Framework for laboratory harmonization of folate measurements in low- and middle-income countries and regions

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The measurement of serum and red blood cell folate, two commonly used biomarkers of folate status in populations, is complicated by analytical and data interpretation challenges. Folate results show poor comparability across laboratories, even using the same analytical technique. The folate microbiologic assay produces accurate results and requires simple instrumentation. Thus, it could be set up and maintained in low- and middle-income country laboratories. However, the assay has to be harmonized through the use of common critical reagents (e.g., microorganism and folate calibrator) in order to produce comparable results across laboratories and over time, so that the same cutoff values can be applied across surveys. There is a limited need for blood folate measurements in a country owing to the periodic nature of surveys. Having a network of regional resource laboratories proficient in conducting the folate microbiologic assay and willing and able to perform service work for other countries will be the most efficient way to create an infrastructure wherein qualified laboratories produce reliable blood folate data. Continuous participation of these laboratories in a certification program can verify and document their proficiency. If the resource laboratories conduct the work on a fee-for-service basis, they could become self-sustaining in the long run.

Keywords: microbiologic assay; folic acid; 5-methyltetrahydrofolate; serum folate; red blood cell folate; cutoff value

Introduction
Folate status can be assessed through dietary intake, blood biomarker concentrations, or a combination of both. The measurement of biochemical indicators is considered to be more objective than dietary assessment, as it is not affected by recall or underreporting bias. The two main biochemical indicators of folate status are serum and red blood cell (RBC) folate, and these indicators have also been recommended by the Biomarkers on Nutrition and Development folate expert panel.\(^1\) RBC folate has recently also been recommended by the World Health Organization (WHO) as a biomarker for neural tube defect (NTD) risk in women of reproductive age.\(^2\) Assessing folate status through the measurement of biochemical indicators is subject to numerous analytical and data interpretation challenges. A recent article on challenges and lessons learned in generating and interpreting nutritional biomarker data from the U.S. National Health and Nutrition Examination Survey provides several examples related to folate as well as general information on laboratory logistics and quality assurance.\(^3\)

There are no validated field techniques to reliably assess folate status in low-resource environments at the point of specimen collection. Blood samples have to be processed, transported, and stored while maintaining uninterrupted cold chain, owing to the labile nature of this vitamin, before they can be analyzed at a central laboratory that has access to continuous electrical power, specific instrumentation and reagents, and well-trained staff. Furthermore, folate results show poor comparability across laboratories, sometimes even using the same analytical technique.\(^4,5\) This makes it difficult to compare folate concentrations across surveys. It
also complicates the use of cutoff values for folate deficiency and insufficiency, resulting in prevalence estimates that either over- or underestimate the true extent of the problem. For these reasons, a designated folate assay has to be chosen that can be reliably set up and maintained in selected low- and middle-income (LMIC) country laboratories. The assay has to be harmonized such that it generates comparable folate concentrations over time and across laboratories. As a result, the same cutoff values can be used to describe folate status in different populations.

Here, we review the challenges in assessing folate status, both from an analytical and a data interpretation perspective. It also lays out a framework for laboratory harmonization of folate measurements using the microbiologic assay setup in a network of regional resource laboratories. The framework includes considerations of the extent of laboratory capacity needed to provide public health support for national surveys, why a network of regional laboratories would be desirable, what the training for the microbiologic assay should include, and why it is critical to have a microbiologic assay kit and a folate certification program for the network laboratories, two vital components of a quality system.

Challenges in assessing folate status

Analytical challenges

Analytical challenges span the preanalytical, analytical, and postanalytical phases. Compared with several other vitamins, folate poses a larger number of analytical challenges. Folate is a generic term for a group of folate derivatives or forms with vitamin activity. Most folate forms are susceptible to decomposition by light, heat, pH, or oxygen, which underlie enzymatic or chemical interconversions. To protect folates from decomposition, the preanalytical phase requires controlled specimen collection, processing, and storage conditions. Exposure of samples to elevated temperature and direct sunlight needs to be avoided, and delays in specimen processing should be minimized. Delayed processing of whole blood exposed to elevated temperature (32 °C) for only 1 day led to a 30% loss of serum folate, and delayed freezing of serum stored at 11 °C for 14 days led to a 22% loss of serum folate. Delayed processing of whole blood exposed to room temperature for 1 and 2 days led to a 10% and 20% loss of RBC folate, respectively. For RBC folate measurement, either washed erythrocytes are used, or—more commonly—a whole-blood hemolysate in ascorbic acid is generated, typically in the field, but alternatively this can also be prepared in the laboratory before the analysis. This step requires great care, because any inaccuracy in the generation of the hemolysate makes the accurate measurement of RBC folate impossible. Furthermore, folates are less stable once erythrocytes have ruptured than in intact whole blood. For example, 10%, 22%, and 49% losses of folate activity have been reported when thawed whole blood was kept at room temperature for 1, 6, and 24 h, respectively. The dilution of thawed whole blood with ascorbic acid needs to be carried out within 1–2 h to avoid a loss of folate. Whole-blood hemolysate in ascorbic acid showed an ~20% loss of folate after being stored at room temperature for 24 hours. Storing serum at −20 °C for 6 months led to an 11% loss of folate (personal communication, Christine Pfeiffer, April 2017). Whole-blood hemolysate stored at −20 °C for 16 months showed relatively comparable folate results. A 10–15% loss of folate has been reported when whole blood was stored at −70 °C for 2 years, while whole-blood hemolysates in ascorbic acid were stable for at least 4 years when stored at −70 °C. Preanalytical requirements for serum and RBC folate are summarized in Table 1.

The calculation of RBC folate is complex and requires information on whole-blood folate, serum folate, and hematocrit if a hemolysate has been used to measure whole-blood folate. This means that three different assays need to be conducted, which is resource intensive. Efforts are underway to explore how much inaccuracy is introduced if simplified approaches are used to estimate RBC folate (e.g., ignoring serum folate in the calculation of RBC folate; deriving hematocrit from the measured hemoglobin). Alternatively, dried blood spots, which are easier to generate in the field, can be used to assess folate status. However, complete drying of the cards before storage in resealable plastic bags with desiccant sachets is critical, and cards can only be kept refrigerated for up to 1 week before being frozen at −20 °C or lower to avoid a loss of folate greater than 10%. Furthermore, folate and hemoglobin have to be measured in the extract of the dried blood spot card, and whole-blood folate results are expressed as hemoglobin–folate (nmol folate per g hemoglobin). While this
### Table 1. Preanalytical specimen handling requirements for serum and red blood cell folate

| Step                     | Requirements                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
|--------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Sample processing        | - Keep evacuated tubes with whole blood (for serum folate) or anticoagulated EDTA blood (for red blood cell folate) cool and protected from light (e.g., cool box with ice packs; avoid tubes touching ice packs, as this could lead to hemolysis).<br>  
- Process blood as soon as possible, but no later than within 2–3 days of blood collection.<br>  
- To obtain serum, allow the whole blood to clot for 30 min–2 h at room temperature, and then centrifuge the tube for 10 min at 1500 × g to separate the serum from the cells.<br>  
- To generate a whole-blood hemolysate, allow the EDTA blood to reach room temperature, mix the tube contents by inversion 8–10 times, pipet 100 µL of well-mixed blood into 1 mL of 1% ascorbic acid solution, and mix well. |                                                                                                                                                                                                                                                                                                                                                   |
| Sample storage           | - Freeze serum as soon as possible, but no later than within 5 days of generation; maintain cold chain.<br>  
- Freeze whole-blood hemolysate as soon as possible, but no later than within 2 h of generation; maintain cold chain.<br>  
- Serum and whole-blood hemolysate can be stored at −20 °C for up to 3 months; avoid freezer with automatic defrost function.<br>  
- For long-term storage, keep serum and whole-blood hemolysate frozen at −70 °C.                                                                                                                                                                                                                                                                      |
| Freeze–thawing         | - Minimize repeated freeze–thawing and length of time sample is exposed to room temperature.<br>  
- Folate is stable in serum and whole-blood hemolysate for up to three freeze–thawing cycles.<br>  
- Avoid more than one thawing cycle for whole blood as well as prolonged thawing time beyond 1–2 hours.                                                                                                                                                                                                                                         |

calculation allows analysis of blood specimens of unknown volume or dilution, the results can only be interpreted if they are converted to RBC folate by multiplying with the mean corpuscular hemoglobin concentration (g hemoglobin per L whole blood).\(^1\)\(^3\)

Serum folate concentrations are \(\sim 10\%\) higher in nonfasted compared with fasted persons,\(^1\)\(^4\) which complicates sample collection in field studies. However, given that this difference is relatively small, it is acceptable to collect nonfasting blood specimens for population estimates.

Some of the analytical challenges to measure blood folate concentrations are the low folate concentrations in serum (ppb range), the fact that folates bind tightly to proteins in circulation and need to be released for the measurement, and the relatively wide dynamic range of folate concentrations (two orders of magnitude) between deficiency and high concentrations.\(^7\) Laboratory methods to measure folate concentrations show poor comparability even when using the same analytical technique.\(^4\)\(^,\)\(^5\)\(^,\)\(^7\)

This is true for both serum and RBC folate, but even more pronounced with RBC folate.

The three main analytical techniques to measure blood folate concentrations are based on the microbiologic assay, protein-binding assays, and chromatographic assays.\(^1\)\(^,\)\(^7\) Of these three techniques, the microbiologic assay is the least expensive, requires relatively simple instrumentation, and is thus best suited for the low-resource setting. However, it is a relatively lengthy manual assay (it takes about 2 days to obtain results), has moderate precision (\(\sim 10\%\) CV), a limited linear range (requires increased dilution for samples with high concentrations and less dilution for samples with deficient concentrations), and is inhibited by the presence of antibiotics or antifolates. Furthermore, the microbiologic assay has not yet been standardized, and thus results from different laboratories may not be comparable.\(^1\)\(^5\)

Protein-binding assays are relatively easy to conduct, because kits are commercially available, and the analysis is fully automated on clinical analyzers. The precision of these assays is typically good (\(\sim 5\%\) CV); however, their accuracy may be questionable owing to the different binding affinities of the folate-binding protein to the various folate forms. These assays may also exhibit matrix effects when the sample needs to be diluted to meet the concentration range of the assay. But possibly the biggest disadvantage of this assay type is lot-to-lot variability, sometimes as a result of manufacturer assay reformulations or recalibrations. These assays were mainly designed to detect folate deficiency in a clinical setting and are therefore less suited for an application in a public health setting where data need to be compared over time and across laboratories.
Chromatography-based assays can achieve the highest specificity, accuracy, and precision if they are carefully validated and controlled. They provide information on individual folate forms that cannot be gained otherwise. These assays are technically complex and expensive to conduct. The main circulating folate form measured by these assays is 5-methyltetrahydrofolate (5-methylTHF); however, other folate forms, such as folic acid and nonmethyl folate forms, are also present in serum and erythrocytes.

Two critical components that provide information about the quality of laboratory assays are proficiency testing programs and certified reference materials. Unfortunately, for folate analysis, neither of these tools yet provide satisfactory answers. Information gleaned from proficiency testing programs on assay comparability may be of limited value because of material commutability issues. Proficiency testing materials often have to be “manipulated” (e.g., addition of preservatives to enhance stability) and thus may behave differently compared with native patient samples. Furthermore, at present, there are no accuracy-based proficiency testing programs available for folate measurement. Laboratory results are most often compared to peer-group means (e.g., same assay platform) or at best to consensus means (across assay platforms, but nonetheless influenced by assays that provide higher or lower results). Neither of these approaches help to improve the interassay variability. No certified reference materials are currently available for serum or whole-blood total folate, the two indicators measured by the microbiologic assay and by protein-binding assays and used to interpret folate status. Available certified reference materials only provide information for one component of total folate, 5-methylTHF, which is the major circulating form of folate. This hampers the validation of many assays, but particularly those that do not discriminate between different folate forms and only measure total folate.

**Data interpretation challenges**

Folate poses significant challenges for data interpretation. A small but easily surmountable challenge is the use of different units of measure. The clinical field more commonly uses conventional units expressed in ng/mL, while research laboratories generally use SI units expressed in nmol/L. Assays that measure individual folate forms, such as 5-methylTHF, need to use SI units because they calculate total folate as the sum of individual folate forms, which can only be done on a molar level. The generally accepted conversion factor to convert from conventional to SI units is 2.266, based on the molecular weight of folic acid. Conversion factors for other folate forms are slightly different (e.g., 5-methylTHF: 2.177). To provide consistency across population surveys, it is advisable to assign folate calibrator concentrations in SI units and thus report folate concentrations for survey samples in SI units.

A more difficult data interpretation challenge is the correct use and interpretation of cutoff values to describe folate status. Cutoffs for risk of megaloblastic anemia have been derived experimentally and describe a clinical manifestation of folate deficiency. Cutoffs for risk of possible deficiency based on rising total homocysteine describe a metabolic folate insufficiency, and they are more tenuous because they have been derived from epidemiologic data. The WHO RBC folate cutoff for insufficiency represents an elevated risk for NTDs in women of reproductive age on the population level. Not only do these cutoffs represent different stages of depletion, they have also been derived using different assays. Thus, before using a particular cutoff, the user needs to know whether their assay produces comparable data to the assay from which the cutoff was derived.

Cutoffs for risk of megaloblastic anemia (serum folate <7 nmol/L; RBC folate <305 nmol/L) have been derived with the traditional microbiologic assay (wild-type microorganism and folic acid calibrator), which is no longer in use. The contemporary microbiologic assay (chloramphenicol-resistant strain) calibrated with 5-methylTHF seems to generate results that are comparable to the traditional microbiologic assay. It is therefore not necessary to adjust the megaloblastic anemia cutoffs when using data generated from the contemporary microbiologic assay calibrated with 5-methylTHF. However, different cutoffs may have to be used for assays that measure either lower or higher levels. Commercially available clinical protein-binding assays often suggest different cutoffs on the basis of small studies performed either in clinically deficient populations or in a healthy group of individuals who are apparently free of the deficiency.
Cutoffs for risk of possible deficiency based on rising total homocysteine (serum folate <10 nmol/L; RBC folate <340 nmol/L) have been derived with the Bio-Rad radioprotein-binding assay, which measured lower than the contemporary microbiologic assay calibrated with 5-methylTHF and is no longer commercially available.\textsuperscript{21} These cutoffs cannot be used with data derived from other assays without knowledge of how the assay in question compares with the Bio-Rad assay. For most assays available today, this information is not known; thus, these cutoffs are of very limited utility.\textsuperscript{6} For consistency across population surveys, it is not advisable to use these cutoffs to interpret the population folate status.

The RBC folate cutoff for elevated risk of NTDs (<906 nmol/L) has been derived with the contemporary microbiologic assay (chloramphenicol-resistant strain) calibrated with folic acid.\textsuperscript{22} This assay generates higher results than the contemporary microbiologic assay calibrated with 5-methylTHF.\textsuperscript{15} Thus, a lower cutoff (<748 nmol/L) has to be used with data generated with the latter assay.\textsuperscript{6,23}

The poor assay comparability, particularly among different platforms of protein-binding assays, poses significant data interpretation challenges for longitudinal assessments and for comparisons among studies, even when comparing the central tendency (e.g., mean, median, or geometric mean) of two populations. While a comprehensive comparability study with currently available assays could be conducted to derive relationships between different assays, this information would become quickly outdated as assays are reformulated. Until assays are better harmonized, it will be difficult to interpret folate concentrations over time and across laboratories. However, for serum folate (where assay comparability is better than for RBC folate), existing data generated with a commercial protein-binding assay could potentially be adjusted to a reference assay (microbiologic assay calibrated with 5-methylTHF) if an appropriate comparison study is conducted and the correlation between the two assays is sufficient to derive a robust regression equation.\textsuperscript{21}

**Framework for laboratory harmonization**

To overcome the above-mentioned analytical and data interpretation challenges, a designated folate assay has to be chosen that can be reliably set up and maintained in selected LMI country laboratories. This folate assay has to be harmonized through the use of common critical reagents (e.g., microorganism and folate calibrator), such that it generates comparable folate concentrations over time and across laboratories. As a result, the same cutoff values can be used to describe folate status in different populations.

**Laboratory capacity needs**

The microbiologic assay is the WHO-recommended laboratory method to assess folate status in populations.\textsuperscript{2} The principle of the assay is that a folate-dependent microorganism (Lactobacillus rhamnosus, formerly called L. casei) grows proportionally to the amount of folate in the sample, and the folate concentration is quantified by measuring the turbidity of the inoculated growth medium after a nearly 2-day incubation at 37 °C.\textsuperscript{24} The microbiologic assay has many advantages that make it a preferred candidate for LMI country laboratories (Table 2) and is currently the only practical choice to obtain comparable results across laboratories and over time.

While many countries may wish to set up a folate laboratory, this approach would likely not be sustainable in the long term. There is a limited need for blood folate measurements in an LMI country because of the periodic nature of surveys. Usually, a nationally representative baseline survey is conducted first to assess the need for an intervention, then a follow-up survey is conducted 1–2 years after the implementation of the intervention to assess its impact. After that, periodic monitoring is conducted roughly every 5 years to verify folate status, possibly only at a sentinel site rather than on a national level. Given that a routine laboratory can typically handle about 10,000 samples per year with a single analyst setup, the periodic surveys would not provide a sufficiently high volume of samples to maintain the assay on a continuous basis. A typical national survey of women of reproductive age may generate approximately 1000–2000 samples. It is easier and more efficient for the laboratory to continuously perform the assay, because each interruption may pose problems and delays when attempting to place the assay back in service. Furthermore, if there is a higher demand for sample analysis, it is more efficient to scale up production in an already proficient and well-equipped...
Table 2. Advantages of the microbiologic assay for use in low- and middle-income country laboratories

| Category       | Advantage                                                                                                                                 |
|----------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Science        | • Measures all biologically active folate forms                                                                                  |
|                | • Appears to be more accurate than many protein binding assays                                                                    |
|                | • Method from which cutoffs for risk of megaloblastic anemia were derived (serum folate <7 nmol/L; red blood cell folate <305 nmol/L) |
|                | • Method from which cutoff for optimal blood folate levels for the prevention of neural tube defects was derived (red blood cell folate <748 nmol/L when 5-methyltetrahydrofolate is used as a calibrator; red blood cell folate <906 nmol/L when folic acid is used as a calibrator) |
|                | • Assay can utilize serum/plasma, whole blood/washed erythrocytes, and dried blood spots                                              |
| Resources      | • Low cost for reagents, supplies, and instrumentation                                                                             |
|                | • Requires only small specimen volume (<50 μL)                                                                                   |
| Complexity     | • Simple assay procedure                                                                                                          |
|                | • Simple instrumentation (microplate reader, incubator, and pipettes)                                                             |
| Logistics      | • Multiple stations can be set up to increase sample throughput                                                                    |
|                | • Manual assay can be automated by introducing an 8-probe sample handler                                                           |
|                | • Performance can be controlled in-house to avoid long-term assay fluctuations that may be misinterpreted as changes in population folate status |
|                | • Assay can be harmonized by using the same microorganism (chloramphenicol-resistant L. rhamnosus) and the same folate calibrator (5-methyltetrahydrofolate) |

laboratory by having multiple analysts conduct the assay in parallel than to perform the assay on a small scale in multiple laboratories.

**Network of regional resource laboratories**

Having a network of regional resource laboratories that are proficient in conducting the folate microbiologic assay and willing and able to perform service work for other countries is the most efficient way to create an infrastructure where qualified laboratories produce reliable blood folate data that can be compared across laboratories and over time. If the resource laboratories conduct the work on a fee-for-service basis, they should be self-sustaining in the long run. However, initially, scientists have to be trained, and laboratories have to be equipped properly, so that they can start functioning as a resource laboratory. Although the required equipment for the folate microbiologic assay is comparatively less expensive than that for other types of assays, financial resources for a microplate reader, 37 °C incubator, stirring hotplate, vortex mixer, heat plate sealer, plate rotator, precision balance, −70 °C freezer, and various adjustable air displacement pipettes, including an 8- or 12-channel pipette and repeater pipette, amount to approximately U.S. $50,000.

It would be desirable to have two or three qualified resource laboratories in each WHO region, for a total of 10–20 laboratories worldwide. Building on local laboratory capacity would help strengthen existing laboratories. As such, there are currently about half a dozen laboratories in different WHO regions that are proficient in conducting the folate microbiologic assay. As part of the International Health Regulations (2005), all WHO Member States work together for global health security. The WHO plays a coordinating role and, together with partners, helps countries build capacities (http://www.who.int/ihr/capacity-strengthening/laboratory/en/). A role for the WHO as an institutional umbrella to help coordinate the network of regional resource laboratories could be explored. The WHO also provides various key documents and resources, such as a Laboratory Quality Management System handbook, a Quality Management System training toolkit, and a Quality Manual template. The Micronutrient Survey Toolkit is an additional resource to help countries design and implement micronutrient surveys (http://surveytoolkit.micronutrient.org/).

The list of requirements for potential resource laboratories is fairly long (Table 3) and focuses on financial and political commitments. The more points from this list are met, the higher the chance to successfully set up a folate microbiologic assay resource laboratory. However, even if these requirements are met, there are complicating factors that can threaten the success of regional resource
Table 3. Requirements for potential folate microbiologic assay resource laboratories

| Category          | Critical and desirable features and requirements                                                                                                                                 |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Organization      | - Organization is well established and recognized for laboratory science and public health work.                                                                             |
|                   | - Organization has track record of successful collaborative projects with other countries and laboratories.                                                              |
|                   | - Management is interested in expanding the laboratory’s portfolio to include the folate microbiologic assay for population monitoring.                                          |
|                   | - Management must provide adequate resources to establish and maintain the laboratory (laboratory space and dedicated permanent staff).                                         |
|                   | - Organization is able to collect funds for fee-for-service activities.                                                                                                         |
| Laboratory        | - Laboratory is experienced with performing quantitative micronutrient analyses in human biological specimens.                                                               |
|                   | - Laboratory can procure necessary laboratory supplies, chemicals, and equipment.                                                                                              |
|                   | - Laboratory is willing and has the capacity to analyze samples from other countries.                                                                                           |
|                   | - Laboratory agrees to undergo regular external verification and certification to document proficiency with the folate microbiologic assay.                                      |
|                   | - Laboratory has a basic understanding of quality assurance tools.                                                                                                             |
|                   | - Laboratory supervisor is actively involved in daily laboratory management.                                                                                                |
| Infrastructure    | - Access to a –80 °C freezer for storage of specimens and quality control pools, folate calibrator, and microorganism inoculum                                                 |
|                   | - Reliable electrical power and back-up generator                                                                                                                              |
|                   | - Basic laboratory equipment, such as a balance.                                                                                                                              |
|                   | - Low UV yellow lighting in area where folate microbiologic assay is conducted                                                                                                  |
|                   | - Good IT infrastructure (e-mail, Internet, word and data processing software)                                                                                                 |
|                   | - Location should be directly serviced by a commercial carrier and be able to obtain dry ice sample shipments without delays                                                  |
| Staff             | - Good technical laboratory skills                                                                                                                                            |
|                   | - Good pipetting skills                                                                                                                                                        |
|                   | - Ability to understand and follow laboratory protocols and standard operating procedures                                                                                      |
|                   | - Experience with improving, validating, and troubleshooting assays is desirable                                                                                            |
|                   | - Proficiency in English for ease of communication                                                                                                                             |

laboratories. Possibly the biggest complication is that not all countries allow their samples to be taken outside of the country for analysis, yet some countries may not have the infrastructure to successfully set up and maintain the folate microbiologic assay. The second biggest complication relates to ensuring the continuity of proficient staffing. Experienced staff are more likely to move on to new responsibilities within the organization or to other organizations, and the loss of “know-how” would be a major setback for the resource laboratory. Having redundancy with proficient staff is a necessity but one often not within reach. Furthermore, resource laboratories may be hesitant to increase their capacity beyond a single analyst setup even if there appears to be more demand, because it is not clear whether the additional demand will persist. A few smaller challenges include delays in analyzing survey samples because of sample backlogs at the resource laboratory and shipping problems due to the need to use dry ice to maintain sample integrity.

Training for folate microbiologic assay

To ensure consistency in protocols and procedures, it is desirable that staff from future resource laboratories be trained in person by a highly experienced laboratory in a controlled setting where good assay performance can be ensured. Typically, two scientists are trained per laboratory to ensure some redundancy from the start of the project. One trainee should be an experienced laboratory analyst who has good laboratory skills at the bench (e.g., pipetting, preparing reagents, and making dilutions) and a good understanding of laboratory protocols. This trainee would be in charge of setting up the assay upon return to the laboratory and training future analysts. Ideally, the second trainee would be a laboratory team leader or supervisor with daily responsibilities to lead a laboratory project.
and staff. This trainee should have a good understanding of laboratory science, assay validation and troubleshooting, and quality assurance. By learning the microbiologic assay together with the laboratory analyst, the laboratory supervisor will develop a deeper understanding of why and how certain steps are done, which will help them later to assist the laboratory analyst with problems and questions. The folate microbiologic assay training in an established and well-functioning quality assurance environment will show the trainees how various logistic aspects are handled in a situation where resources are less limited (e.g., preparation and use of quality control (QC) materials, tracking of specimens from “cradle to grave,” documentation of laboratory work through standard operating procedures, data review and approval steps, instrument maintenance and documentation, personnel training, and documentation). This gives the laboratory supervisor trainee an opportunity to think about how they could implement and facilitate key aspects of a quality assurance system in their setting with more limited resources. Being in a more powerful position that allows decision making and advocacy, the laboratory supervisor should serve as a liaison to the organization’s management and relate to them laboratory needs and challenges to develop and maintain a culture where high-quality laboratory work is appreciated and supported.

The folate microbiologic assay training typically takes about 3 weeks to allow trainees to first observe various procedures and then conduct the procedures themselves. The training also includes aspects of how to prepare the microorganism inoculum and the folate calibrator. Lastly, numerous quality assurance topics are covered, such as the preparation and use of bench QC materials, maintenance and verification of pipettes and other equipment, data review and interpretation, and assay validation and troubleshooting. The timing of the training should be chosen such that the future resource laboratory has access to all supplies and equipment necessary to promptly set up the folate microbiologic assay upon returning from the training. To ensure successful implementation of the folate microbiologic assay in the new resource laboratory, the trainees must follow certain posttraining requirements: set up the assay within 1 month of returning from the training, send assay performance data to the trainers, and participate in an initial assay certification to document proficiency. If a microbiologic assay kit can be made commercially available, and training videos documenting specific procedural steps are available to allow prospective trainees to review the materials before the training as well as after returning from the training, the length of the training could potentially be shortened to 1 week, which would significantly lower the associated costs.

Microbiologic assay kit

While all of the supplies needed to set up the folate microbiologic assay are commercially available, this is not in form of a ready-to-use kit and thus requires substantial work and experience by the laboratory to generate the set of reagents needed to conduct the assay. To facilitate the work of the resource laboratories and ensure consistent quality of reagents, a microbiologic assay kit should be developed that contains the following key components: microorganism, folate calibrator, and other pre aliquoted reagents that need to be added to the growth medium at the time of preparation (chloramphenicol, manganese sulfate, and ascorbic acid) (Fig. 1). These three components need to be stored frozen at −70 °C and shipped on dry ice. Should open-market procurement of the growth medium (stored at ambient temperature) become difficult, this could also be part of the assay kit. Lastly, QC materials (stored frozen at −70 °C and shipped on dry ice) could be made available.

Growth medium. There is only a limited number of manufacturers who produce the growth medium, and recently there have been delays in being able to procure the growth medium from one manufacturer due to technical difficulties. There is a possibility that this manufacturer may stop producing the growth medium. Efforts are currently underway to communicate to the manufacturers the need for uninterrupted availability of the growth medium to ensure a successful implementation of resource laboratories using the folate microbiologic assay. To avoid the need for individual negotiations between resource laboratories and the manufacturer(s), the inclusion of the growth medium into the assay kit could be considered. One bottle of growth medium powder generates sufficient reagent to measure approximately 700–800 samples, and the shelf life of the powdered growth medium is approximately 2 years. Depending on the size of the folate survey, a few bottles of growth medium
Figure 1. Folate microbiologic assay kit.

would be sufficient to analyze approximately 2000 survey samples.

**Microorganism.** The chloramphenicol-resistant *L. rhamnosus* (ATCC 27773 or NCIB 10463) can be procured from the American Type Culture Collection (Manassas, VA). From this, a cryoprotected inoculum has to be prepared that is aliquoted and frozen for daily use (typically <1 mL/vial). Some difficulties associated with the production of the microorganism inoculum are that the batch needs to have good growth properties, shows a low background in folate-free medium, and generates reproducible daily calibration curves; furthermore, the batch-to-batch variability of the inoculum has to be minimized to avoid undesired effects on the assay performance. One vial of microorganism inoculum is needed for each assay to inoculate the 200 mL of growth medium.

**Folate calibrator.** Difficulties associated with the generation of the folate calibrator are the sensitive nature of folates (folates easily decompose in the presence of oxygen, heat, and/or light); the need to determine the concentration of the folate stock solution spectrophotometrically; the need to prepare accurate folate intermediate solutions that are aliquoted and frozen for daily use; and the fact that the microorganism responds differently to folic acid or 5-methylTHF as a calibrator. Even though 5-methylTHF is the major folate form in both serum and RBCs, laboratories have traditionally used folic acid as a calibrator because it is more stable and easier to handle. However, given that the microorganism responds with stronger growth to 5-methylTHF compared with folic acid, using 5-methylTHF as a calibrator provides more accurate results.

**QC materials.** The availability of large well-characterized pools of QC material and predefined QC rules for the acceptance and rejection of assay results is a cornerstone of a strong quality assurance program. When a laboratory prepares a large batch of QC materials in house and then carefully characterizes the folate concentration in that material over a period of 10–20 assays, the material can be used over multiple years and multiple studies to verify and document stable assay performance, provided the material is safely stored at −70 °C. However, it requires experience to be able to select appropriate
materials and to generate a high-quality homogeneous product, particularly given the sensitivity of folates to decomposition. If the QC materials are of insufficient quality, they may hamper instead of help with troubleshooting assay problems. A further complication with the in-house generation of QC materials is that the commercial availability of blood products from blood banks is generally more limited in LMI countries. Clinical laboratories often use commercial QC materials to verify the performance of their assays. However, this typically does not allow them to perform long-term monitoring for assay shifts using the same QC material. It would therefore be beneficial if sufficient amounts of high-quality QC materials could be made available to the resource laboratories that analyze survey samples.

The availability of a folate microbiologic assay kit would help to minimize between-laboratory variability, and it would greatly simplify operations in the resource laboratories. For example, upon conclusion of the training, the trainees could be provided with a start-up assay kit that contains limited amounts of ready-to-use microorganism inoculum, 5-methylTHF calibrator, and the other reagents needed for the growth medium preparation, as well as QC pools with known folate target values and acceptability limits. All of this may allow the resource laboratory to more easily and more quickly set up the folate microbiologic assay. Later, when the resource laboratory is approached to conduct fee-for-service folate analysis for a survey, the resource laboratory could purchase a survey assay kit containing the necessary amounts of the above three components to complete the analysis for that particular survey. They would also have to purchase the growth medium and QC pools.

**Folate certification program for folate microbiologic assay laboratories**

To verify and document that resource laboratories are proficient in conducting the folate microbiologic assay, a folate assay certification program needs to be developed, and assay performance criteria for bias and precision need to be defined (Fig. 2). Each potential resource laboratory needs to undergo an initial assay certification in which they analyze a predefined number of biological samples over multiple days and the results are compared to predetermined performance criteria. If performance criteria are met, the resource laboratory obtains a folate assay proficiency certificate valid for 1 year. The resource laboratory signs up for an annual certification program and twice a year obtains a predefined set of biological samples that are analyzed over multiple days. If the resource laboratory again meets the performance criteria, they obtain a new folate assay proficiency certificate valid for 1 year. This annual recertification ensures that measurement quality is maintained over time.

**Enhanced capacity of regional resource laboratories**

A few aspects that could amplify the public health impact of regional resource laboratories are worth mentioning. First, each resource laboratory could eventually become a trainer of other laboratories in the region, after having several years of continued experience and documented proficiency with the folate microbiologic assay. Thus, the original training of the regional resource laboratory would in essence be a “train-the-trainer” situation. Second, with available resources, periodic technical workshops could be conducted for the regional resource laboratories to update them on new technologies, quality assurance issues, and other pertinent laboratory science aspects. This may serve as an incentive to retain talented laboratory staff at their current institution and thus help with the sustainability of the folate microbiologic assay in LMI countries. Third, the responsibilities of regional resource laboratories could be broadened by expanding their capacity to assess other micronutrients in addition to folate. Owing to the common biochemical pathways of folate and vitamin B\(_{12}\), assessing vitamin B\(_{12}\) status together with folate status would greatly improve the interpretation of B vitamin status in the population. Vitamin B\(_{12}\) can also be analyzed by the microbiologic assay, which may facilitate the setup owing to the compatible equipment required for both folate and vitamin B\(_{12}\). Other micronutrients that are typically assessed as part of national micronutrient surveys are vitamin A, iron, and iodine. Including these additional micronutrients into the training and certification program would require additional resources, as would equipping laboratories with the necessary instrumentation.

**Consensus statement and next steps**

To move the folate laboratory harmonization project forward in LMI countries, initial resources need to be committed to developing and making
available a folate microbiologic assay kit, to developing and implementing a folate microbiologic assay certification program, and to selecting and training suitable laboratories that can build a network of regional resource laboratories. The intent is that the framework will be sustained at the country and regional level once established with some level of global oversight and yearly proficiency certification.

**Research gaps and needs**

Most of the research gaps related to the framework of laboratory harmonization revolve around technical issues to simplify the maintenance, operation, and interpretation of the folate microbiologic assay:

- Can a stable microbiologic assay kit be produced that can be stored refrigerated or even at ambient temperature for at least 1 year? This may include a lyophilized form of the microorganism that can be easily reactivated by dissolving it in growth medium to generate the inoculum needed for a larger number of assays. Similarly, the use of a lyophilized calibrator could be explored. Lastly, the stable assay kit could contain preweighed quantities of other reagents that need to be added to the growth medium at the time of preparation.

- Can the harmonized folate microbiologic assay achieve comparable between-laboratory variability as commercial protein-binding assays conducted on a single instrument platform?

- Can folic acid (better stability) be used as a calibrator instead of 5-methylTHF if all laboratories use the same microorganism and results are mathematically adjusted to be equivalent to 5-methylTHF calibration?

- Can RBC folate be accurately assessed from a whole-blood folate measurement without having to separately measure serum folate and hematocrit and by using the available hemoglobin data?

**Summary**

Assessing folate status through the measurement of biochemical indicators is subject to numerous analytical and data interpretation challenges. The microbiologic assay is the WHO
recommended laboratory method to assess folate status in populations. If the assay is harmonized through the use of common critical reagents, most importantly the folate calibrator and microorganism, and if other critical reagents that are difficult to procure are provided (i.e., growth medium and QC pools), the microbiologic assay could be a practical choice to obtain comparable results across laboratories and over time. The availability of a microbiologic assay kit that contains these critical reagents would greatly facilitate laboratory operations. Because of the limited need for blood folate measurements in an LMI country due to the periodic nature of surveys, having a network of regional resource laboratories that are proficient in conducting the folate microbiologic assay and willing and able to perform service work for other countries could be a sustainable way to create an infrastructure where qualified laboratories produce reliable blood folate data. To verify and document the resource laboratory’s achievement and maintenance of proficiency in conducting the folate microbiologic assay, the laboratory would undergo an annual evaluation and certification. The capacity of the regional resource laboratories could be further enhanced if they themselves become trainer laboratories and if their responsibilities can be broadened to provide laboratory service work for additional micronutrients beyond folate.

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Competing interests

The authors declare no competing interests.

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