QUALITY ASSESSMENT MANUAL FOR LABORATORIES USING THE CDC FOLATE MICROBIOLOGIC ASSAY KIT

Version 1

2021

THIS QUALITY ASSESSMEMENT (QA) MANUAL WAS GENERATED BY THE CDC NATIONAL CENTER FOR ENVIRONMENTAL HEALTH, DIVISION OF LABORATORY SCIENCES, NUTRITIONAL BIOMARKERS BRANCH.

LIST OF ABBREVIATIONS

ALTM	All laboratory trimmed mean
CAP	College of American Pathologists
CLIA	Clinical Laboratory Improvement Act
CMS	US Centers for Medicare & Medicaid Services
CV	Coefficient of variation
EQA	External quality assessment
HCT	Hematocrit
IATA	International Air Transport Association
LOD	Limit of detection
MBA	Microbiologic assay
NBB	Nutritional Biomarkers Branch
NIBSC	National Institute for Biological Standards and Control
NIST	National Institute for Standards and Technology
QA	Quality assessment
QC	Quality control
PT	Proficiency testing
RBC	Red blood cell
SD	Standard deviation
SOP	Standard operating procedure
UK NEQAS	United Kingdom National External Quality Assessment Service
WB	Whole blood

INTRODUCTION

Welcome to the Quality Assessment (QA) Manual for the folate microbiologic assay (MBA). This QA manual is specifically geared towards laboratories using the CDC folate MBA kit and complements the Folate Microbiologic Assay Training Manual. Whereas the training manual details the procedure of how to analyze samples with the folate MBA, this QA Manual provides additional information on sample collection, processing and storage; sample analysis and review; data reporting and external QA. A QA program ensures that the final results reported by the laboratory supervisor are accurate and of the highest quality. To achieve high quality results, full staff participation is required. QA addresses the following aspects: 1) avoidance of mistakes, 2) consistency of performance, 3) data integrity, and 4) opportunities for training.

A complete QA system covers aspects throughout the lifecycle of a sample, starting with sample collection and concluding with the reporting of results. This includes issues related to 1) internal quality control (QC) through the use of bench and blind QC samples; 2) equipment monitoring, maintenance, and function checks; and 3) external quality assessment via participation in performance verification or proficiency testing programs. Documentation of institutional policies and laboratory procedures, their review and approval through the chain of command, proper staff training and competency assessment, and periodic laboratory audits should also be part of any comprehensive QA system, but these aspects are only briefly covered in this QA manual.

Regardless of resources, the quality of laboratory analyses can only be ensured if internal QC is conducted as part of every run that contains any survey or research samples and if the validity of the assay is independently verified in an external performance verification program prior to starting with any survey or research sample analysis. These minimum requirements should be followed even in low-resource environments to ensure the accuracy of data.

SECTION 1. GETTING READY FOR SURVEY SAMPLE ANALYSIS

Planning for micronutrient surveys can be a lengthy process often involving a number of external and in-country agencies and organizations such as the Ministry of Health, international organizations and funding partners, the local implementing partner, potential consultants, and the main analytical laboratory selected to conduct some or all biomarker analyses. Over a period of months or years, these partners plan the numerous activities which must be coordinated to ensure efficient collection of samples from the target population and accurate determination and reporting of data for each micronutrient of interest. Typically, the main analytical laboratory takes at least temporary ownership of the survey samples and needs to develop processes to track and document sample information. The long-term fate of residual specimens (left-over after laboratory analyses are completed) and of potential pristine back-up specimens should also be part of the discussions. Thus, it is advisable that the analytical laboratory is involved early enough in the planning process.

Examples of activities relevant to the analytical laboratory during the planning phase of micronutrient surveys are listed below:

- Coordination among survey planners on survey content and logistics to include:
    - Target population group
    - Specimen matrix and volume
    - Samples size
    - Sample shipment logistics (e.g., availability of dry ice, required forms)
    - Timeline of sample collection and arrival
    - Sample inventory and proper storage
- Defining and executing agreement between analytical laboratory and customer for service work
    - Cost per test
    - Timeline
    - Hierarchy of analyses when specimen volume is limited
    - Data reporting format
    - Frequency of reporting
    - Required documentation (e.g., material transfer agreement, approved human subjects protocol)
- Verifying that laboratory documentation is in place
    - Approved standard operating procedures (SOPs) for sample collection
    - Approved SOPs for laboratory analysis
    - Laboratory staff training records
    - Laboratory instrument maintenance records
    - Laboratory instrument log sheets for function checks (e.g., refrigerator and freezer temperature log sheets)
    - Certificate of assay proficiency

- Verifying that laboratory informatics issues are in place
    - Spreadsheet for QC results
    - Spreadsheet for participant results
    - Process for data review, approval and tracking
    - Defining retention time for laboratory records

SECTION 2. SAMPLE COLLECTION, PROCESSING AND STORAGE

Many important factors must be considered when obtaining high-quality samples that are used in studies to assess nutrition status. Recognizing these factors and following strict procedures for collection, storage, management and transport of human samples can reduce pre-analytical contamination risk, ensure analyte integrity and thus promote accurate assessment of nutrition status.

An informative guide, ***Improving the Collection and Management of Human Samples Used for Measuring Environmental Chemicals and Nutrition Indicators,*** has been prepared by CDC's Division of Laboratory Sciences and is available at: https://www.cdc.gov/biomonitoring/human_samples.html. This resource, which is intended for use by epidemiologists, laboratory staff and other health scientists involved in the design and implementation of human biomonitoring studies, describes considerations to help ensure the quality of samples throughout collection, storage and shipment and highlights the importance of involving laboratory personnel from the earliest stages of study design throughout all steps of the investigative process.

The above referenced document contains sample collection and management information applicable to various biomonitoring methods as well as best practices for the collection, storage and shipment of samples required for specific biomarker tests.

The following sub-sections provide specific information on preanalytical factors that influence the assessment of folate status as well as protocols for specimen collection, processing and shipment for serum/plasma and whole blood folate based on those developed and used by the CDC Nutritional Biomarkers Laboratory. Also included are a list of references providing additional information regarding sample collection, processing and storage specific to folate status assessment.

2.A. PREANALYTICAL FACTORS INFLUENCING FOLATE STATUS ASSESSMENT

Folate forms are sensitive to degradation by heat, oxidation, light and either acidic or alkaline pH values; thus, special care needs to be taken during sample collection, processing and storage to avoid folate losses.

Sample collection

Variables	Serum folate	Red blood cell (RBC) folate
Fasting	Fasting is essential for individual but probably not for population (~10% average difference between overnight fasted and <3 h fasted) (Haynes 2013).	Fasting is not required (Haynes 2013).
Venous vs. capillary blood	No data	Finger stick (capillary) blood samples compare well to venous blood samples if folate concentration is normalized to hemoglobin (O'Broin 1997).
Influence of anticoagulants	Serum is preferred over plasma which might contain fibrinogen clots; in general, both matrices provide similar results (O'Broin 1980; Fazili 2004; Fazili 2013).	EDTA whole blood is used; other anticoagulants are not customary.

Sample processing

Variables	Serum folate	Red blood cell (RBC) folate
General requirements	Protect evacuated tubes with whole blood from light and keep cool (avoid freezing to keep RBCs intact); prompt processing of whole blood and freezing of serum is recommended.	Protect evacuated tubes with anticoagulated EDTA whole blood from light and keep cool (avoid freezing to keep RBC intact). Prepare hemolysate with ascorbic acid (1% w/v) by using accurate pipetting. Prompt processing and freezing of prepared hemolysate is recommended. Measure hematocrit (HCT) to correct for packed cells. Subtract the contribution of serum folate when calculating RBC folate concentration (preferred).

Variables	Serum folate	Red blood cell (RBC) folate
Delayed processing	Protect from light and keep refrigerated; prepare serum within 1 d, but no later than within 2–3 d of blood collection. Storage of unprocessed whole blood at room temperature is unacceptable, particularly if storage time is greater than a few hours (10–20% loss after 6–12 h at 37°C [Zhang 1998]; 30% loss after 1 d at 37°C [Drammeh 2008]).	Whole blood is stable for several days refrigerated. Prepare hemolysate with ascorbic acid (1% w/v) within 1 d, but no later than 4 d of blood collection (folate recovery is >90% at 4°C [O'Broin 1980; O'Broin 1997]). Storage of unprocessed whole blood at elevated temperature is unacceptable (~10% loss after 1 d at 22°C [Fazili 2012]); 20-30% loss after 1 d at 37°C [O'Broin 1997; Drammeh 2008]).

Sample storage

Variables	Serum folate	Red blood cell (RBC) folate
Storage stability	Protect from light – stable for 1-2 d at room temperature in serum but not in EDTA plasma (EDTA accelerates folate degradation by 2% per h [Hannisdal 2009]). Folate is stable for 1 week at refrigerator temperatures (Drammeh 2008; Clement 2009). Folate is stable for a few years at -70°C (Pfeiffer 2010). Ascorbic acid (0.5% w/v) can be added before storage to improve stability (O'Broin 1980).	Hemolysate with ascorbic acid (1% w/v) is stable for several weeks at -22°C (O'Broin 1997) and several years at -70°C (Pfeiffer 2010). Moderate folate losses can occur if whole blood is stored frozen (<20% loss after 2 y at -70°C [Fazili 2012]).
Freeze/thaw stability	There is little folate deterioration with 3 or fewer freeze/thaw cycles (Drammeh 2008).	There is little folate deterioration in hemolysates through 3 freeze/thaw cycles (Fazili 2012) but significant folate loss in whole blood already at 2 cycles (Fazili 2012). Significant folate losses can occur if frozen whole blood is subjected to prolonged thawing times (O'Broin 1997; Fazili 2012).

Modified from Bailey et al. 2015. Complete citation for this reference and all those indicated in the table are listed on the following page.

References

Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory III JF, Mills JL, Pfeiffer CM, et al. Biomarkers of nutrition for development - folate review. J Nutr 2015;145:1636S–80S.

Clement NF, Kendall BS. Effect of light on vitamin B12 and folate. Lab Med 2009;40: 657–659.

Drammeh BS, Schleicher RL, Pfeiffer CM, Jain RB, Zhang M, Nguyen PH. Effects of delayed sample processing and freezing on serum concentrations of selected nutritional indicators. Clin Chem 2008;54(11):1883–91.

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(12):2378–81.

Fazili Z, Sternberg MR, Pfeiffer CM. Assessing the influence of 5,10-methylenetetrahydro folate reductase polymorphism on folate stability during long-term frozen storage, thawing, and repeated freeze/thawing of whole blood. Clin Chim Acta 2012;413(11-12):966–72.

Fazili Z, Whitehead Jr RD, Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. Anal Bioanal Chem 2013;405:4549–60.

Hannisdal R, Ueland PM, Eussen SJ, Svardal A, Hustad S. Analytical recovery of folate degradation products formed in human serum and plasma at room temperature. J Nutr 2009;139:1415–8.

Haynes BM, Pfeiffer CM, Sternberg MR, Schleicher RL. Selected physiologic variables are weakly to moderately associated with 29 biomarkers of diet and nutrition, NHANES 2003-2006. J Nutr 2013;143 (Suppl):1001S–10S.

O'Broin SD, Kelleher BP, Davoren A, Gunter EW. Field-study screening of blood folate concentrations: specimen stability and finger-stick sampling. Am J Clin Nutr 1997;66:1398–1405.

O'Broin JD, Temperley IJ, Scott JM. Erythrocyte, plasma, and serum folate: specimen stability before microbiological assay. Clin Chem 1980;26:522–4.

Pfeiffer CM, Fazili Z, Zhang M. Folate analytical methodology. In: Bailey L. B. (ed.) Folate in Health and Disease. Second edition. CRC Press: Taylor & Francis Group, Boca Raton, FL. 2010. p. 517–74.

Zhang DB, Elswick RK, Miller WG, Bailey JL. Effect of serum-clot contact time on clinical chemistry laboratory results. Clin Chem 1998;44:1325–33.

2.B. SPECIMEN COLLECTION/PROCESSING/SHIPPING PROTOCOL – SERUM/PLASMA FOLATE

Use of a consistently executed protocol for specimen collection and processing is essential to eliminate data variability caused by erratic technique.

Materials needed:

- Usual blood collection supplies
- Red or tiger top Vacutainers (for serum - preferred) or K2EDTA Vacutainers (for EDTA plasma)
- Pre-printed specimen labels with barcode (preferred)
- Empty pre-labeled 2-mL cryovials (to store the plasma or serum in); make sure that the label is applied to the cryovial in the correct orientation so that the barcode can be read (ladder, not picket-fence)
- Racks to hold sample vials
- Cardboard or plastic storage boxes, 9x9 or 10x10 array
- Marker pen
- Styrofoam insulated shippers
- Dry ice
- Cold packs
- Package sealing tape (clear type, such as 3M, preferred)

Collection: Follow standard venipuncture collection technique to collect blood. Mix the EDTA Vacutainer well by inversion at least 8-10 times after collection to ensure thorough mixing of the anticoagulant with the blood. Place red/tiger top Vacutainer and/or the EDTA Vacutainer into a coolbox that contains cold packs to keep it cool and protected from light during transport back to the laboratory. Make sure that you put some bubble wrap or other material in between the Vacutainers and the cold packs to avoid unintentional hemolysis of red cells. Discard the blood collection supplies properly in accordance with your institutional regulations.

Processing: Return to the laboratory promptly. The whole blood should be processed the same day it was collected. If that is not possible, maintain the EDTA Vacutainer in the refrigerator protected from light for up to a maximum of 2 days before processing. The red/tiger top Vacutainer should be processed the day of collection. This may require that each team is equipped with a portable centrifuge and freezer so that samples are processed in the field or in the closest regional laboratory on the day of collection.

<u>Processing for serum:</u>

After allowing the red/tiger top Vacutainer to clot for 30 min to 2 h maximum at room temperature, centrifuge it for 10 min at 1000–1500 g-force (or relative centrifugal force RCF) to separate the serum from the cells; this corresponds to different revolutions per min or RPM settings depending on the radius of the rotor (check centrifuge rotor manual for RCF to RPM conversion table); a fixed speed portable centrifuge operating at 3500 RPM is often

selected to facilitate the process in the field. Pipet 1.0 mL of serum into a pre-labeled 2.0-mL cryovial. If needed, pipet the remaining serum (avoid contamination with RBCs) into another 2.0-mL cryovial and label for appropriate assay. After using all the necessary serum, properly discard used Vacutainer and any blood collection supplies in biohazard bags and sharps containers until they are discarded in accordance with local institutional regulations.

Processing for EDTA plasma:

Allow the EDTA Vacutainer to reach room temperature and re-mix its contents by inversion at least 8-10 times. Centrifuge the Vacutainer for 10 minutes at 1000–1500 g-force to separate the plasma from the cells. Carefully pipet 1.0 mL of plasma (or whatever volume is required for the analysis) into a pre-labeled 2.0-mL cryovial. If needed, pipet the remaining plasma (avoid contamination with RBCs and buffy coat) into another 2.0-mL cryovial and label for appropriate assay. After using all the necessary plasma, properly discard used Vacutainer and any blood collection supplies in accordance with your institutional regulations.

Storage: Place all serum and/or plasma samples according to specimen type and aliquot number in storage boxes labeled appropriately for study and freeze all samples at -70°C (e.g. Box 1 = RBC folate, aliquot 1; Box 2 = RBC folate, aliquot 2; Box 3 = serum vial, etc.). When filling the box, mark the top left corner with a marker pen to indicate starting point. Continue to fill each row, left to right, until box is filled. As survey participant samples are collected and processed, create a log sheet for each box listing the participant ID, date collected, location of the vials in the box, and any pertinent information which may affect assay results such as short draw, fasting status if known, hemolysis, lipemia, etc.

Transport: Wrap the frozen pre-labeled storage box with absorbent paper, place it into a Ziplock bag and seal the bag. Place the Ziplock bag in the bottom of the shipping box. If necessary, use sheets of bubble wrap to ensure specimens remain in a vertical position. Fill the Styrofoam-lined shipping box with dry ice. Allow one pound of dry ice for every 2 hours in transport. Close the Styrofoam lid. Place a copy of the study log sheet describing these samples between this lid and the outer cardboard lid. Seal the outer carton lid. Notify the laboratory at your destination the day the shipment is mailed.

2.C. SPECIMEN COLLECTION/PROCESSING/SHIPPING PROTOCOL – RED BLOOD CELL FOLATE

Use of a consistently executed protocol for specimen collection and processing is essential to eliminate data variability caused by erratic technique. Proper use of the pipette is critical as this ensures accuracy of the RBC folate level. This is the biggest source of error for the pre-analytical phase.

A hematocrit result is needed to calculate RBC folate from whole blood and serum folate and needs to be obtained for each survey participant. Typically, the hematocrit is determined in the field at the time the whole blood sample is processed and, if so, needs to be transferred to the laboratory which analyzes the whole blood folate.

Materials needed:

- Usual blood collection supplies
- 4-mL K2EDTA Vacutainers
- Pre-printed specimen labels with barcode (preferred)
- 2-mL cryovials containing 1 mL of 1 g/dL ascorbic acid diluent (1% w/v); prepared ahead of time and stored frozen at -70°C for up to 6 months
- Alternatively: L-Ascorbic acid can be pre-weighed into 50-mL Falcon tubes (0.3 g/tube) and the tubes can be stored at room temperature, protected from light, for up to 1 year. The ascorbic acid solution (1% w/v) can be prepared freshly on a daily basis, as needed, by adding 30 mL of deionized water to the Falcon tube. Any left-over ascorbic acid solution should be discarded at the end of the day. In this case, a 1,000-µL pipette is also needed to dispense 1 mL of the ascorbic acid diluent into the 2-mL cryovials.
- 100-µL micropipet and disposable tips (to add EDTA whole blood to ascorbic acid vial for lysis of erythrocytes)
- Empty pre-labeled 2-mL cryovials (to store the whole blood lysate in); make sure that the label is applied to the cryovial in the correct orientation so that the barcode can be read (ladder, not picket-fence)
- Cardboard or plastic storage boxes, 9x9 or 10x10 array
- Racks
- Marker pen
- Styrofoam insulated shippers
- Dry ice
- Cold packs
- Package sealing tape (clear type, such as 3M, preferred)

Collection: Follow standard venipuncture collection technique to collect blood in a 4-mL EDTA Vacutainer (spray-dried K2EDTA is preferred). Mix the EDTA Vacutainer well by inversion at least 8–10 times after collection to ensure thorough mixing of the anticoagulant with the blood. Place the EDTA Vacutainer into a coolbox that contains cold packs to keep it cool and protected from light during transport back to the laboratory. Make sure though

that you separate the Vacutainer from the cold packs through bubble wrap or other material to avoid unintentional hemolysis of red cells. Discard the blood collection supplies properly in accordance with your institutional regulations.

Processing: Return to the laboratory promptly. Ideally, the whole blood should be lysed the same day as it was collected. However, if that is not possible, maintain the EDTA Vacutainer tube in the refrigerator protected from light for up to a maximum of 2 days before lysis. When you are ready to lyse the whole blood, remove one vial with ascorbic acid diluent per participant from the laboratory freezer and allow it to thaw at room temperature. Alternatively, prepare fresh 1% ascorbic acid solution and accurately pipet 1 mL into a pre-labeled 2-mL cryovial. Allow the EDTA Vacutainer to reach room temperature and re-mix its contents by inversion at least 8-10 times. Remove the stopper of the EDTA Vacutainer and pipet 100 µL of well-mixed blood into the vial containing the ascorbic acid; mix well. Determine the HCT by using a portion of the remainder EDTA whole blood. If EDTA plasma is needed for analysis, re-stopper the EDTA Vacutainer carefully and centrifuge it for 10 minutes at 1000–1500 g-force (or relative centrifugal force RCF) to separate the plasma from the cells; this corresponds to different revolutions per min or RPM settings depending on the radius of the rotor (check centrifuge rotor manual for RCF to RPM conversion table); a fixed speed portable centrifuge operating at 3500 RPM is often selected to facilitate the process in the field. If plasma is not needed, discard the EDTA Vacutainer properly in accordance with your institutional regulations.

Storage: Place all RBC folate samples (lysed whole blood) according to aliquot number in storage boxes labeled appropriately for study and freeze all samples at -70°C (e.g. Box 1 = RBC folate, aliquot 1; Box 2 = RBC folate, aliquot 2). When filling the box, mark the top left corner with a marker pen to indicate starting point. Continue to fill each row, left to right, until box is filled. As survey participant's samples are collected and processed, create a log sheet for each box listing the participant ID, date collected, location of the vials in the box, and any pertinent information which may affect assay results such as short draw, fasting status if known, hemolysis, lipemia, etc. Include the participant hematocrit result for correct calculation of the RBC folate concentration.

Transport: Wrap the frozen pre-labeled storage box with absorbent paper, place it into a Ziplock bag and seal the bag. Place the Ziplock bag in the bottom of the shipping box. If necessary, use sheets of bubble wrap to ensure specimens remain in a vertical position. Fill the Styrofoam-lined shipping box with dry ice. Allow one pound of dry ice for every 2 hours in transport. Close the Styrofoam lid. Place a copy of the study log sheet describing these samples between this lid and the outer cardboard lid. Seal the outer carton lid. Notify the laboratory at your destination the day the shipment is mailed.

2.D. ADDITIONAL RESOURCES

Readers interested in background information on folate biology and biomarker specific issues, can refer to the first reference listed below. Issues related to folate analytical methods, including more details on sample collection, processing, and storage, can be found in the second reference. The third reference provides a brief overview for nutritional biomarkers beyond folate.

Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory III JF, Mills JL, Pfeiffer CM, et al. Biomarkers of nutrition for development – folate review. J Nutr 2015;145:1636S–80S.

Pfeiffer CM, Fazili Z, Zhang M. Folate analytical methodology. In: Bailey LB (ed.) Folate in Health and Disease. Second edition. CRC Press: Taylor & Francis Group, Boca Raton, FL. 2010. pp. 554–555 (sample collection); pp. 555–556 (sample processing); pp. 556–57 (sample storage).

Pfeiffer CM, Schleicher RL, Caldwell KL. Biochemical Indices. In: Cabellero B (ed.) Encyclopedia of Human Nutrition. Third edition. Waltham, MA: Academic Press. 2013. Volume 1, pp. 156–74.

SECTION 3. SAMPLE ANALYSIS AND DATA REVIEW

3.A. SET UP THE FOLATE MICROBIOLOGIC ASSAY

The folate MBA start-up kit can be used to set the assay up at the local laboratory. It contains materials for 20 runs including quality control (QC) samples with known target values and training samples to allow an initial evaluation of the laboratory results compared to CDC results.

- Prior to conducting any runs, familiarize yourself with the instrumentation used for this assay, set up instrument log sheets to track usage, maintenance, and function checks, and verify and document the accuracy and precision of your pipettes. For additional information on these steps, see section 3.C.
- The focus of the first few runs should be on obtaining a proper calibration curve, a low assay blank, and reasonable CVs for the replicate of each calibrator point and sample dilution. This is also the time to set up the plate reader software to correctly calculate results based on the calibration curve and the sample dilution factor. For troubleshooting assistance with assay problems, please refer to section 3.D.
- The focus of the next few runs should be on obtaining QC results reasonably close to the CDC target values and on further improving the sample handling and pipetting techniques to achieve good assay precision.
- Finally, the next 10 (or more) runs should be carried out to characterize the QC samples and analyze the training samples. For additional information on these steps, see section 3.B.
- At a minimum, confirmation should be obtained from CDC about likely acceptable assay performance based on the QC and training samples prior to starting the analysis of survey samples.
- Ideally, the laboratory should participate in the folate method performance verification program and obtain a certificate of acceptable assay performance prior to starting the analysis of survey samples. For additional information on this program, see section 5.C.

3.B. INTERNAL QUALITY CONTROL

There are multiple reasons for using QC samples in every run performed by the laboratory:

- QC gives confidence and validity to the results,
- Monitors the performance and output (especially drift) of a process,
- Indicates when problems/deficiencies exist,
- Helps in decision making about results (i.e., acceptable criteria), and
- Provides the statistical basis from which one can judge the results.

When selecting and using QC materials, note the following:

- Composition should be same as patient samples,
- The laboratory procedure manual or the manufacturer's manual should specify appropriate QC materials for analysis,
- QC materials should be handled as patient samples,
- Mean and standard deviation (SD) of QC material must be established before use, and
- QC materials with different concentrations must have different lot numbers.

To help laboratories with limited resources and difficulties generating their own QC materials, the folate MBA kit contains 2 QC pools at different folate concentrations.

Characterization of QC materials

While the QC pools included in the folate MBA kit have an assigned target value (mean) and variability (SD) by the CDC folate MBA, the resulting acceptability criteria (mean ± 2 SD and mean ± 3 SD) may be too tight for other laboratories. Thus, each laboratory should analyze the QC pools to determine their own assay variability.

- When confident that the assay performs reasonably well, use materials from the folate MBA start-up kit to characterize QC pools and analyze training samples over 10 or more runs.
- Calculate the mean and SD for each pool from the 10 characterization runs.
- Ideally, the mean should be within ±5% of the CDC target value and the SD should be less than 2 times the CDC SD.
- If your assay performs within ±5% of the CDC assay, calculate the acceptability criteria for your assay by using the CDC mean as the target value and your laboratory SD to reflect your assay variability:
    - $Mean_{CDC} \pm 2\ SD_{Laboratory}$
    - $Mean_{CDC} \pm 3\ SD_{Laboratory}$
- Otherwise, calculate the acceptability criteria by using your laboratory mean and laboratory SD; however, do not accept a difference of more than ±10% from the CDC target value without troubleshooting:

- $Mean_{Laboratory} \pm 2\ SD_{Laboratory}$
- $Mean_{Laboratory} \pm 3\ SD_{Laboratory}$

- QC limits can be determined using an ***interactive Excel file "QC characterization and tracking template" (available upon request)***:
  - Data are to be added to the blue-shaded cells only and include the date of the run, QC results for replicates 1 and 2, the R^2 of the calibration curve, analyst name or ID, and any pertinent comments.
  - Formulas for automatic calculation of characterization statistics have been programmed into the cells of rows 6–12: characterization mean, SD, coefficient of variation (CV), mean ± 2 SD, and mean ± 3 SD, range mean, range SD, range mean + 2SD, and range mean + 3SD.
  - An example for QC characterization for whole blood folate is shown below.

WB folate low QC data_QC characterization and tracking

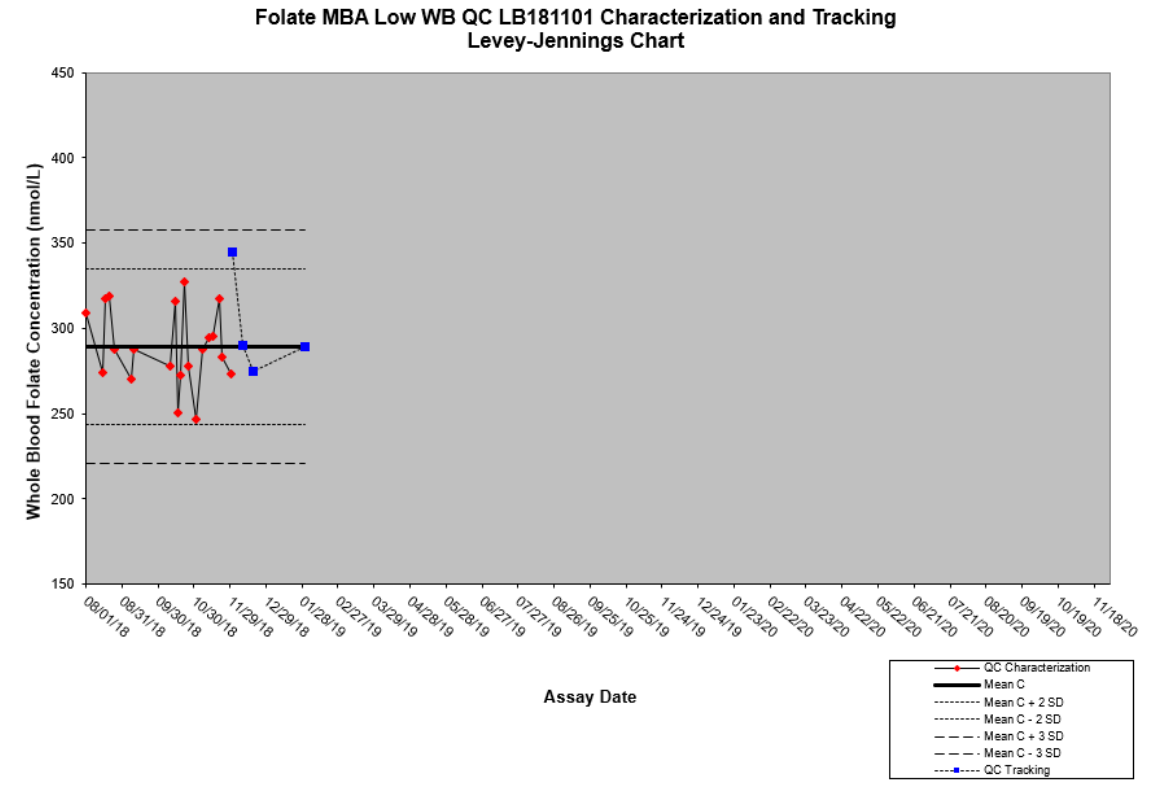
Characterization were over 20 days from 8/1/2018 -11/30/2018
QC tracking began on 12/1/2018

Mean c (nmol/L):	289.3	Range mean (absolute)	17.35
SD:	22.7	Range SD	12.99
% CV:	7.9	Range mean + 2SD	43.3
Mean c - 2SD	243.8	Range mean + 3SD	56.3
Mean c + 2SD	334.7		
Mean c - 3SD	221.1		
Mean c + 3SD	357.4		

Number of days	assay	Date	Low QC_LB181101					Mean c	Mean c - 2SD	Mean c + 2SD	Mean c - 3SD	Mean c + 3SD	R^2	Analyst	Comments
			Repl 1	Repl 2	Mean	Range	Range abs								
1	Char	08/01/18	298	320	309	22.0	22.0	289.3	243.8	334.7	221.1	357.4	0.9980		
2	Char	08/15/18	279	269	274	-10.0	10.0	289.3	243.8	334.7	221.1	357.4	0.9990		
3	Char	08/17/18	315	320	318	5.0	5.0	289.3	243.8	334.7	221.1	357.4	1.0000		
4	Char	08/20/18	328	310	319	-18.0	18.0	289.3	243.8	334.7	221.1	357.4	0.9500		
5	Char	08/25/18	298	278	288	-20.0	20.0	289.3	243.8	334.7	221.1	357.4	0.9800		
6	Char	09/08/18	280	260	270	-20.0	20.0	289.3	243.8	334.7	221.1	357.4	0.9600		
7	Char	09/10/18	294	281	288	-13.0	13.0	289.3	243.8	334.7	221.1	357.4	0.9700		
8	Char	10/10/18	269	287	278	18.0	18.0	289.3	243.8	334.7	221.1	357.4	0.9990		
9	Char	10/15/18	320	311	316	-9.0	9.0	289.3	243.8	334.7	221.1	357.4	0.9980		
10	Char	10/17/18	256	245	251	-11.0	11.0	289.3	243.8	334.7	221.1	357.4	0.9500		
11	Char	10/19/18	298	247	273	-51.0	51.0	289.3	243.8	334.7	221.1	357.4	1.0000		
12	Char	10/22/18	330	325	328	-5.0	5.0	289.3	243.8	334.7	221.1	357.4	0.9970		
13	Char	10/25/18	258	298	278	40.0	40.0	289.3	243.8	334.7	221.1	357.4	0.9960		
14	Char	11/01/18	246	248	247	2.0	2.0	289.3	243.8	334.7	221.1	357.4	0.9850		
15	Char	11/06/18	287	289	288	2.0	2.0	289.3	243.8	334.7	221.1	357.4	0.9870		
16	Char	11/12/18	279	310	295	31.0	31.0	289.3	243.8	334.7	221.1	357.4	0.9540		
17	Char	11/15/18	312	278	295	-34.0	34.0	289.3	243.8	334.7	221.1	357.4	0.9630		
18	Char	11/20/18	310	325	318	15.0	15.0	289.3	243.8	334.7	221.1	357.4	0.9510		
19	Char	11/22/18	289	278	284	-11.0	11.0	289.3	243.8	334.7	221.1	357.4	0.9870		
20	Char	11/30/18	268	278	273	10.0	10.0	289.3	243.8	334.7	221.1	357.4	0.9630		

Tracking of QC results

- Bench QC pools contained in the folate MBA kit are incorporated into each run in an **open** way (analyst knows the identity and concentration expected in the sample). No survey samples should be analyzed without the presence of bench QC samples in every run.
- At least 2 levels of bench QC pools should be used.
- During each run, these 2 levels of bench QCs are analyzed in duplicate together with unknown survey samples by distributing the QC samples across the number of sample plates.
- The tracking of QC results can be accomplished using the same ***interactive Excel file "QC characterization and tracking template" (available upon request)***.
    - The date of analysis, along with the QC results, the R^2 for the calibration curve, the analyst name or ID, and any pertinent comments are to be entered for each run into the blue-shaded cells only.
    - The QC data are plotted automatically in the QC chart, which allows easy visual inspection to assess whether the acceptability limits are met.
    - An example for QC tracking for whole blood folate is shown below.



Rules to evaluate QC results

- If the QC results are not within the acceptability limits, the run should not be accepted; the analyst should determine the cause of the QC failure and take appropriate steps to address the problem and then repeat the run.
- **QC Means** are used to judge the quality of the run. The system is declared "out-of-control (OOC)" if any of the following events occur:
    - 3S rule: A single run mean for one or more pools falls outside the upper or lower 3SD limit.
    - 2S rule: The run means of two or more pools fall either both above or both below 2SD limit.
    - Trending rule:
        - Warning if 2–4 successive run means for a single pool fall either all above or all below 2S limit
        - Ten successive run means for a single pool fall either all above or all below the center line, establishing a trend. The 10^{th} run is declared "out of control".
    - If a run is declared "out of control", do not report any results from the run, repeat the entire run.
- Range is used as orientation, not to judge the quality of a run. If one or more of the following conditions apply, conduct a careful review to decide whether the run needs to be repeated.
    - A single within-run range falls above the range mean + 3SD.
    - The within-run ranges for two or more pools fall above the range mean + 2SD.
    - Two successive within-run ranges for a single pool fall above the range mean + 2SD.
    - Eight successive within-run ranges for a single pool are above or below the center line.

Rules to evaluate sample results

1. Sample results are considered invalid and analysis should be repeated if:
   n=4 and CV >15%

   n=3 and CV >10%

   n <3
2. If raw folate concentration <0.05 or >1 nmol/L, repeat the sample with lower dilution or higher dilution.
3. If potential inhibition is confirmed for a sample, don't report the result, report "No Reportable Result".
4. If serum folate concentration <7 nmol/L, potential deficiency, repeat the sample at lower dilution. If confirmed with lower/higher result ≤25%, report the 2nd result.
5. If WB folate concentration <127 nmol/L, potential deficiency, repeat the sample at lower dilution. If confirmed with lower/higher result ≤25%, report the 2nd result.
6. If RBC folate concentration <305 nmol/L, it is considered folate deficiency.
7. If wrong sample color or volume is observed in WB lysate vial, e.g. bright red color means wrong solution instead of 1% ascorbic acid was used to make the lysate, don't need to analyze the sample, report "No Reportable Result". If ascorbic acid was not added into the vial to prepare lysate, don't analyze the sample, report "No Reportable Result".
8. If serum folate data is not available, use imputed serum folate value of 18 nmol/L.
9. If HCT data is not available, use imputed HCT value of 40%.

Additional information

Additional information about analyzing and interpreting biomarker data can be found in: Pfeiffer CM, Lacher DA, Schleicher RL, Johnson CL, and Yetley EA. Challenges and lessons learned in generating and interpreting NHANES nutritional biomarker data. Adv Nutr 2017;8:290–307.

- This publication contains information regarding the inclusion of blind QC pools and "field-split" participant samples in analytical run (p. 302).
- Also included is a graphic detailing a variety of tools that can be used to ensure the quality of laboratory data (Figure 1).

3.C. EQUIPMENT MAINTENANCE AND FUNCTION CHECKS

General:

Laboratory equipment should be checked regularly to ensure acceptable performance. Maintenance (including preventive maintenance) and function checks are to be documented in an equipment log located near the equipment. The frequency of maintenance and function checks should follow manufacturer's recommendations, when available. Manufacturer's recommendations should be included in the equipment log.

Each analytical procedure outlines the equipment maintenance and function checks for proper method performance and acceptable results from the checks. These checks must be made at the interval specified in the documentation of the procedure. Maintenance and function checks are to be documented in the equipment log. Failure of a function check is to be documented in the equipment log along with remedial action. For any calibration, calibration verification, or maintenance procedure, a log should be kept with the date, finding, and who performed the work.

Pipettes:

- The accurate calibration of pipettes used for quantitative measurements should be verified at least twice a year either by using a manufacturer pipette calibration service and/or by performing a weight-based verification in-house by the analyst. If there are no specific in-house guidelines or requirements, manufacturer specifications regarding accuracy and precision should be followed.
- Calibration verification should also be performed by the analyst when the QCs fail performance specifications, a new lot of calibrators is prepared, new pipettes are put in service, a new analyst starts using the pipettes, etc. Calibration verification should be properly documented.
- ***In-house calibration verification for single or multi-channel pipettes*** should be performed according to the following steps ***(template Excel files available upon request)***:

    1. Set pipette to target volume.
    2. Place weighing boat on balance and tare to 0.000 g; pipette water into boat using 10 different tips (single channel) or 10 different tips for each of the 8 or 12 channels (multi-channel). Tare to 0.000 g after each addition of water.
    3. Enter the 10 measurements into the water weight column of the Excel file.
    4. Check room temperature and enter correct water density from the water density table.
    5. Evaluate mean and CV of the calculated volumes.

In-House Pipette Calibration Verification

1. Set pipette to target volume
2. Place weighing boat on balance and tare to 0.000g; pipette water into boat using ten different tips and record weights
3. Tare to 0.000g after each addition of water
4. Enter the 10 measurements into Water Weight column in the following table
5. Check room temperature and enter correct water density from Water Density table
6. Evaluate Mean and CV of Calculated Volumes using pre-defined rules for accuracy and precision

Pipette SN: 221321 **Size:** 400 uL **Brand :** Eppendorf

Date: 12/11/2018 **Analyst:** MX

Tip Number	Target Volume (µL)	Water Weight (mg)	Water Density (mg/µL) at 22°C	Calculated Volume (µL)
1	400.0	398.00	0.99777	398.9
2		399.00		399.9
3		398.00		398.9
4		399.00		399.9
5		397.00		397.9
6		398.00		398.9
7		399.00		399.9
8		400.00		400.9
9		399.00		399.9
10		398.00		398.9

Temperature °C	Water Density (g/mL, mg/µL)
15	0.99910
20	0.99821
22	0.99777
23	0.99754
24	0.99730
25	0.99705
26	0.99679
27	0.99652
28	0.99624
29	0.99595
30	0.99565
31	0.99534
32	0.99503
33	0.99471
34	0.99437
35	0.99403

Evaluation

Mean (µL)	399.4	
Target (µL)	400.0	
SD	0.852	
Inaccuracy	-0.15%	Pass
Imprecision (CV)	0.21%	Pass

Pipette Calibration Rules

Test Volume	Inaccuracy		Imprecision (CV%)	
≤ 50 µL	≤ 2%	pass	≤ 2%	pass
	> 2%	fail	> 2%	fail
> 50 µL	≤ 1%	pass	≤ 2%	pass
	> 1%	fail	> 2%	fail

Reviewed by:
Date:

- A ***pipette log sheet*** should be kept documenting calibration verification of all pipettes used for preparing the folate calibrator or for diluting and transferring samples ***(template Excel file available upon request)***.

Calibration Verification - Pipettes used for preparing folate MBA calibrator

Brand	Eppendorf	Eppendorf	Eppendorf	Eppendorf
SN	xxxxxx	xxxxxx	xxxxxx	xxxxxx
Single/ Multiple channel	Single	Single	Single	Single
Manual/Electronic	Manual	Manual	Manual	Manual
Size	100-1000 uL	100-1000 uL	10-100 uL	10-100 uL
Volume used in MBA	400 uL	250 uL	100 uL	50 uL
Calibration verification date	06/01/19	06/01/19	06/01/19	06/01/19
Calibration verification date	11/28/19	11/28/19	11/28/19	11/28/19
Calibration verification date				
Calibration verification date				
Calibration verification date				
Calibration verification date				

Plate reader:

- Spectrophotometers should be verified regularly to ensure appropriate wavelength and accurate readings. This may be accomplished through an instrument verification program or using the instrument manufacturer's instruction. For Bio-Tek plate readers, the System Test and Absorbance Test Plate can be used. A protocol for performing calibration verification of a Bio-Tek plate reader using the Absorbance Plate test is detailed in **Appendix A**.
- A log sheet, such as that included in **Appendix B**, should be used to record daily use of the plate reader and to note comments regarding calibration verification, problems, maintenance and repairs.

Incubator:

- In general, there are no calibration or function checks required for this equipment.
- Temperature should be monitored when used and noted on a log sheet such as that included in **Appendix C**.
    - It is important that the temperature is even throughout the incubator.
    - Check the temperature at several spots and assure that the interior fan (usually included for this purpose) is working properly.

Refrigeration equipment:

- It is important to monitor storage equipment with temperature-dependent components/functions.
- High and low alarm points should be set for freezers to define and help maintain an acceptable temperature range.
    - Maintain freezer logs (see **Appendix D** for an example). Check and note on a log sheet at least weekly to ensure stable temperatures for assay materials requiring storage at low temperatures (i.e., -20°C or -70°C).
    - Refrigerator temperatures should also be checked weekly.
    - Freezers and refrigerators should also be monitored regularly for excessive ice deposit and inoperative cooling fans.

Balances and test weights:

- Balances should be calibrated annually by a certification service.
- Calibration should be verified prior to use by use of a calibrated test weight (i.e. S-class NIST traceable test weight) similar in weight to the chosen test substance.
    - Accuracy of the measured test weight should be within $\pm$1% of the test weight size.

- Take corrective action in the event of test weight failure.

- Additional guidelines for balance use and maintenance are provided in **Appendix E**.

3.D. TROUBLESHOOTING GUIDE FOR THE CDC FOLATE MICROBIOLOGIC ASSAY

To assist in identifying probable causes of problems detected with the folate MBA and determining potential solutions, please consult the troubleshooting guide included below.

Problem: No or low growth of microorganism in assay

Potential Cause: Microorganisms are dead or partially dead

Underlying Issue	Solution
The frozen microorganism was thawed for a prolonged period of time before adding into medium.	Add microorganism into medium within 5–10 min after thawing.
The vials containing microorganism were thawed during transportation or storage.	Keep vials containing microorganism frozen during transportation and storage.
Microorganisms were accidently added into hot medium during preparation.	Add microorganism into medium after cooling down to room temperature.

Potential Cause: Culture medium problem

Underlying Issue	Solution
The wrong medium was used.	Check medium catalog number.
The medium was expired.	Check expiration date.
A reagent was omitted in medium preparation, e.g. microorganism, ascorbic acid, or manganese sulfate.	Check whether all required reagents were added to the medium.

Potential Cause: Incubation problem

Underlying Issue	Solution
The incubator temperature was too high, too low, uneven or unsteady.	Check and monitor incubator temperature.
The incubation time was too short or too long.	Record incubation time and follow required incubation time.
An incorrect type of plate or sealing membranes was used.	Use non-treated plates and recommended sealing membrane.

Problem: Contamination (higher ODs in all blank wells)

Potential Cause: Folate contamination originating from the high-concentration calibrator

Underlying Issue	Solution
The assay medium and/or sodium ascorbate solution was contaminated during preparation.	Dilute calibrator in a separate room or area than where sample preparation is conducted.
The water used for reagent preparation is contaminated.	Use a separate sink from the sample preparation area to discard left-over calibrator and pour the solution directly into a drain.
Lab supplies and/or working bench (e.g. beakers, stirring bars, pipettes, plates and sealing membranes) are contaminated.	Thoroughly wash beakers and stirring bars with bleach (~10%) and rinse with plenty of water. Change bench covers and wipe pipettes with diluted bleach (~10%), then with a moist towel.
Lab coat or gloves are contaminated.	Frequently change gloves and often wash lab coat.
The original bottle of medium, sodium ascorbate or Tween-80 is contaminated.	Discard the contaminated bottle and use fresh chemicals to prepare fresh solutions.

Potential Cause: Contamination caused by other organism(s):

Underlying Issue	Solution
Antibiotic (chloramphenicol) was not added to medium or degraded antibiotic was used in the medium preparation.	Make sure antibiotic added into the medium is functional.

Problem: QC failed

Potential Cause: Sample and/or standard preparation errors leading to high QC results

Underlying Issue	Solution
The folate concentration in the calibrator working solution is inaccurate (too low) due to use of less volume for the 100-µL stock solution or the diluted calibrators, or the use of more volume for the sodium ascorbate solution in the 50-mL volumetric flask.	All pipettes should be calibrated at least twice a year. Frequently check accuracy of pipettes on the balance. Use correct pipetting technique.

Underlying Issue	Solution
The calibrator degraded due to early thawing and prolonged exposure to room temperature, inappropriate storage conditions, exposure to light, or freeze/thaw cycle(s).	Make sure calibrator stock is stored appropriately and not expired. Never refreeze and reuse thawed calibrator. Thaw calibrator stock solution ~15–30 min before use. Do not let it sit too long on the bench. Protect calibrator from direct light exposure.
The sample dilution was inaccurate. More sample volume or less of sodium ascorbate solution volume was used in glass dilution tubes.	Check dilution and pipette accuracy. Use correct pipetting technique.
The pipetting of calibrator working solutions and/or diluted samples into plates was inaccurate.	All pipettes should be calibrated at least twice a year. Frequently check accuracy of pipettes on the balance. Use correct pipetting technique.
The sample and calibrator plates were not mixed and handled in the same way.	All plates should be handled in the same way after incubation. Mix plates well on 360 rotator and read within ~1 min after mixing.

Potential Cause: Sample and/or standard preparation errors leading to low QC results

Underlying Issue	Solution
The folate concentration in the calibrator working solution was inaccurate (too high) due to use of more volume for stock solution or the diluted calibrators or use of less volume for the sodium ascorbate solution in the 50-mL volumetric flask.	Check pipette accuracy. Use correct pipetting technique.
The sample folate was degraded due to thawing too long at room temperature, inappropriate storage conditions, direct exposure to light, or too many freeze/thaw cycles.	Make sure samples are handled and stored appropriately. Protect samples from direct light exposure.
The sample dilution was inaccurate due to use of less sample volume or more sodium ascorbate solution volume in the glass dilution tube.	Check dilution and pipette accuracy. Use correct pipetting technique.

Underlying Issue	Solution
The pipetting of calibrator working solutions and/or diluted samples into plates was inaccurate.	All pipettes should be calibrated at least twice a year. Frequently check accuracy of pipettes on the balance. Use correct pipetting technique.
The sample and calibrator plates were not mixed and handled in the same way.	All plates should be handled in the same way after incubation. Mix plates well on 360 rotator and read within ~1 min after mixing.

Problem: Numerous samples have high CV among the 4 replicates

Underlying Issue	Solution
Too many wells leaked after incubation. Leaking wells showed darker brown color or less volume.	Carefully seal plates.
Bubbles or particles were present in the wells before reading.	Thoroughly pop all bubbles before reading. Check if there is any particle in wells. If so, mask the wells.
The pipetting of 50 µL and 100 µL diluted sample into the wells was inaccurate.	Check pipette accuracy and use correct pipetting technique.
The plates were not mixed well.	Mix plates well and read at ~1 min after mixing.
The reader does not read evenly.	Check reader function.

Problem: Wrong sample concentration is reported

Underlying Issue	Solution
There was an error in sample dilution due to inaccurate pipettes and/or technique.	All pipettes should be calibrated and correct pipetting technique should be used. Dilute samples according to the sample dilution table.
There was an error in entering the dilution factor in Gen5.	Note any irregular dilution(s) and enter dilutions used into Gen5.
The wrong sample ID was entered into Gen5.	Carefully enter sample IDs into Gen5.
The plate number was improperly labeled.	Carefully label plate number and add diluted sample into correct plate.
The incorrect plate was picked up and read as a different plate number in Gen5.	Read plate as correct plate number in Gen5.
Mistakes were made in preparing whole blood lysate (e.g. sodium ascorbate used	Report errors to the customer who provided the samples for analysis.

Underlying Issue	Solution
instead of ascorbic acid solution) or ascorbic acid was omitted when preparing the lysate.	

Problem: Wrong calibration curve shape

Underlying Issue	Solution
OD at the highest calibration point was lower than usual (0.9–1.0) due to inaccurate dilution or pipetting or shorter incubation time.	See solution in “QC failed - High QC results”.
OD at the highest calibration point was higher than usual (0.9–1.0) or the high end of the calibration curve was flat due to inaccurate dilution, inaccurate pipetting or over-incubation.	See solution in “QC failed - Low QC results”. Record incubation start and stop time. Stop incubation, mix and read at appropriate time. Check accuracy of the pipettes and pipetting technique.
ODs in blank wells were higher than usual (~0.1-0.2) due to contamination.	See solution in “Contamination”.
The curve was not shaped as a smooth polynomial 3 curve due to a calibrator dilution and/or pipetting error.	Check if pipettes with wrong volume settings are used in calibrator dilution or if inaccurate pipetting technique was used.

SECTION 4. DATA REPORTING

4.A. DOCUMENTATION OF ANALYTICAL RUNS

Proper and consistent documentation of laboratory activities and information is an essential component of good laboratory practices.

- Minimum information to be noted for each analytical run should include the date of the run, calibration results, QC results, and analyst name or ID who prepared the specimens.
- Ideally, records should be kept of everything that might affect the results of the analyses.
- These records will make it simpler to determine the source of a problem and to correct analytical problems.
- Consistent run report templates (can be made available upon request) are of value for organizing analytical run information, raw data and patient and QC results.
- An example of the results page of the whole blood folate run report is included below:

Whole blood folate results report form

Run Date:	12/12/2018	Sample QC Rules:	If n=4, CV>15%, repeat the sample
Run No:	1		If n=3, CV>10%, repeat the sample
Analyst:	SR		If n<3, repeat the sample
Analyte:	Whole bllod folate (WBF)		If raw folate concentration > 1 nmol/L, repeat with higher dilution
Study Name:	Training		If raw folate concentration < 0.05 nmol/L, repeat with lower dilution
Sample Group #:			If potential inhibition, repeat with higher dilution; if confirmed, no repotable result
Instrument & SN:	BioTek PowerWave		5MeTHF conversion factor: 1 ng/mL = 2.179 nmol/L

No	Sample #	Sample ID	Number of replicates N	Concentration in nmol/L	CV of replicates in %	Comment 1	Comment 2
1	SPL1	2016-0055 -001-L16	4	538.4	2.1		
2	SPL2	2016-0055 -001-L16	4	559.4	2.5		
3	SPL3	2016-0055 -001-L16	4	580.9	11.5		
4	SPL4	2016-0055 -001-L16	4	540.5	2.6	> regular dilution	
5	SPL5	2016-0055 -002-L16	4	245.8	7.3	< regular dilution	
6	SPL6	2016-0055 -002-L16	4	242.0	4.5		
7	SPL7	2016-0055 -002-L16	3	248.2	3.9		
8	SPL8	2016-0055 -002-L16	4	243.8	6.7		
9	SPL9	2016-0055 -005-L14	2	472.1	8.7		
11	SPL19	example 10	4	1634.3	4.0	Repeated at higher dilution, report 1st valid result	
12	SPL20	example 11	4	291.4	42.5	potential inhibition	
14	SPL21	example 12	0	?????	?????	No ascorbic acid is added. No Reportable result	
16	SPL22	example 13	4	158.0	30.0	WBL wrong color. No reportable result	
17	SPL23	example 14	4	233.0	11.0	WBL volume obviously wrong from 1.1 mL, no reportable result	
18	SPL11	example 2	1	572.6	?????	Repeat with regular dilution	
19	SPL12	example 3	0	?????	?????	Repeat with regular dilution	
20	SPL14	example 5	3	553.4	35.0	Repeat with regular dilution	
21	SPL15	example 6	4	450.3	5.3		
22	SPL16	example 7	4	540.6	32.3	Repeat with regular or lower dilution	
23	SPL17	example 8	2	1255.0	1.0	Repeat with more dilution	
24	SPL18	example 9	4	149.0	5.7	Repeated at lower dilution, report 1st valid result	
25	hiQC1	HB181102	4	600.0	9.6		
26	hiQC2	HB181102	4	990.0	6.0		
27	lowQC1	LB181101	4	326.0	4.0		
28	lowQC2	LB181101	4	358.0	2.3		

4.B. CALCULATION OF RBC FOLATE CONCENTRATION

The calculation of RBC folate via the traditional formula provides the most accurate RBC folate concentration and thus a good reflection of body stores, however it requires results for whole blood lysate folate, serum folate, and hematocrit (in %).

$$\text{RBC folate, nmol/L} = \frac{(\text{Whole blood lysate folate} * 11) - \text{Serum folate} * (1-\text{HCT}/100)}{\text{HCT}/100}$$

To avoid human error, an Excel spreadsheet with programmed formulas should be used to calculate RBC folate concentrations (template available upon request).

As in the example below, data are added to the fields for HCT, WBLF and SFOL; RBC folate is automatically calculated using a programmed formula in the locked RBCF field.

Study name:	Study X				RBCF nmol/L = ((WBLF*11) - SFOL*(1-Hct/100)) / Hct/100			
Analyte:	Red blood cell folate (RBCF)				WBLF: whole blood lysate folate			
Run Date:	10/28/2018 - 12/24/2018				WBF: whole blood folate = WBLF*11 (original dilution)			
Analyst:	MZ & SJ				SFOL: serum folate			
					RBCF: red blood cell folate			
					Folate unit: nmol/L			
Sample ID	**HCT**	**WBLF**		**WBF**	**SFOL**		**RBCF**	**Comment**
	%	**Assay date**	**nmol/L**	**nmol/L**	**Assay date**	**nmol/L**	**nmol/L**	
2157892	40.0	11/24/18	9.1	100	10/28/18	12.0	232	
2589635	33.8	12/01/18	42.2	464	10/28/18	69.9	1236	
5894125	42.3	12/01/18	52.0	572	10/25/18	37.9	1301	
5896244	40.4	12/01/18	43.3	476	10/25/18	60.4	1089	
4577893	43.8	12/01/18	33.5	368	10/25/18	18.0	817	
5849625	44.6	12/01/18	61.8	680	10/25/18	18.0	1502	
4789654	39.0	12/24/18	33.6	370	12/01/18	71.9	836	
4879523	33.8	12/24/18	42.2	464	12/01/18	69.9	1236	
5879541	40.4	12/24/18	43.3	476	12/01/18	60.4	1089	

SECTION 5. EXTERNAL QUALITY ASSESSMENT (EQA)

5.A. PURPOSE OF EQA

- EQA is a valuable and important tool for laboratories to assess how their values compare to other methods and laboratories and therefore to assess the quality of their results.
- A laboratory may perform consistently based on their internal QA program, but only by participating in an external QA program can the laboratory compare their results to those of other laboratories.
- The US Centers for Medicare & Medicaid Services (CMS) maintains a list of official CLIA (Clinical Laboratory Improvement Act) approved proficiency testing programs https://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/Proficiency_Testing_Providers
    - In these programs laboratory performance is rated according to pass/fail criteria.
    - EQA programs are not regulatory in nature, but they provide valuable feedback to laboratories.
- Some common EQA/PT programs for nutritional indicators include:
    - College of American Pathologists (CAP) https://www.cap.org/laboratory-improvement/proficiency-testing
    - United Kingdom National External Quality Assessment Service (UK NEQAS) https://ukneqas.org.uk/programmes/

5.B. OVERVIEW OF A TYPICAL EQA/PT PROGRAM

- These programs provide quality control samples to participating laboratories several times per year (the number of samples and frequency varies by program).
- The unknown samples are analyzed by the participating laboratories and the results are reported to the organizer within a predefined reporting period for performance assessment.
- The organizer compiles all results and generates reports that are distributed to the participating laboratories.
- The EQA program sets criteria for acceptability of the participating laboratory's results based on different criteria.
- Typically, laboratory results are compared to an all laboratory trimmed mean (ALTM), or a method-specific mean (if there are larger method differences), and therefore the interpretation of results is not necessarily accuracy-based.
- If a particular analyte has been standardized and/or if target values for the EQA materials have been assigned by an accuracy-based method, then the EQA program can verify the accuracy of results.
- The laboratory results are considered acceptable if they fall within the range of acceptability.

  Typically, 80% of laboratory results must fall within predefined acceptability limits for the laboratory to successfully pass an EQA challenge.

5.C. PERFORMANCE VERIFICATION PROGRAM FOR FOLATE MBA

CDC's Nutritional Biomarkers Branch (NBB) established the folate performance verification program in 2019. The program provides an independent assessment of analytical performance for laboratories working to determine population folate status. The folate microbiologic assay used in the folate performance verification program was selected based on a WHO recommendation (WHO, 2015). It produces comparable data across laboratories when the laboratories use the same key reagents, such as the calibrator and microorganism (Zhang 2018). To facilitate the use of common key reagents, CDC offers assay materials containing common key reagents for epidemiologic use in population surveys.

The performance verification program for the folate microbiologic assay assesses the performance and continued proficiency of regional resource laboratories engaged in public health work. The program is voluntary and non-regulatory and does not provide laboratory accreditation. Participation can either be on a one-time basis to assess method performance (e.g., upcoming survey) or on a continuous basis, where the 40 samples are analyzed over the course of one year (e.g., 10 samples per quarter). The program uses objective quality goals based on biologic variation to assess the acceptability of method performance (Fraser 1997).

Target participants:

Laboratories engaged in public health work assessing folate status in populations.

Program goals:

Maintain the quality of folate measurements and document continued laboratory proficiency in performing the folate microbiologic assay through an annual performance report.

Featured analytes: Whole blood folate only
Serum and whole blood folate

Participant requirements:

- Receive 40 samples per year and 8 quality control (QC) samples per year
- Analyze 10 samples and 2 QC samples per quarter in duplicate and return data to CDC after each of the four (4) challenges
- Receive a brief summary report after each challenge and a comprehensive annual performance report showing the measurement imprecision and the percent difference compared to the CDC CLIA-approved folate microbiologic assay; the annual performance report is valid for one year
- Alternatively, analyze all 40 samples in duplicate over a shorter period of time for a onetime assessment of method comparability to CDC

Shipping requirements:

Participating laboratories must be able to receive frozen samples packed in dry ice, retrieve samples from customs at the airport, and pay for any costs required by customs.

5.D. ADDITIONAL VERIFICATION OF THE FOLATE MBA

- An alternative means of validating a biomarker assay such as the folate MBA is through the use of international reference materials.

- Common sources for international reference materials for nutritional indicators are:
    - National Institute of Standards and Technology (NIST) (https://www.nist.gov/srm)
    - National Institute for Biological Standards and Control (NIBSC) https://www.nibsc.org/standardisation.aspx
    - Institute for Reference Materials and Measurements https://ec.europa.eu/jrc/en/reference-materials

- Reference materials for serum and whole blood total folate are currently available through the NIBSC. Reference materials from NIST have no certified total folate value assignment.

- The **NIBSC Code 03/178 1st International Standard For Vitamin B12 and Serum Folate** (https://www.nibsc.org/documents/ifu/03-178.pdf; see **Appendix F** for Specification sheet) has been available since fall 2005. Pertinent information regarding the use of this standard for assay verification is included below:
    - This is a 1-level material with an assigned content of 12.1 nmol/L (5.33 ng/mL) total folate (when reconstituted with 1.0 mL distilled/de-ionized water) as measured by LC-MS/MS.
    - Analyze reconstituted material in duplicate as unknowns against working standards. The measured concentrations should be within ±20% of the assigned content. There may be method biases.

- The **NIBSC Code 95/528 1st International Standard 1996 Whole blood folate** (https://www.nibsc.org/documents/ifu/95-528.pdf; see **Appendix G** for Specification sheet) is the only reference material for whole blood folate. Pertinent information regarding the use of this standard for assay verification is included below:
    - This is a 1-level material with an assigned content of 13 ng/ampoule whole blood folate (consensus value by microbiologic assays and radioassays).
    - Analyze reconstituted material in duplicate as unknowns against working standards. The measured concentrations should be within ±20% of the assigned content. There may be method biases.

SECTION 6. DOCUMENTATION OF POLICIES AND PROCEDURES

Documentation is the general term for all written procedures followed by all the staff working on the analyses. These procedures should include any safety precautions specific to the procedure, a listing of all materials, reagents and equipment required as well as a step-by-step description of the analytical procedure. Such documentation, often referred to as standard operation procedures (SOP) helps ensure that all analyses are performed in a consistent, repeatable and efficient manner.

6.A. SAFETY PRECAUTIONS

- General laboratory safety precautions and those specific to all methods and procedures used in the laboratory should be well documented.

- The laboratory chief should ensure that each laboratory analyst has read and is familiar with the safety precautions involved in each procedure.

6.B. WRITTEN PROCEDURE MANUAL

- A written procedure for the performance of all analytical methods used in the laboratory must be readily available and followed by laboratory personnel.
- Each procedure must be approved, signed and dated by the laboratory director.
- Any changes must also be approved, signed and dated by the laboratory director.
- To assure uniformity and minimize differences between analysts, all staff involved in the analysis must be aware of any changes so that everyone follows the same procedures.

6.C. LABELING OF REAGENTS AND SAMPLES

Laboratory:

- Reagents, solutions, and other supplies must be labeled appropriately to indicate the identity of contents, the concentration, the preparation and expiration dates, name of preparer, recommended storage requirements and any other pertinent information for proper use.
- Chemical containers should also be marked with the receipt and open date.

Field:

- The field and laboratory staff involved in the surveys should be well-trained for proper labeling of vacutainers/vials used for blood collection, urine collection, sample storage, etc.
- The cryovial boxes used for storing samples should be labeled appropriately and stored in freezers.
- All samples collected and stored should be documented using electronic lists.
- Bar-coded labeling of tubes, vials and boxes is preferred whenever possible.

Shipping of samples:

- Follow the International Air Transport Association (IATA) regulations when packing styrofoam boxes to ship samples on dry ice to other laboratories for analysis.
- Label boxes with a dry ice label indicating the quantity of dry ice and with other labels as applicable.
- Include a shipping list with the samples, which includes the study name, date of sample collection, number of samples, and any other relevant information.
- Maintain proper documentation of samples shipped to various laboratories for analysis (both sender and receiver).

SECTION 7. PROPER STAFF TRAINING

- Qualified, well-trained, and competent personnel are essential for good laboratory performance.
- New staff members should be trained appropriately to perform the assay with the best possible accuracy and precision.

  The performance of staff is evaluated by:

  - direct observation of test performance, specimen handling, specimen processing and testing
  - direct observation of the recording and reporting of test results
  - review of QC results, proficiency testing results and preventive maintenance records
  - direct observation of performance of instrument maintenance and function checks
  - review of blind QC data
  - assessment of problem-solving skills.
- Evaluation is performed by the laboratory chief or his designee.
- If necessary, additional training should be provided to enhance the technical skills.

SECTION 8. LABORATORY AUDITS

Laboratory audits verify compliance with technical and operational procedures. They should be performed periodically in preparation for formal accreditation or renewal or as an internal review of policies, processes and procedures. Such audits can range from an overall assessment of laboratory safety and performance to more directed observations focusing on a specific process (i.e., sample collection) or procedure (i.e., folate MBA).

Accordingly, the audit should focus on a review of laboratory specific documents (such as SOPs), records (assay and equipment log sheets) and direct observations of procedures (routine conduct of an assay procedure by technical staff) to identify inconsistencies between approved policies and actual performance and to identify areas for improvement or needing clarification. To maintain objectivity, it is preferred that the audit be conducted by a regulatory entity of the institution, representative of another laboratory within the department, etc. rather than the supervisor or other staff member of the laboratory being audited.

A written report should be prepared upon completion of the audit and shared with all laboratory members so that corrective actions may be taken. The laboratory audit report should be an objective presentation of facts and observations rather than subjective listing of comments and opinions. A checklist format rating items reviewed/observations made on a graded scale of adherence to protocol is an efficient and objective means of obtaining and sharing information obtained during the audit. A written narrative may also be used in addition or in lieu of the checklist.

SECTION 9. REFERENCES AND ADDITIONAL RESOURCES

Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory II JF, Mills JL, Pfeiffer CM, et al. Biomarkers of nutrition for development: folate review. J Nutr 2015; 145:1636S–80S.

Pfeiffer CM, Fazili Z, Zhang M. Folate analytical methodology. In *Folate in Health and Disease*. L. B. Bailey, Ed. CRC Press: Boca Raton, FL. 2010, pp 517–74.

Pfeiffer CM, Zhang M, Lacher DA, Molloy AM, Tamura T, Yetley EA, Picciano MF, Johnson CL. Comparison of serum and red blood cell folate microbiologic assays for national population surveys. J Nutr 2011; 141:1402–9.

Pfeiffer CM, Sternberg MR, Hamner HC, Crider KS, Lacher DA, Rogers LM, Bailey RL, Yetley EA. Applying inappropriate cutoffs leads to misinterpretation of folate status in the US population. Am J Clin Nutr 2016; 104;1607–15.

Pfeiffer CM, Zhang M, Jabbar S. Framework for laboratory harmonization of folate measurements in low- and middle-income countries and regions. Ann N Y Acad Sci 2018; 1414:96–108.

World Health Organization. Guideline: optimal serum and red blood cell folate concentrations in women of reproductive age for prevention of neural tube defect. Geneva, Switzerland: World Health Organization, 2015.

Zhang M, Sternberg MR, Pfeiffer CM. Harmonizing the calibrator and microorganism used in the folate microbiological assay increases the comparability of serum and whole-blood folate results in a CDC round-robin study. J Nutr 2018; 148:807–17.

APPENDICES

APPENDIX A. BIO-TEK MICROPLATE READER CALIBRATION VERIFICATION PROTOCOL

Set up an Absorbance Test Plate in Gen5

1) From the desktop, open the Gen5 software.
2) On the Gen5 main menu, click "**System Menu**" on the left side.
3) Once the program opens, click "**System**" from the top menu bar.
4) Under the "**System**" menu, click "**Diagnostics**", choose "**Test plate**", and click the "**Add/modify plates…**" option.
5) A box will pop up and give you a list of test plates already saved in the system.
6) To add a new plate, click "**Add…**" on the right side of the window.
7) Choose the plate type using the part number (P/N), enter the serial number of the test plate, enter the certification dates, and enter the date of next certification.
8) Using the Absorbance Test Plate Calibration Certificate supplied in the box with the test plate, enter the values of the Absorbance OD standards.
9) Next, select anywhere from 1 to 4 Peak Wavelength Accuracy tests that you want the system to perform every time you run the test. Enter the expected peak wavelength (using the values from the certificate provided) and enter the test range.
10) Click "**OK**" and you will see a list of all the test plates you have added to the system.
11) Click "**CLOSE**" to close the window and then exit the program.

Run the Absorbance Test Plate calibration check in Gen5

1) From the desktop, open the Gen5 software.
2) On the Gen5 main menu, click "**System Menu**" on the left side.
3) Once the program opens, click "**System**" from the top menu bar.
4) Under the "**System**" menu, click "**Diagnostics**", choose "**Test plate**", and click the "**Run…**" option.
5) A box will pop up with test plate options. Enter your name under "User:" and make sure that "**Perform Peak Wavelength Test**" is checked.
6) Hold the "**Ctrl**" key on the keyboard and select all the wavelengths that you want to test in the calibration test.
7) Enter any comments for the run under the "**Comments**" section and click "**Start Test**" when you are done.
8) Place the test plate in the BioTek plate reader and click "**OK**" when the screen prompts you to do so.
9) The program will perform the peak wavelength tests and test the selected wavelength in duplicates.
10) The screen will prompt you to **rotate the plate 180^{o}.** Do so, and then click "**OK**" to continue the program.
11) When completed, a window will pop up with "**Test Plate Status**" that displays the results. Remove the test plate now and place back in the casing.
12) You can now save the result in the proper folder and print/sign the result for your records.

Save/print Absorbance Test Plate Results in Gen5

1) At the end of a Test Plate Run, a window will pop up with "**Test Plate Status**".
2) From here you can view, save, and print the results. To save, click "**Save As**" in the top right.
3) Save the file in the appropriate folder of your choosing under the following format: "**20XX-MM-DD Reader I Absorbance Test Plate Result_Initials.txt**"
4) To print the result, click "**Print…**" in the top right and select the printer. Click "**Properties**" to the right of the printer selection and be sure to choose front and back printing to save paper. It may be listed as "**Duplex Printing**". If so, choose "**Long Edge**".
5) Click "**OK**" and print.
6) If the computer is not connected to a printer, print the result to "PDF Writer" and email it to yourself.
7) Give to supervisor to sign the results on the designated line and save for record keeping.

View diagnostics history of Absorbance Test Plate results in Gen5

You can always go back and retrieve the information from previous Test Plate runs in order to save the files, reprint them, and analyze them.

1) From the desktop, open the Gen5 software.
2) On the Gen5 main menu, click "**System Menu**" on the left side.
3) Once the program opens, click "**System**" from the top menu bar.
4) Under the "**System**" menu, click "**Diagnostics**", choose "**History…**".
5) A window will pop up and display the Diagnostics history for that machine on that computer only. The date of the test, type of test, instrument ID, Pass/Fail result, and user name will appear.
6) To reprint the information, double click on one of the items and the "**Test Plate History**" will appear.
7) Click the "**Print…**" button on the top left and select the printer and properties.
8) To save the result as a text file, click "**Save As…**" and save using the format on page 4, step 3.

*It is best to print the result from the Gen5 program and not from the text file, as the formatting is different in the text file. The Gen5 program highlights important information when printed out from the program.

APPENDIX B. PLATE READER LOG SHEET

An example log sheet for a plate reader is included below:

Daily Plate Reader Logsheet for Year 2019

Instrument: Plate Reader (PowerWave) **Serial #:** xxxxx **Purchase Date**: 1/2/2012
Location: Building x, Room xxx
Insert your initials on the date when the instrument is used

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Jan																															
Feb																															
Mar																															
April																															
May																															
June																															
July																															
Aug																															
Sep																															
Oct																															
Nov																															
Dec																															

Notes (e.g., problems, repairs, maintenance, calibration):

APPENDIX C. INCUBATOR LOG SHEET

An example log sheet for a 37°C incubator is included below:

Daily Incubator Logsheet for Year 2019

Instrument: Fisher Incubator **Serial #:** xxxxx **Purchase Date:** 1/2/2001
Location: Building x, Room xxx **Temp. Range**: 37°C ± 2°C
Check incubator temperature prior to usage and add your initials if the temp is within range; if not, corrective action is needed and specified under "Notes"

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Jan																															
Feb																															
Mar																															
April																															
May																															
June																															
July																															
Aug																															
Sep																															
Oct																															
Nov																															
Dec																															

Notes (e.g., problems, repairs, maintenance, calibration):

APPENDIX D. FREEZER LOG SHEET

An example log sheet for a freezer is included below:

Daily Freezer Logsheet for Year 2019

Instrument: Thermo TSE400A **Serial #:** xxxxxxx **Purchase Date:** 1/2/2001
Location: Building x, Room xxx **Optimal Temp. Range**: 65°C ± 20°C
Check temperature at least once a week and enter it into the logsheet and add your initials

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Jan																															
Feb																															
Mar																															
April																															
May																															
June																															
July																															
Aug																															
Sep																															
Oct																															
Nov																															
Dec																															

Notes (e.g., problems, repairs, maintenance, calibration):

APPENDIX E. BALANCE USE AND MAINTENANCE GUIDELINES

The following guidelines are modified from the Standard Operating Procedure for Balances and Test Weights used by the CDC Nutritional Biomarkers Branch:

ANALYTICAL AND PRECISION BALANCES

- **WEIGHING PROCEDURE**
    1) Place the weighing boat or desired container in the center of the weighing pan.
    2) Press tare to zero the weight of the tare container.
    3) Load the substance onto the tare container with care.
    4) Record the weight when the balance shows a stable weight along with the circle symbol.
- **CALIBRATION**
    - Balances should be calibrated annually by a certification service and the resultant certificates should be filed on the network and in the lab.
- **CALIBRATION VERIFICATION**
    - Prior to using a balance, a calibrated S-class NIST traceable test weight, similar in weight to the amount of the desired substance, is weighed on the balance to verify the balance accuracy. Accuracy of the measured test weight should be approximately within ±1% of the test weight size. Take corrective action as needed if the weight fails.
    - If a balance fails the calibration pre-test, check the log sheet to see who used it for that particular weight range since the last verification and notify the person/method. Also, write up a corrective action.
- **MAINTENANCE AND GENERAL GUIDELINES**
    1) Check that the balance is properly leveled on a vibration-free table and away from draft and air vents.
    2) Clean the balance, especially the weighing pan, before and after each use.
    3) Avoid oscillations during weighing by releasing and arresting the pan gently.
    4) Place no object on the pan nor add any weights until the balance is arrested.
    5) Place the object in the center of the pan.
    6) Add weights systematically, in decreasing order of magnitude, starting with a weight larger than required.
    7) Do not exceed the maximum permissible load of the balance.
    8) Do not weigh warm objects until they have returned to room temperature. Warm objects can generate a current of air inside the balance, which can result in inaccurate readings. Cold objects can also condense moisture, which can increase the weight.
    9) Do not weigh substances that fume or absorb moisture.

10) Avoid unnecessary rubbing or wiping of the weighing bottle with poor conduction material. This can generate static electricity, which can create attraction or repulsion within various parts of the balance, resulting in inaccurate readings.
11) While taking readings, make sure that there is nothing on the outside of the weighing container or on the pan.
12) Keep the draft shield doors closed when the balance is not in use.

TEST WEIGHTS

- **OPERATION**

1) Ensure the weighing pan on the balance is clean before placing the test weight.
2) Choose the calibrated test weight that is similar in weight to the amount of substance that you intend to measure.
3) Handle the test weight using clean gloves and tweezer provided and gently place it in the center of the weighing pan.
4) Record the weight when the balance shows a stable weight along with the circle symbol.
5) Check if the measured weight is within ±1% of the test weight size, and if so, record it on the log sheet and proceed to weighing the desired substances. If not, repeat the procedure to confirm. If the weight fails after confirmation, take corrective action before proceeding.

- **CALIBRATION**

  - Weight sets should be certified each year and the resultant certificates filed on the network and in the lab.

- **CALIBRATION VERIFICATION**

  - Verification of individual test weights is done by analyst before each use. Accuracy of the measured test weight should be approximately within ±1% of the weight size to proceed. Take corrective action as needed if the weight fails.

- **MAINTENANCE**

1) Keep the test weights in their original boxes at room temperature.
2) Use provided gloves or any clean gloves and a tweezer to handle the test weights.
3) Do not drop the test weights or place them on possibly contaminated surfaces.
4) Check that the test weight is clean and has no damage before each use.
5) Notify team lead or supervisor if the test weights are damaged or need to be replaced.

APPENDIX F. WHO INTERNATIONAL STANDARD FOR B12/FOLATE

Medicines & Healthcare products Regulatory Agency

NIBSC
Confidence in Biological Medicines

WHO International Standard
Vitamin B12, Serum Folate and HoloTC
NIBSC code: 03/178
Instructions for use
(Version 3.0, Dated 26/10/2015)

1. INTENDED USE

The assay of blood levels of the vitamins B_{12} and folate is the current routine procedure for determining deficiency of these vitamins. Deficiency can result in a number of clinical conditions including megaloblastic and pernicious anaemia.

The International Standard (IS) for serum B_{12} and serum folate, 03/178, was assayed using a wide range of methods in 24 laboratories in 7 countries. Methods included a range of commercial analysers and, for serum folate, candidate reference methods of isotope-dilution tandem mass spectrometry coupled to liquid chromatography (LC/MS/MS). The inclusion of three serum samples in the study, with different B_{12} and folate levels, demonstrated a considerable reduction in inter-laboratory variability when the B_{12} and folate levels in the samples were determined relative to the IS with assigned B_{12} and folate values rather than to the analysers' calibration.
Since the IS was first established, commercial assays for holotranscobalamin (holoTC), the active portion of B12, have become available. There is evidence that holoTC is a better marker of early B12 deficiency than total B12. This prompted another international collaborative study to assign a holoTC value to the IS for B12 and serum folate, 03/178. Twelve laboratories in 8 countries participated.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The IS for serum B_{12} and serum folate, 03/178, has an assigned value of 12.1 nmol/L total folate, made up of 9.75 nmol/L 5MeTHF (5-methyltetrahydrofolate; coefficient of variation (CV) 5.5%), 1.59 nmol/L 5FoTHF (5-formyltetrahydrofolate; CV 4.2%) and 0.74 nmol/L FA (folic acid; CV 31.6%), when reconstituted with 1.0 mL distilled/deionised water, as determined using LC/MS/MS. The total folate content of 12.1 nmol/L is equivalent to 5.33 ng/mL, using a conventional conversion factor of 2.266.

The IS for serum B_{12} and serum folate, 03/178, has an assigned consensus value of 480 pg vitamin B_{12} (480 pg/mL when reconstituted with 1.0 mL distilled/deionised water). The preparation will be re-evaluated when a reference measurement procedure has been established.

The IS 03/178 has an assigned consensus holoTC value of 107 pmol/L when reconstituted with 1.0 mL (0.107 pmol/ampoule).

The variance in the ampoule contents was determined to be 0.08%.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each ampoule contains the lyophilized residue of ~1 mL human serum.

5. STORAGE

Unopened ampoules should be stored at -20°C or below.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
Reconstitute the contents with 1.0 mL distilled/deionised water on the day of assay.

8. STABILITY

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label. For information specific to a particular biological standard, contact the Technical Information Officer or, where known, the appropriate NIBSC scientist.

NIBSC follows the policy of WHO with respect to its reference materials.

Accelerated degradation studies on 03/178 indicate that the lyophilized material will be adequately stable at -20°C with respect to B_{12}, holoTC and folate content. Once reconstituted, users should determine the stability of the material according to their own conditions of storage and use.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

9. REFERENCES

SJ Thorpe, A Heath, S Blackmore, A Lee, M Hamilton, S O'Broin, BC Nelson and C Pfeiffer. An international standard for serum vitamin B_{12} and serum folate: international collaborative study to evaluate a batch of lyophilized serum for B_{12} and folate content. Clin Chem Lab Med 45, 380-386 (2007).

10. ACKNOWLEDGEMENTS

We thank the participants of the collaborative studies.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Lyophilisate		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling: See caution, Section 2	
Other (specify):	Contains material of human origin		
Toxicological properties			
Effects of inhalation:	Not established, avoid inhalation		
Effects of ingestion:	Not established, avoid ingestion		
Effects of skin absorption:	Not established, avoid contact with skin		
Suggested First Aid			
Inhalation:	Seek medical advice		
Ingestion:	Seek medical advice		
Contact with eyes:	Wash with copious amounts of water. Seek medical advice		
Contact with skin:	Wash thoroughly with water.		
Action on Spillage and Method of Disposal			
Spillage of contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.			

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.08g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

APPENDIX G. WHO INTERNATIONAL STANDARD FOR WHOLE BLOOD FOLATE

WHO International Standard
Folate, Whole Blood Haemolysate
NIBSC code: 95/528
Instructions for use
(Version 4.0, Dated 04/04/2008)

1. INTENDED USE

The standard is for use in microbiological assays and binding assays for whole blood folate.

The whole blood preparation, 95/528, was evaluated by 13 laboratories in 5 countries. The preparation was assayed using microbiological assays and radio-assays against in-house standards. On the basis of the results, and with the agreement of the participants of the collaborative study, the World Health Organization (WHO) Expert Committee on Biological Standardization established 95/528 as the 1st International Standard for whole blood folate, with an ASSIGNED CONTENT OF 13 ng/AMPOULE.

2. CAUTION

This preparation is not for administration to humans.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

THE FOLATE CONCENTRATION OF THE RECONSTITUTED MATERIAL IS 13 ng/ml.

4. CONTENTS

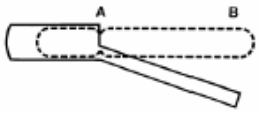
Country of origin of biological material: United Kingdom.
Whole blood from two donors (individual donations tested and found negative for HBsAg, anti-HIV and anti-HCV; obtained from North London Regional Transfusion Centre, Colindale, London, UK) was used to prepare the standard. The blood was pooled, then haemolysed and diluted (1 in 10) in 0.5% (w/v) ascorbic acid solution, dispensed into ampoules and freeze-dried. Secondary desiccation was then carried out to remove residual moisture.

5. STORAGE

Store unopened ampoules at or below -20°C.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure ampoule is scored all round at the narrow part of the neck, with a diamond or tungsten carbide tipped glass knife file or other suitable implement before attempting to open. Place the ampoule in the ampoule opener, positioning the score at position 'A'; shown in the diagram below. Surround the ampoule with cloth or layers of tissue paper. Grip the ampoule and holder in the hand and squeeze at point 'B'. The ampoule will snap open. Take care to avoid cuts and projectile glass fragments that enter eyes. Take care that no material is lost from the ampoule and that no glass falls into the ampoule.



Side view of ampoule opening device containing an ampoule positioned ready to open. 'A' is the score mark and 'B' the point of applied pressure. Care should be taken on opening to prevent loss of contents.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.

Reconstitute the contents of each ampoule with 1.0 ml distilled water. Store at 4°C (short term only since the preparation does not contain sodium azide).

8. STABILITY

It is the policy of WHO not to assign an expiry date to their international reference materials. Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label. They remain valid with the assigned potency and status until withdrawn or amended. Accelerated degradation studies have indicated that this standard is suitably stable, when stored at -20°C or below, for the assigned value to remain valid until the standard is withdrawn or replaced. These studies have also shown that the standard is suitably stable for shipment at ambient temperature without any effect on the assigned value.

For information specific to a particular biological standard, contact the Technical Information officer or, where known, the appropriate NIBSC scientist.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

SJ Thorpe, D Sands, AB Heath, MS Hamilton, S Blackmore and T Barrowcliffe. An International Standard for whole blood folate: evaluation of a lyophilised haemolysate in an international collaborative study. Clin Chem Lab Med 2004; 42:533-539.

10. ACKNOWLEDGEMENTS

We thank the participants of the collaborative study to assign unitage.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG
WHO International Laboratory for Biological Standards, UK Official Medicines Control Laboratory
T +44 (0)1707 641000
nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: Lyophilisate		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling: See caution, Section 2	
Other (specify):	Contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.02g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards