7 that contain pre-aliquoted 495 µL of solvent #1 and 55 uL of internal standard mix (Mix B). Next, aliquots of 275 µL deionized water is added to make a final volume of 1.1 mL in each well. To well #1 (Reagent blank) 825 µL of solvent #1 and 275 uL of deionized water are added. To well #2 (zero-calibrator) 770 µL of solvent #1, 275 µL of deionized water, and 55 µL of internal standard mixture (Mix B) are added.

All calculations are based on results obtained from the daily calibration curve. Area ratios between the analyte area and the internal standard area from single analysis of each calibrator are calculated and a linear regression equation is generated for the 6-point calibration curve (not forced through zero). The concentrations for each calibration curve are listed below:

MET: 0, 1, 2, 4, 20, and 100 nmol/L (S0, S1, S2, S3, S4 & S5)
PGA, FOT, THF, and MYT: 0, 0.5, 1, 2, 10, and 50 nmol/L (S0, S1, S2, S3, S4 & S5)

At the end of each run, the calibration curve is reanalyzed as unknowns. The measured concentrations of these calibrators should agree within ± 15% of their set values for each compound.

b. Calibration of the MS instrument

1) The tuning and mass calibration of the API 4000 and 4000 QTRAP quadrupole 1 and quadrupole 3 is performed by infusing a solution of polypropylene glycol [PPG 0.1mmol/L] via a Harvard syringe pump at 10 µL/min flow rate using the Manual Tune (Q1 and Q3 scans) or Auto Resolution option. Usually instrument calibration is performed during bi-annually scheduled preventative maintenance by an Applied Biosystems Service Engineer unless otherwise required.
2) The PPG calibration solution kit contains a PPG standard 2000 vial for positive ion mode calibration and a PPG standard 3000 vial for negative ion mode calibration.
3) Preparation of the PPG standard 2000: API 4000 uses a 1:50 dilution; add 0.4 mL of PPG standard for positive ion calibration to 19.6 mL of PPG dilution solvent (see section 6a for how to prepare dilution solvent).
4) PPG 3000 is used directly without any dilution.
5) Follow the manufacturer's hardware manual for instructions and notes in **Addendum 3** for calibration with PPGs.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A run consists of the following sequence of samples: reagent blank, 6 calibrators (including a zero-calibrator), first set of QCs, patient samples, and second set of QCs. The assay can be run in the 96-well plate format (routine runs) or in a single cartridge format (R & D). Samples can either be prepared manually (R&D or trouble shooting) or by automated pipetting (routine runs) for SPE. SPE can either be carried out manually (R&D or trouble shooting) or automated using the Gilson instrument (routine runs).

a. Preliminaries

1) Prepare buffers and mobile phase (can be made ahead of time).
2) 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).
3) Prepare fresh 0.1% ascorbic acid for **Mix A** and **Mix B**.
4) Add 1% acetic acid to the pre-made mobile phase only before use.
5) Allow frozen serum or whole blood hemolysates (QC or unknown patient samples), stock standard and internal standard solutions to thaw and reach ambient temperature (this takes about 40 min.).
6) Label 1.5-mL microcentrifuge vials for blank, standards, controls, and patient samples (manual preparation for occasional R&D runs) or mark the 96 well-plate rows for the number of samples to be analyzed (automated preparation for routine runs).
7) Vortex thoroughly stock standard solutions, QC specimens, and unknown patient specimens before pipetting.
8) Prepare mixture of standards (**Mix A**) first, then mixture of internal standards (**Mix B**) as described in section 7a.
9) Prepare calibrator **S5** as follows:
    - 950 µL sample solvent #1 + 50 µL from Mix A.
10) From calibrator **S5** prepare calibrators from **S4, S3, S2 & S1** as follows:
    - 800 µL (vial S4), 960 µL (vial S3), 980 µL (vial S2) and 990 µL (vial S1) of sample solvent #1
    - Add 200 µL (vial S4), 40 µL (vial S3), 20 µL (vial S2), and 10 µL (vial S1) from calibrator **S5.**
11) Vortex all calibrators to ensure adequate mixing.

b. Manual Sample Preparation for Solid Phase Extraction (occasional R&D runs)

1) Arrange labeled vials in pre-marked racks.
2) Reagent blank vial: add 825 µL solvent #1 (vial 1).
3) **Zero-calibrator vial** (**S0**): add 770 µL solvent #1 (vial 2).
4) **Calibrator vials (S1-S5**): add 495 µL solvent #1 (vials 3-7).
5) To all the above vials add 275 µL deionized water (vials 1-7).
6) **Calibrator vials**: add 275 µL of each calibrator (S1 to S5; vials 3-7).
7) QC and patient sample vials: add 770 µL solvent #1 and 275 µL specimen (vial 8 and beyond).
8) To all of the above vials except reagent blank add 55 µL of internal standard mix (mixed calibrator B), so that the final volume is 1.1 mL (vials 2 and beyond).
9) Vortex all tubes and incubate at 4ºC for 20-30 min.
10) Alternatively use 96 well-plate (typical) to prepare calibration curve (exactly as above) and unknown specimens starting calibration curve from row #1.

c. Automated Sample Preparation for Solid Phase Extraction on Microlab Star^{Let}-Hamilton (routine runs)

1) Check the tip racks for 1000 µL and 200 µL tips on Tip Carrier-Tip _CAR_480_A00, at location 1-5 on worktable, and restock if needed.
2) Wash the sample buffer and water troughs thoroughly with warm water and then rinse with deonized water.
3) Load these troughs onto Reagent Carrier- RGT_CAR_3R_A01 at location 7 on worktable. In trough at position 1, add 1% ammonium formate buffer (solvent #1) and in trough at position 2 add deionized water.
4) Arrange calibrators, one set of QC samples and unknown specimens on Sample Carrier-SAMP_CAR_32_A00 at location 8-9 on worktable.
5) Place 8 glass tubes (16x100) containing internal standard mixture (Mix B) on Sample Carrier -SAMP_CAR_32_A00 at location 11on worktable.
6) Place a 2-mL 96-well collection plate for sample collection on destination rack (18209010l) located between 12 to17 on worktable.
7) Go to the **Microlab Star Run** method icon on desk top, double click to open a work station window.
8) Go to the open file menu, locate the Folate Method folder, and open the method to run. (File→ Open→Folate 2007/Folate 2008 (choose relevant folder) →double click on the folder to open method→[for example Folate serum method] → Hit start {●Green icon})→Initialize→Edit tip count window appears→ Select Labware → Here ensure First and Last tip counts are correct →Go next → Prompt window appears → Here select Number of samples -Start position should be always at 8 (for a routine run) and minimum sample number is set as 10 and maximum number of samples depends on the daily run size.
9) Alternatively (R & D only) open the **Microlab Star^{Let}** through Method Editor if method needs updates, changes or evaluations to check correct performance.
10) After the dilutions are performed based on the method script, remove the destination plate, cover it with the 96-well plastic seal, and incubate it at 4ºC for 20 min (serum only) prior to SPE.
11) The WB method on **Microlab Star^{Let}** is split into two sub-methods
    - The WB sample aliquot (275 µL + 55 µL IS mix) are prepared on day-1. Calibration curve (275 µL each cal. + 55 µL IS mix) including reagent and zero blanks are also prepared on day-1 on a 96 well-plate. The sample plate is then incubated at 37°C/4h and stored at-20°C until next morning. On day-2 the sample in the plate are diluted with buffer/water according to the standard protocol prior to SPE on the Gilson.

d. Automated Solid Phase Extraction Using Gilson 215 SPE (routine runs)

1) Preparation of the Gilson 215 SPE system:
    - Method prime syringes and solvent ports: (Method Type = Fixed Tray sequential, configuration = 215 SPE with solvent station at CDC)
    - On the monitor that is connected with computer for Gilson, from desktop right double click on 735 Sampler Software icon and choose Application, and enter by saying OK
    - Go to the File menu pull down and select New

- Application Window appears, select application and enter OK here.
- Next window is "Choose application mode", here click on T icon (to select the desired Tray).
- We have methods for 2 Trays as *Micronutrient Tray 1, Micronutrient Tray 2,* and *Micronutrient Tray 2 with tracking.* Micronutrient Tray 1 is specified for 1-mL volume racks and Micronutrient Tray 2 and Micronutrient Tray 2 with tracking is specified for 2-mL volume racks (collection plates).
- The current method uses Micronutrient Tray 2 with tracking, highlight and select it by entering OK. (Tray arrangement should be selected as default at fixed)
- From the main menu window go to Process and select Insert Steps, Insert Steps window appears with Method applications.
- Select "Prime Syringe and Solvent Port" method and enter insert, click OK.
- Next window appears for run method, here save applications with a simple and suitable name (for example 100208 as month/date/year), click OK and go back to main menu window.
- From the main menu window go to options and deselect Simulate.
- Now go to green icon and click on it to start the run.

Description: Prime pump at a flow rate of 6 mL/min, for a total of 15 mL solvent from the reservoir. Prime transfer ports at 6 mL/min with 5 mL of each solvent #1 at port 1 solvent #2 at port 2, and solvent #3 at port 3.

> ***NOTE**: On Gilson-2 in lab 1207 we use plastic bottles for solvent #1, # 2 & # 3 (instead of glass bottles on Gilson-1/Rm1201) and 8 probe directly aspirates solvents from these bottles, thus does not need to be primed. We only perform initial purge/prime through reservoir (60% MEOH). On Gilson-2 we add only 150 mL of solvents in these bottles for each run and the leftover is discarded at the end of the run.*
>
> *Instead Gilson-1 is a different design and has Valvemat connected that uses lines to pull liquid from the glass solvent bottles and hence needs line priming with each solvent prior to run. We use ~500 mL solvents in these bottles.*

2) Loading sample rack and collection rack on the Gilson 215 SPE system:

- Load: Prepare the calibration standards and patient samples as described above in section 7a and section 8a-c and pipette 1.05 mL into the 2-mL sample rack for each sample (manual prep).
- Typically samples are already prepared on a 96 well-plate for the load step.
- Use a 96-well block embedded with 100 mg phenyl sorbent (BondElut or Matrix, Varian) with rack 804 or 805 for SPE (typical). While the 96-well block is more expensive than individual custom cartridges, it provides a perfect Z-arm seal at the top of the plate during the SPE procedure is efficient and involves less preparation time.
- Keep the collection plate on the front end of the 805 rack.
- Alternatively, individual SPE cartridges can be used:

- o Place the 2-mL 96-well plate containing the samples on the 205H tray and another fresh 2-mL 96-well plate as collection plate on a custom tray (Versatubes) for collection of the extracted samples.
- o Keep the collection plate on the front end of the custom rack (Versatubes).
- o Load Versaplate with as many cartridges as are needed for the samples in each run (customized).
- o Make sure the cartridges are pushed down and leveled uniformly to ensure proper sealing under the Gilson Z-arm. The cartridge extractor tool can be used manually to check for uniform height across the rows.
- o Position the Versaplate with cartridges on top at the back of the custom tray.
- o Run time for each row (8 samples) is ~25 min. It takes ~ 2.5 h to extract 6 rows of samples (48 samples) on Gilson that includes 2 sets of QC, 7 calibrators and 35 unknown specimens (routine).

3) Running the Gilson 215 SPE System:

- After lines and syringes have been primed, start the application again.
- To start the application, go to Process from main menu window and select "Insert Steps".
- Select the Method Versaplate Final 2 mL with volume track and spevac/Method 96-well Block 805 with volume track spevac.
- Deselect the autoscale function to visualize the full racks image.
- The sample rack at 205H will appear in yellow color; highlight and select in this rack as many rows as needed for the sample extraction with the mouse.
- Click on insert and say OK.
- The start method window appears.
- The syringe and solvent port method application that might be still on the screen can be highlighted with mouse and deleted or left there.
- If left on the main screen then right click on the first row of your current Method that needs to be started (Method Versaplate Final 2 mL with volume track and spevac) and hit start step (this is the first function that appears on right click of mouse) to select the sequence of the rows
- Go to the green icon and press to start the run.

The Gilson 215 SPE system processes 8 wells simultaneously (approx 25 min/row). All SPE steps, such as cartridge conditioning, sample loading, cartridge washing and sample elution from the cartridge are performed automatically.

Description:
Condition: In 3 Steps, 1.5 mL methanol at solvent bottle 1; 1.5 mL acetonitrile at solvent bottle 2; and 1 mL solvent @ 1
Load: 1 mL from tray at plate front-1 rack
Wash: In 2 steps, 1.5 mL each with solvent @ 2
Elute: Single step into Versaplate collect with 1.0 mL solvent @ 3

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: 2x1mL of methanol and 2x1mL 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 some clean ones.
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. Manual Sample Preparation for LC/MS/MS Analysis (occasional R&D runs)

1) Arrange and label 1.5-mL microcentrifuge vials as many as needed.
2) Using 1-mL Eppendorf pipette with disposable tips, pipette SPE eluate from the collection rack into these vials.
3) Using 1-mL disposable syringes and 0.45 µm filters, filter 250-300 µL of the eluate directly into 250-uL 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.

g. Automated Sample Preparation for LC/MS/MS Analysis (96-well plate format for routine runs)

1) After SPE is completed, transfer 700 uL of the extracted sample into a fresh Captiva filter plate that is placed on top of a fresh autosampler collection plate (31 mm Nunc plate) using a transfer method on the Tecan liquid handler.
2) The samples are then filtered through the Captiva filter plate using a vacuum manifold (IST) at 5 mm pressure for 5 min.
3) The autosampler collection plates are sealed with the pierceable seal and arranged on the HP1100 or HP1200 96-well model autosampler for LC/MS/MS analysis.

h. LC/MS/MS Instrument Preparation

1) <u>HPLC Line Purging and Priming:</u>

   Before every run, the HPLC lines are purged and the column is primed with a series of solvents.

   - Open the purge valve of the binary pump (black knob) 2 or 3 turns

- Operating through the handset module of the HP1100 click on setting menu and select binary pump
- Select Channels B1 (methanol: water 90:10) for pump and hit enter
- Enter 5.0 mL/min as flow rate and enter done
- Start the pump by entering ON/OFF key
- Let the pump purge for 5-10 min; pressure should be <5 bar
- After 10 min enter 0.0 mL/min flow rate and close the purge valve
- Enter 500 µL/min flow rate and let the column prime for 5-10 min
- Record the pressure into the logbook; pressure (~80 bar typical)
- Go to flow rate 0.0 mL/min and open the purge value again
- Go to setting menu and select the binary pump again
- Enter 0.0% for Channel B1.
- Then select Channel A1 and enter 100% (mobile phase - A1)
- Go to flow rate 5 mL/min
- Let the lines purge for 5-10 min with the mobile phase, pressure should be <5 bar; record the pressure in the logbook. (if the pressure during purging is >6-7 bar, the white frit in the purge valve needs to be replaced)
- Go to flow rate 0.0 mL/min and close the purge value
- Enter again 250 µL/min flow rate and let the column prime for another 10 min; record the pressure into the logbook, it should be (~75 bar typical) when the column is relatively new and up to >110 bar when column is relatively old (~3000 samples injected in normal flow)
- After this purging and priming the pump is ready for analysis.
- Close pump by entering **ON/OFF** key to turn it off or leave it running at 250 µL/min flow rate of mobile phase until samples are ready to be run.

2) Mass Spectrometer Preparation and Daily Check:

MS/MS method parameters for each folate species and the corresponding internal standards are listed in **Addendum 4**. Before every run, the Turbo Ion Spray (TIS) source is taken apart, the curtain plate is removed, and the Orifice plate is cleaned with methanol dabbed lint-free Kim wipes. The curtain plate is first cleaned with warm water or soap & water (if too dirty) then wiped with methanol dabbed lint-free kimpwipe. The ion spray needle is checked for any blocks by flushing methanol within the tubing using a 1-mL syringe (once a month or as needed). The Turbo Ion Spray source is put back in place and from the desktop Analyst 1.4.2 software is started. At least two sample solvent injections are run as a daily routine before the actual run is started. To verify that the system is working OK, either a zero-calibrator (mixture of internal standards) or the low QC sample from the previous run can be injected prior to the analysis of next run (as a instrument check).

3) Prepare run by loading method: Folate MRM Method 2008.dam

- First thing to do before the Analyst software is started,
  - **o** On desk top go the service icon. Click on stop service to Analyst.
  - **o** Then click on start service to Analyst.
- Next start by opening Analyst software from desktop (Analyst icon).
- Select your project directory (for example “Folate2008”)
- Go to build acquisition batch mode and highlight it.
- Then go to the file open menu and double click on it.

- Click on the acquisition batch file folder.
- Pull one of the previously saved the acquisition batch files and open it.
- Enter a new date in the data file column (for example “Se100108”) and save it as a new batch (for example “FolateSe090103”).
- Now make all necessary changes to the new batch or make changes to the quantitation method (Qunt. Method needs changes only if the sample set is larger or smaller then the previous batch).
- Thoroughly check your vials for correct positions.
- Cross check whether the method is associated with the correct quantitation method. Make necessary changes if needed (depends on your sample size).
- Save the batch again and submit it for analysis.
- Always submit 2 or 3 solvents injection and a zero-calibrator or low QC (from the previous run) before starting the new sample run to ensure that the system is working properly, the background is low and clean and all MRM transitions are as expected.
- If all looks OK for the test run, submit the new sample set.
- All LC-MS/MS batches for MRM runs should be associated with the correct Method name for example: **Folate_MRM Method_2008.dam**

4) HPLC Rinse Method & Shutdown Method

- HPLC rinse method: HPLC_Rinse batch should be associated with HPLC_Rinse method. This method runs in Q1 scan for 20 min in isocratic (90:10 methanol:water, Channel B1) solvent system.
  - **o** After the batch is loaded for analysis, go to acquire mode again and highlight it.
  - **o** Go to file menu and open acquisition batch files.
  - **o** Select the HPLC_Rinse batch file and open it.
  - **o** Enter new date and save it by replacing and submit the batch, then close it.
  - **o** Typically multiple methods can be submitted at the same time as the analysis method by selecting √ multiple icon
- Shutdown Method: Shutdown method batch should be associated with Shutdown method and runs in MRM scan for 1 min in isocratic solvent system (90:10 methanol: water, Channel B1)
  - **o** Go to the acquire mode and open file from file menu.
  - **o** Select acquisition batch again and open Shutdown batch file.
  - **o** Enter new date and save it by replacing and submit the shutdown batch, then close it.
  - **o** Typically shutdown method can be submitted at the same time as the analysis method by selecting √ multiple icon and selecting it from the method drop down menu.

i. Processing and Reporting a Run

1) Reviewing the run: When the batch run is finished acquiring the data, the data is viewed by opening the data file from Analyst 1.4.2 software. Chromatograms for each folate form (respective transition) are checked for retention time, peak shapes/separation/intensity and/or any co-eluting interferences.

2) Quantitation and integration of the completed data file:

- From the Analyst 1.4.2 software go to Quantitate icon and double click on it, Quantitation Wizard icon becomes available.
- Upon a double click on Quantitation wizard icon, a Create Quantitation Set-Select Sample window appears.
- All data files are available and can be viewed in this window.
- Double click on the instrument folder (typical) or the data folder
- All data files become available, double click on the required data file, the individual samples show up in the Available Sample window column.
- From here highlight the number of individual samples that need to be processed for integration and with the shift key on your key board highlight as many samples as needed for the results table and move with the help of double arrows (→→) those samples to the Selected Sample window column (typical). Otherwise if all samples from the data file are needed for results generation simply click on Add All icon.
- After selection of samples, click on Next and Choose setting window appears with options of Settings/Create Quantitation/Query
- Using pull down menu, bring Summary and select None in Default Query page (since Quantitation Method is already made prior to sample analysis and is associated with the datafile), then click on Next.
- Create Quantitation Set-Select Method window appears, click here on Choose existing method (For example Folate_SEQuant Method_API400.qmf) or (All Folate_ WB_ nmol/L.qmf) then next click on finish.
- A results table with auto integration is generated.
- To save this table, go to file and click on save as; Save Quantitation Results window appears; give name as Folate/matrix/month/day/year (**FolateSe100208-No spaces**). Quantitation result files are saved as rdb files. The same name as to the data file should be given to results file.
- Now review all peaks with a double click on any sample in the table.
- Review calibrators, QC's and unknowns for any necessary integration corrections and make changes either using manual option or auto integration option. Auto integration is preferred whenever possible.
- To see the chromatogram parameters click on show/hide parameters icon.
- Check bunching factor (should be left at 2 or 3/THF & MYT can be set at 1), number of smoothing should be 3. Make changes if necessary and accept it by selecting Apply & save it.
- Right click on mouse and select Update method.
- The Qunatitation Peak Review window appears; select No to any change to manually integrated peaks as the updating auto integration should only apply to auto integrated peaks.
- After these changes and review save the results file again.
- Print the results for each analyte for review and documentation (Research) or print & save to a PDF folder on your network drive for future.
- To process the results on another PC, copy the data file and batch file via a USB flash drive to the network (Q drive) in the Analyst folder\Project\ data\batch (typical).
- To import the results file to database, go to the table settings with a right click on mouse and select either "Serum results table for import "or "WB results table for import". A new results table is created with settings information that is needed for importing the results to the database.
- Next click on File, in the drop down menu select Exportis save the table with Import

3) Calculate the results using Excel:
   - The final integrated results can either be directly imported to the FrontEnds Access 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 FrontEnds Access 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.

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. At this point, the individual results from each folate species have to be added up to obtain a whole blood TFOL result. This whole blood TFOL can now be converted to a RBC folate result, but taking the hematocrit and the serum TFOL of the sample into consideration:

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

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. If the serum folate value is not available, assume a value of 18 nmol/L. When the serum value is low compared to the RBC value (or not available), 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 when necessary, and cleaned on a monthly basis. Filters in the solvent bottles are replaced when needed (6-8 months-routinely). The pre-column filters are replaced after ~200 injections. The curtain plate is cleaned on daily basis with water first and then wiped with lint free Kimwipe dabbed in

methanol. Orifice plate is also wiped daily with methanol dabbed lint-free Kimwipe. Preventative maintenance is performed by service engineers on all major equipment (HPLC, Gilson, Tecan, Hamilton) at least once a year and on MS twice 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 are inserted prior to the arrival of the samples in the Inorganic Toxicology and Nutrition Branch. These specimens are prepared at two levels so as to emulate the 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.

The results from the QC samples are checked after each run. The system is declared “in control” if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared “out of control”:

- 1_{3s} Any of the three QC results are outside the 3s limit
- 2_{2s} Two of the three QC results in the run are outside the 2s limit (same side of mean)
- R_{4s} Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean

- 10_x Ten sequential QC results (across pools and across runs) are on the same side of the mean

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 re-evaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in the ACCESS database. For runs that are not imported into ACCESS (exception, research-type runs), QC results are stored electronically in the analyte-specific folder on \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Data handling\Excel runs. A hardcopy of the QC results from each run is also kept by the analyst.

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

- 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.
- Run folate standards in Q1 scan mode to see if molecular ion is detected.
- Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator.
- Check the autosampler for proper sample injections.
- Look for sample preparation errors, i.e., if the analyst forgot to add internal standard, specimen, right volume of buffer etc.
- Check the calibrations of the pipettes.
- 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.
- Do not report analytical results for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

- The most common causes of imprecision are intermittently inaccurate micro-pipettors or pipetting errors.
- Stock standards, internal standards and specimens should be vortex-mixed thoroughly before pipetting.
- Handling stocks and internal standards in step-wise sequential manner will minimize the chances of cross-contamination.
- 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.
- Also change of gloves after preparations of stock and working standards and internal standards are recommended to avoid any contamination.
- 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.
- Buffers should be made fresh daily and pH should be checked.
- 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.

- Acetic acid (1 mL/100 mL) should be added to sample solvent #3 and 0.5% to the mobile phase only before use.
- Hemolyzed serum samples may give falsely elevated values.
- The entire sample preparation and calibration should be prepared in yellow sub-dued light.
- Multiple freeze/thaw cycles of specimens for extended time at room temperature will cause degradation of folates and should be avoided.
- 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.
- Exposure to strong sunlight for more than eight hours may cause 10-20% serum folate degradation.
- 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.
- HPLC system (lines and column) should be purged and primed properly.
- 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).

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. **The ruggedness testing findings for this method are presented in Addendum 5.** Please refer to Chapter 21 of the 2008 DLS Policies and Procedures Manual for further information on ruggedness testing.

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 (8). Clinical reference ranges reported for RBC folate are 317-1422 nmol/L with the CPB radioassay (8). Reference ranges derived from previous NHANES surveys have been determined with the BioRad assay, which runs on average ~35% lower than the microbiologic assay and the LC/MS/MS method. In our hands, the LC/MS/MS method and the microbiologic assay give comparable results. New reference ranges based on LC/MS/MS will have to be determined with NHANES 2007-2008 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. 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)

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX by the supervisor of any serum folate result that is <7 nmol/L or RBC folate result that is <317 nmol/L. Copies of FAX sent concerning abnormal results are kept in a notebook by the supervisor for the duration of the study.

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, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically several times weekly to the Westat ISIS computer and then are transferred 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 Microsoft Access 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 are kept on files in \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Data handling\Excel runs on the DLS LAN.

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

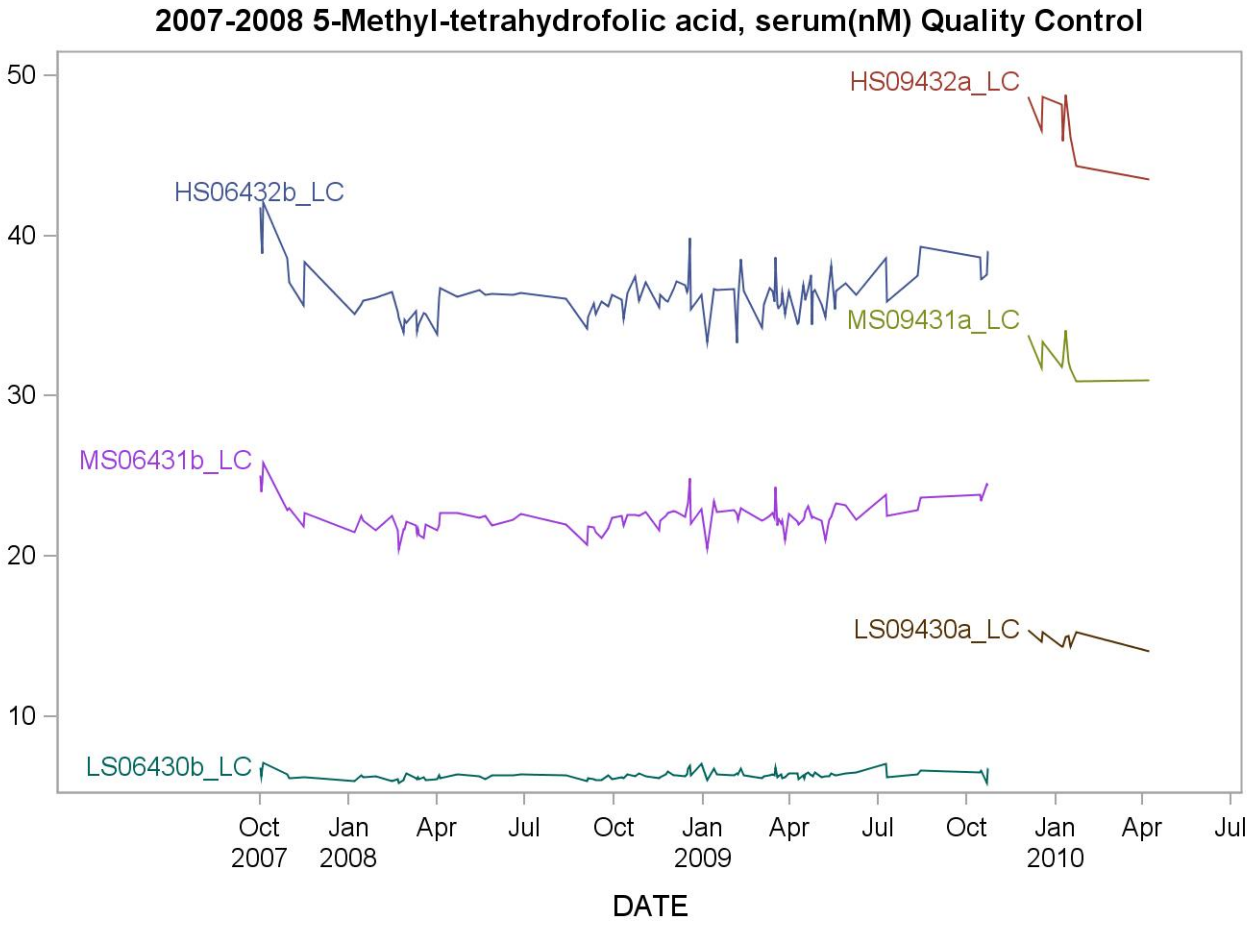
containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

19. Summary Statistics and QC Graphs

See next pages.

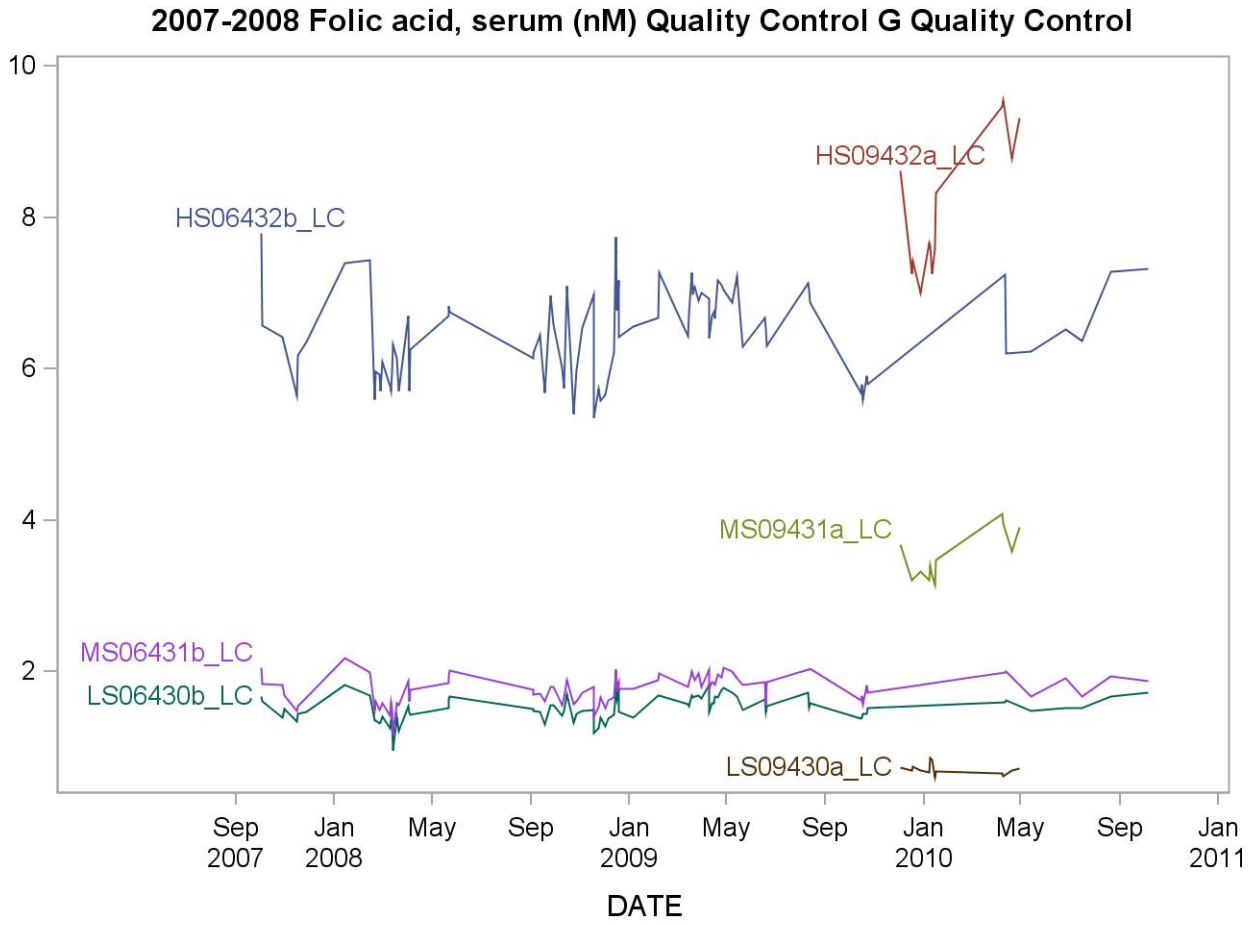
Summary Statistics for 5-Methyl-tetrahydrofolic acid, serum(nM)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS06432b_LC	110	01OCT07	23OCT09	36.380	1.594	4.4
LS06430b_LC	110	01OCT07	23OCT09	6.340	0.257	4.1
MS06431b_LC	110	01OCT07	23OCT09	22.529	0.921	4.1
HS09432a_LC	10	03DEC09	08APR10	46.815	1.868	4.0
LS09430a_LC	10	03DEC09	08APR10	14.790	0.465	3.1
MS09431a_LC	10	03DEC09	08APR10	32.275	1.119	3.5



Summary Statistics for Folic acid, serum (nM) Quality Control G

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS06432b_LC	93	02OCT07	06OCT10	6.452	0.588	9.1
LS06430b_LC	93	02OCT07	06OCT10	1.519	0.160	10.5
MS06431b_LC	93	02OCT07	06OCT10	1.771	0.186	10.5
HS09432a_LC	13	03DEC09	29APR10	8.144	0.907	11.1
LS09430a_LC	13	03DEC09	29APR10	0.708	0.073	10.3
MS09431a_LC	13	03DEC09	29APR10	3.499	0.318	9.1



References

1. Pfeiffer CM, Fazili Z, McCoy L, Zhang M, and Gunter EW. Determination of folate vitamers in human serum by stable-isotope dilution tandem mass spectrometry and comparison to radioassay and microbiologic assay. Clin Chem 2004; 50: 423-32.
2. Fazili Z, Pfeiffer CM. Measurement of folates in serum and conventionally prepared whole blood lysates: Application of an automated 96-well plate isotope-dilution tandem mass spectrometry method. Clin Chem 2004; 50: 2378-81.
3. Fazili Z, Pfeiffer CM., Zhang M., and Jain R. Erythrocyte folate extraction and quantitative determination by liquid chromatography-tandem mass spectrometry: Comparison of results with microbiologic assay. Clin Chem 2005; 51: 2318-25.
4. Fazili Z, Pfeiffer CM, Zhang M. Comparison of serum folate species analyzed by LC-MS/MS with total folate measured by microbiologic assay and Bio-Rad radioassay. Clin Chem 2007;53:781-4.
5. Fazili Z, Pfeiffer CM, Zhang M, Ram JB, Koontz D. Influence of 5, 10-methylene-tetrahydrofolate reductase polymorphism on whole blood folate measured by LC-MS/MS, microbiologic assay and BioRad assay. Clin Chem 2008; 54: 197-201.
6. Pfeiffer CM, Gregory JF III. Enzymatic deconjugation of erythrocyte polyglutamyl folates during preparation of folate assay: investigation with reversed-phase liquid chromatography. Clin Chem 1996; 42: 1847-54.
7. Mastropaolo W, Wilson MA. Effect of light on serum B12 and folate stability. Clin Chem 1993;39(5):913.
8. Tietz NW. Clinical Guide to Laboratory Tests. Third Edition. 1995. WB Saunders Company.

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 (Addendum 1-5)

Addendum 1

Formulas to calculate the molar concentration of the folate stock solutions

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

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 4441.4/27600 = **105.7**
Concentration (µmol/L) = 0.661 x 10 x 1000 x1000 /27600 = **239.5**

Addendum 2

Approximate target concentrations of the various folate species in QC pools

Matrix	Levels	MET	FOT	PGA	THF	MYT	TFOL
Serum (nmol/L)	Low QC	5*	1	1	1	1	7
	Medium QC	15	2.5	2.5	2.5	2.5	25
	High QC	30	5	5	5	5	50
WB lysate (nmol/L)	Low QC	5*	1	1	1	1	11[&]
	Medium QC	15	2.5	2.5	2.5	2.5	25
	High QC	30	5	5	5	5	50

***** as low as possible

[&] 11 nmol/L WB lysate folate corresponds to 122 nmol/L WB folate and 305 nmol/L RBC folate; this is the same as 140 ng/mL RBC folate (based on MET molar conversion of 2.179 and 0.4 HCT).

Addendum 3

Instructions for API 4000 calibration using PPG solutions

1. Open Analyst 1.4.2 software from the desk top.
2. High light Tune mode icon and under this click on Resolution Optimization feature.
3. "Resolution optimization options" window appears.
4. Select PPG's Pos against standard option by scrolling the pull down menu.
5. In Instrument option select **Quad 1** first and select resolution as **Unit**.
6. Click on positive polarity and check if reference is as PPG's Pos. calibration ref.
7. Check mark the mass Calibration on Success and hit start.
8. The Analyst will run the calibration for some time and upon completion Save Resolution Optimization by saying Yes
9. Now after the Quad 1 is calibrated deselect it and select Quad 3 and hit start to calibrate the Q3.
10. Save by clicking yes upon completion.

Note: Only **Quad 3** can be either selected as **High** or **Unit** for resolution based on sample and sensitivity desired. In case the samples have heavy matrix we may consider High resolution to get the peaks resolved. This change may though increase the background and signal intensity as well. **Quad 1** should always stay a **Unit** Resolution.

Addendum 4

Typical MRM Method Parameters (analysis in positive ion mode)

Analyte (Transition)	*DP	*CE	CXP
MET (*m/z* 460 ->*m/z* 313)	40	27	12
$^{13}C_5$-MET (*m/z* 465->*m/z* 313)	56	29	12
PGA (*m/z* 442-> *m/z* 295)	40	30	12
$^{13}C_5$-PGA (*m/z* 447->*m/z* 295)	45	30	12
FOT (*m/z* 474->*m/z* 327)	41	27	10
$^{13}C_5$-FOT (*m/z* 479->*m/z* 327)	60	29	10
THF (*m/z* 446->*m/z* 299)	60	27	12
$^{13}C_5$ THF (*m/z* 451->*m/z* 299)	55	27	12
5,10 CH=THF (*m/z* 456->*m/z* 412)	90	39	16
$^{13}C_5$-5,10 CH=THF (*m/z* 460->*m/z* 416)	86	37	16

DP, declustering potential; CE, collision energy; CXP, collision cell exit 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: 200 msec; ion spray voltage: 5 200 V; source temperature: 550 ^{o}C; curtain gas: 20 psi; gas 1: 20 psi; gas 2: 40 psi; CAD gas: 4.0 psi.

* DP and CE are voltages that are linked and can be adjusted based on analyte response and purity. If background is noisy use high DP to reduce adduct and dimer formation and choose low CE. If more sensitivity is desired use low DP and high CE.

Addendum 5

Ruggedness Testing – Folate Species by LC-MS/MS

Detailed information can be found at:

\\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA\Method Validation and Verification\Test Method Verification\Non-kit assays\FOL - LCMSMS

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.

1. **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 species 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 species 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.