FOLATE MICROBIOLOGIC ASSAY

TRAINING MANUAL

Version 1

2018

THIS TRAINING MANUAL WAS GENERATED AS PART OF A COLLABORATIVE PROJECT BETWEEN THE CDC NATIONAL CENTER FOR ENVIRONMENTAL HEALTH, DIVISION OF LABORATORY SCIENCES, NUTRITIONAL BIOMARKERS BRANCH AND THE CDC FOUNDATION, NUTRITION INTERNATIONAL, AND THE BILL & MELINDA GATES FOUNDATION

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LIST OF ABBREVIATIONS

5-methyl-THF	5-methyltetrahydrofolate
CDC	Centers for Disease Control and Prevention
CV	Coefficient of variation
Hct	Hematocrit
L. rhamnosus	*Lactobacillus rhamnosus*
MBA	Microbiologic assay
MO	Microorganism
OD	Optical density
QC	Quality control
RBC	Red blood cell
SA	Sodium ascorbate
CAL	Calibrator
WBL	Whole blood lysate
WHO	World Health Organization

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INTRODUCTION

Welcome to the Folate Microbiologic Assay Training Manual. The purpose of this manual is to outline and detail steps of the folate microbiologic assay. For best results, the training manual can be used in conjunction with the training video and a set of training posters. All three of these tools are laid out similarly. Also included in the training manual are procedures for reviewing and calculating results, tips for optimizing assay performance and information on supply and equipment needs.

When women of reproductive age have low blood folate concentrations, their pregnancies have an increased risk of developing neural tube birth defects. Surveys that determine folate status help countries assess the extent of these folate deficiencies. Then, they can determine if interventions, such as fortification or supplementation, are needed.

Knowing the optimal blood folate concentration needed for women of reproductive age to reduce the risk of neural tube birth defects is key to interpreting the data. A recent guideline published by the World Health Organization provides this information and recommends the use of the microbiologic assay to assess folate status in population surveys (WHO 2015).

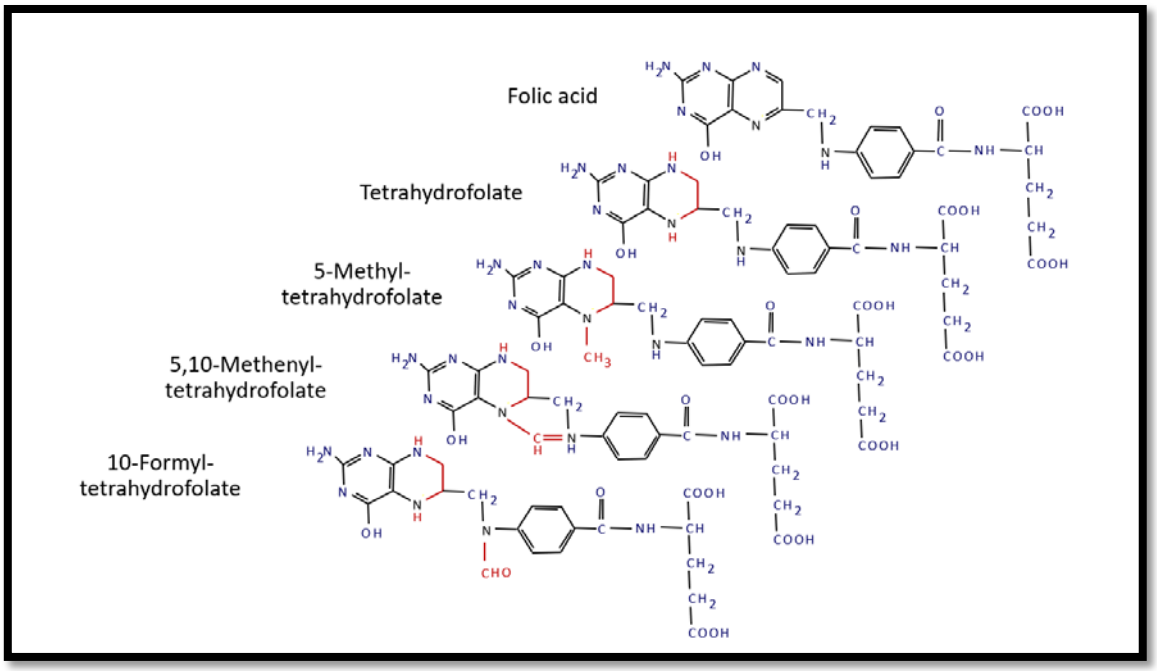
Different folate assays produce different results (Pfeiffer et al., 2016). This makes it difficult to interpret data from multiple sources or use the same cutoff value to assess folate deficiency or insufficiency.

The folate microbiologic assay is the assay of choice for population surveys because it responds similarly to all biologically active forms of folate (chart page 8), requires a small sample volume, and does not use expensive supplies or instrumentation, making it particularly suitable for low-resource settings.

Furthermore, recent findings show that when different laboratories use the same critical reagents—such as the microorganism and calibrator—in their microbiologic assay, the comparability of their results improves (Zhang et al., 2018).

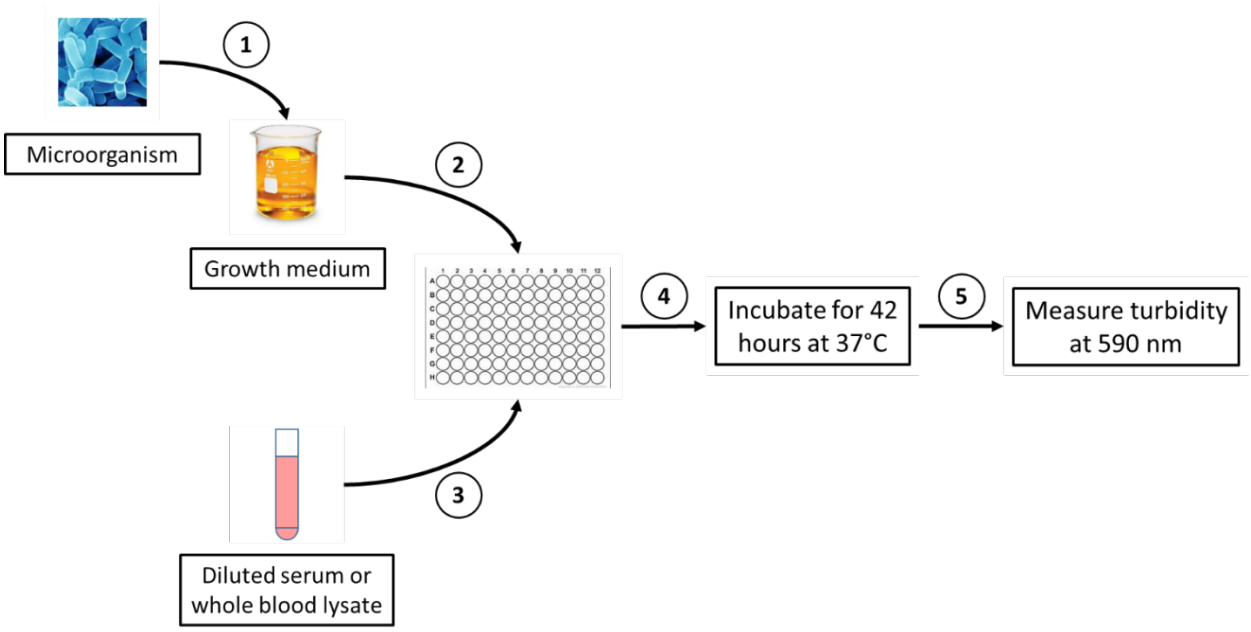
In performing the folate microbiologic assay, please remember to consider all biological specimens to be potentially infectious. Therefore, it is critical that you observe universal precautions, wear appropriate protective personal equipment, and follow good laboratory practices when handling chemicals.

BIOLOGICALLY ACTIVE FORMS OF FOLATE



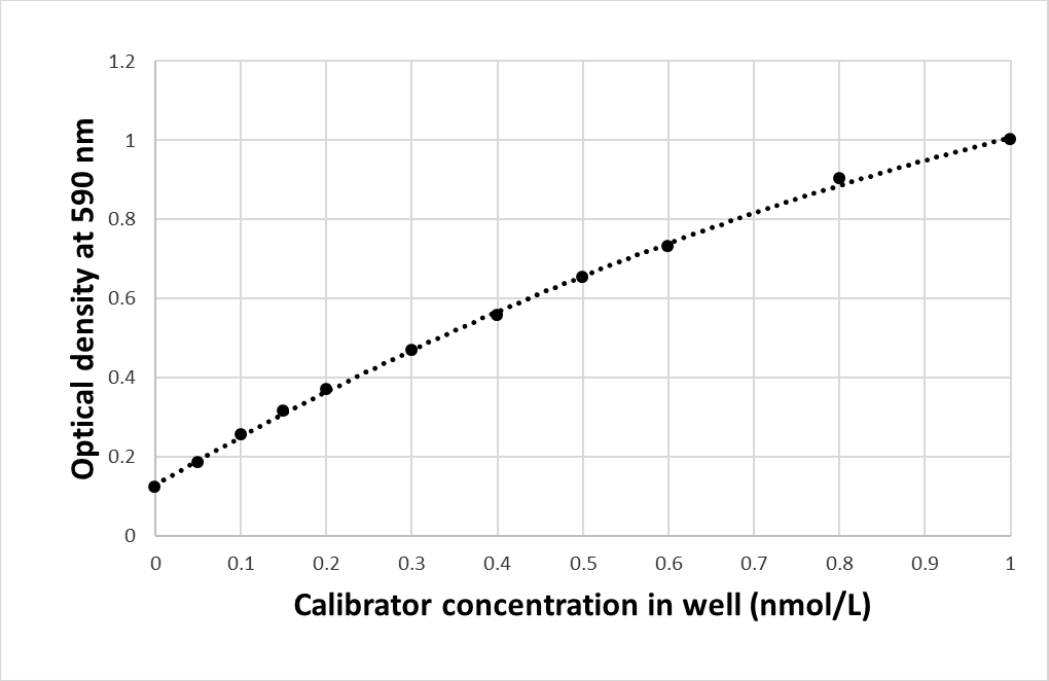
PRINCIPLE OF THE FOLATE MICROBIOLOGIC ASSAY

- First add the microorganism (*Lactobacillus rhamnosus*) to growth medium containing all the nutrients the microorganism needs to grow, except for folate (step 1).
- Then, the inoculated growth medium and diluted serum or whole blood lysate are added to 96-well plates (steps 2 and 3).
- Incubate the plates for 42 hours at 37°C (step 4).
- Assess the folate concentration by measuring the turbidity of the inoculated medium at 590 nm in a microplate reader (step 5).



- The growth of the microorganism is proportional to the amount of folate present in the sample.

- To calibrate the assay, we prepare a separate calibrator plate using 5-methyltetrahydrofolate, the main folate form in serum and in most whole blood samples.



Calibrator: 5-methyltetrahydrofolate

NOTE: ***Parts 1 through 6 are conducted on day 1, while parts 7 through 9 are conducted on day 3 of the folate microbiologic assay.***

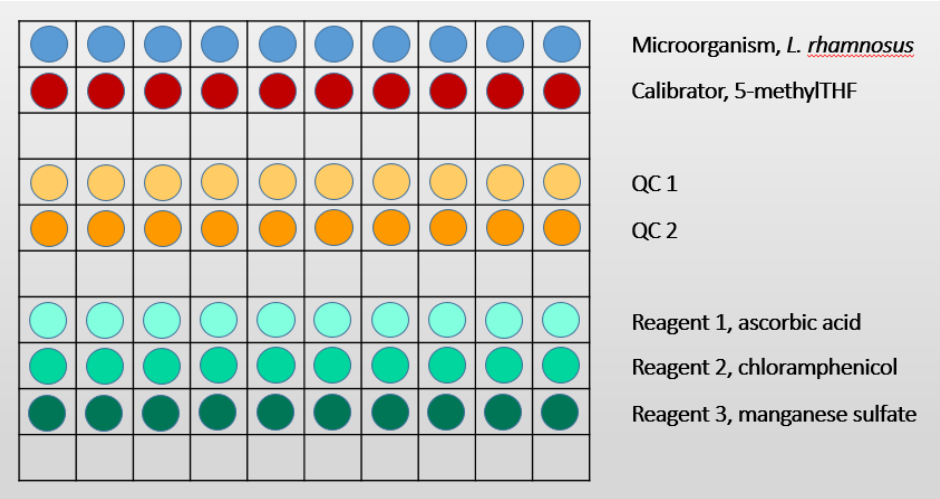
PART 1. PREPARE REAGENTS

1.1 FOLATE MICROBIOLOGIC ASSAY KIT

- To facilitate the set-up and maintenance of the microbiologic assay in low-resource public health laboratories, the CDC has assembled a frozen assay kit containing the following components:
  - Microorganism *(Lactobacillus rhamnosus)*
  - Calibrator stock solution (5-methyl-tetrahydrofolate)
  - Quality control materials (2 levels)
  - Reagents (3) needed for the preparation of the growth medium
    - Ascorbic acid
    - Chloramphenicol
    - Manganese sulfate

- Not provided with the kit are the following items:
  - Growth medium (Folic Acid Casei)
  - Tween-80
  - Sodium ascorbate
  - Deionized water

NOTE: ***Each assay kit box contains materials for 10 runs (10 vials/material).***



1.2 PREPARE REAGENTS FRESH FOR EACH RUN

Two reagents need to be prepared freshly for each run: 0.5% sodium ascorbate and growth medium containing microorganism.

- **Sodium ascorbate solution (0.5%, 500 mL)**
  Add 2.5 g of sodium ascorbate into a 500-mL beaker.
  Add 500 mL of distilled water, mix well. Cover the beaker with foil.

- **Assay growth medium (200 mL)**
  Weigh 14.1 g of Folic Acid Casei Medium, add it into a 400-mL beaker.
  Add 200 mL of distilled water, mix well. Cover with foil.

  Heat solution to boiling, keep boiling for 3 min. Cool down to room temperature.

  Add 1 vial of ascorbic acid stock solution* (1 mL per vial).
  Add 1 vial of chloramphenicol stock solution* (2 mL per vial).
  Add 1 vial of manganese sulfate stock solution* (1 mL per vial).
  Add 60 µL of Tween-80.
  Mix well for a few minutes.

  Thaw 1 vial of *L. rhamnosus**; add 700 µL (or amount specified in assay kit) to the medium.
  Stir gently, cover the beaker with foil.

***NOTE:** To avoid wasting growth medium, scale the amount of growth medium prepared to the number of plates included in a run based on information provided in Table 1.*

Table 1. Amounts of chemicals needed for certain volumes of growth medium

Number of plates	Medium needed (mL)	Folic Acid Casei medium (g)	DI water (mL)	Tween-80 (µL)	Ascorbic acid* (µL)	Chloramphenicol* (µL)	sulfate* (µL)
7-8	200	14.1	200	60	1000	2000	1000
5-6	150	10.6	150	45	750	1500	750
2-4	110	7.8	110	33	550	1100	550

*Component of Folate Microbiologic Assay Kit

1.3 EQUIPMENT AND SUPPLIES

The major equipment and supplies needed for the folate microbiologic assay are listed below. See **Appendix A** for additional details.

- Microplate reader
- Microplate heat sealer (not essential, but makes plate sealing much easier and avoids having to apply a lot of manual pressure)
- 37°C incubator
- 360° rotator
- Vortex mixer
- Stirring hotplate
- Various pipettes and corresponding pipette tips
- General laboratory equipment, such as a balance, freezer, and refrigerator
- 96-well microplates
- Sealing membrane
- General laboratory supplies such as glassware, racks, and various plastic and glass vials
- General laboratory safety supplies such as gloves, lab coat, safety glasses, biohazard autoclave bags and waste pans, bench covers and bleach

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PART 2. SET UP AND DILUTE SAMPLES

2.1 ASSAY RUN SHEET

Keep a run sheet (such as below) to make note of medium lot #, calibrator lot #, sample dilution factor, or any other specific information.

Folate Microbiologic Assay Run Sheet

Run Date:				
Analyst name:				
Analyte:	**Serum Folate**		**Whole blood folate**	
Study samples:				
Calibrator lot #:				
Microorganism lot #:				
Growth medium (Himedia) lot #:				
Instrument Model & SN:	**Bio-Tek PowerWave, xxxx**			
Start incubation time:				
Stop incubation time:				

Additional notes:

2.2 THAW SAMPLES AND SET-UP RACKS

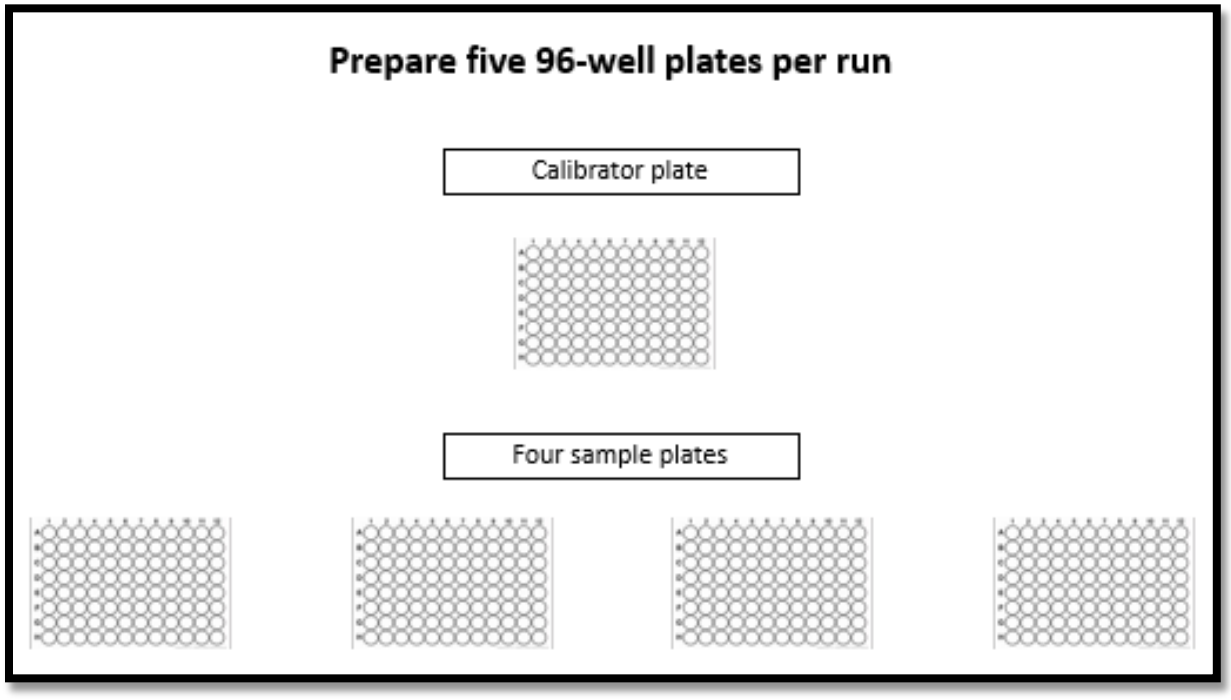
Thaw samples and QCs

- Thaw frozen unknown samples (serum or whole blood lysate) in 12x4 racks and add QC and blank samples to each rack.

Set-up racks

- To minimize errors during sample dilution, use a parallel setup for sample vials coming from the collection site (unknown samples) and for the glass tubes that contain the diluted samples (i.e., 12 samples/row, same number of rows per rack).
- Label the same number of glass tubes by 1, 2, 3…, as you have samples/run and place them in 12x6 glass tube racks.
- Each rack (12x4 and 12x6) holds 48 samples, which fit into two 96-well plates.

An experienced analyst can comfortably prepare five 96-well plates per run: one calibrator plate (#1) and four sample plates (#2–5). In a run with five plates, each sample plate contains one QC sample, 22 unknown samples, and one blank sample used as a negative control. Thus, each run consists of two low and two high QC samples, 88 unknown samples, and four blanks (see **Appendix B** for detailed layout).



2.3 SAMPLE DILUTION

- For regular dilutions, dilute serum samples at ~1:100 and whole blood lysate samples at ~1:140 as indicated below. The whole blood lysate samples are whole blood diluted 1:11 with ascorbic acid. Thus, the regular whole blood dilution factor is 11*140=1540. Alternative dilutions for samples anticipated to contain very low (lower dilution) or high (higher dilution) concentrations of serum or whole blood folate are outlined in the **Table 2**.

- For serum samples, add 1,475 µL of 0.5% sodium ascorbate into each glass tube using a 25-mL automated repeater pipette.

- For whole blood lysate samples, add 2,075 µL of 0.5% sodium ascorbate into each glass tube using the 25-mL automated repeater pipette.

- Thoroughly vortex-mix each sample vial before transferring 15 µL of unknown or QC sample into the glass tubes containing the appropriate amount of sodium ascorbate using a single channel manual pipette (10–100 µL range). Ensure that there are no air bubbles in the pipette tip.

- As you finish with one sample, move the sample vial to the second 12x4 rack (initially empty) and move the glass tube to the second 12x6 rack (initially empty). This helps to keep track of which samples have been pipetted and which still need to be pipetted.

- When completed, cap the glass tubes in the 12x6 rack; keep them on the bench protected from light.

- Move the original sample vials in the 12x4 rack to the refrigerator and keep them there until later when you scan the sample IDs. Do not change the sequence of the sample vials on the rack. After the samples have been scanned, return the vials to the freezer for future analysis, if needed. Always make sure to keep sample exposure to room temperature and light to a minimum.

- Record assay information on the run sheet.

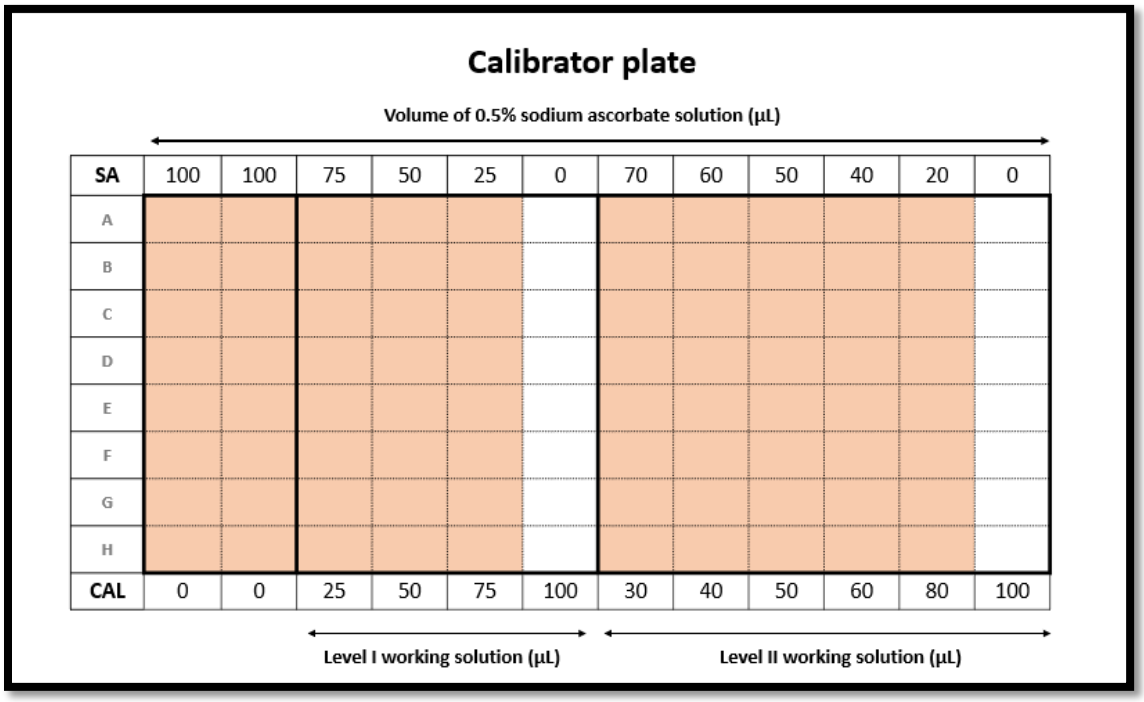
Table 2. Alternative sample dilution for serum and whole blood lysate (WBL) samples

Matrix	Dilution	Sample (µL)	Sodium ascorbate (µL)	Total volume (µL)	Dilution factor in software		Note
					100 µL added	50 µL added	
Serum	~30	15	450	465	31.0	62.0	Lower than regular dilution
	~50	15	750	765	51.0	102.0	Lower than regular dilution
	~100	**15**	**1475**	**1490**	**99.3**	**198.7**	**Regular dilution**
	~200	15	1475	1490	198.7	397.3	Higher than regular dilution. Make 1:2 pre-dilution: 150 µL serum + 150 µL SA
WBL (1:11)	~50	15	750	765	561.0	1122.0	Lower than regular dilution
	~70	15	1025	1040	762.7	1525.3	Lower than regular dilution
	~140	**15**	**2075**	**2090**	**1532.7**	**3065.3**	**Regular dilution**
	~280	15	2075	2090	3065.3	6130.7	Higher than regular dilution. Make 1:2 pre-dilution: 150 µL WBL + 150 µL SA

PART 3: PREPARE 96-WELL PLATES AND ADD SAMPLES

3.1 LABEL CALIBRATOR PLATE

- Label each plate on the front with the date and analyst initial.
- Write the plate number on the upper left corner and on the front.
- Plate #1 is used for the calibrator and subsequent plates are used for samples.
- The assay is conducted by using 8 replicates for each calibrator concentration.
- Write the different volumes of sodium ascorbate (SA) at the top of each column and the different volumes of calibrator (CAL) at the bottom of each column on the plate.



3.2 LABEL SAMPLE PLATES

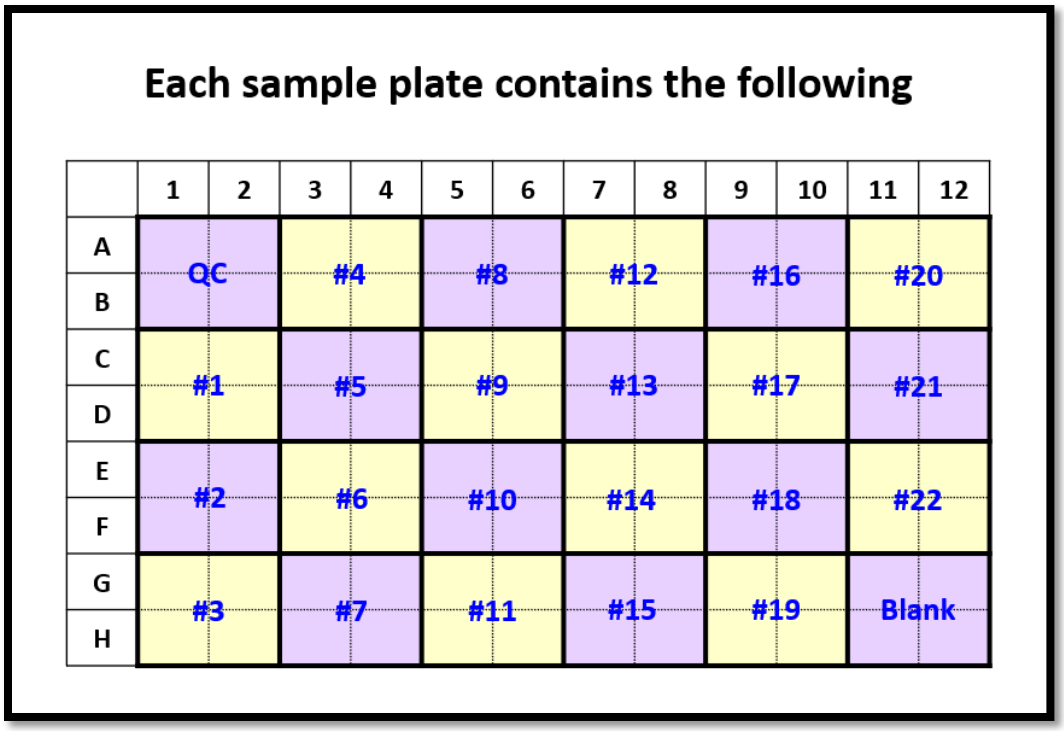
- The assay is conducted by using 4 replicates for each sample at 2 different dilutions (2 replicates/dilution). A total of 24 samples can be analyzed per sample plate including at least 1 QC sample and 1 blank.

- To help with correct transfer of diluted samples into the plate, divide the plate into columns and rows as shown here.

- Each sample will occupy four wells.

- Use a marker pen to label the sample # on the sample plates.

- A complete set-up of 4 sample plates is included in **Appendix B**.



3.3 ADD GROWTH MEDIUM

The first two reagents that need to be added to the 96-well plates are the growth medium and the sodium ascorbate solution.

Growth medium: Use a 12-channel electronic pipette (50–1,250 µL) with the repetitive pipetting function and volume set to 200 µL to add 200 µL of growth medium to all plates.

Plate 1 -- Calibrator plate

Add growth medium (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200	200	200	200	200	200	200	200	200	200	200	200
B	200	200	200	200	200	200	200	200	200	200	200	200
C	200	200	200	200	200	200	200	200	200	200	200	200
D	200	200	200	200	200	200	200	200	200	200	200	200
E	200	200	200	200	200	200	200	200	200	200	200	200
F	200	200	200	200	200	200	200	200	200	200	200	200
G	200	200	200	200	200	200	200	200	200	200	200	200
H	200	200	200	200	200	200	200	200	200	200	200	200

Plate 2 – 5 -- Sample plates

Add growth medium (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200	200	200	200	200	200	200	200	200	200	200	200
B	200	200	200	200	200	200	200	200	200	200	200	200
C	200	200	200	200	200	200	200	200	200	200	200	200
D	200	200	200	200	200	200	200	200	200	200	200	200
E	200	200	200	200	200	200	200	200	200	200	200	200
F	200	200	200	200	200	200	200	200	200	200	200	200
G	200	200	200	200	200	200	200	200	200	200	200	200
H	200	200	200	200	200	200	200	200	200	200	200	200

3.4 ADD SODIUM ASCORBATE

Sodium ascorbate: To add sodium ascorbate to plate #1, the calibrator plate, use a smaller 12-channel electronic pipette (20-300 µL) with the single pipetting function and 8 tips. Transfer appropriate volumes into each column as shown here.

Calibrator plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	75	50	25	0	70	60	50	40	20	0
B	100	100	75	50	25	0	70	60	50	40	20	0
C	100	100	75	50	25	0	70	60	50	40	20	0
D	100	100	75	50	25	0	70	60	50	40	20	0
E	100	100	75	50	25	0	70	60	50	40	20	0
F	100	100	75	50	25	0	70	60	50	40	20	0
G	100	100	75	50	25	0	70	60	50	40	20	0
H	100	100	75	50	25	0	70	60	50	40	20	0

Volume of 0.5% sodium ascorbate solution (µL)

Next, use the same 12-channel electronic pipette but now with the repetitive pipetting function and volume set to 50 µL to add sodium ascorbate into plates #2-5 (sample plates), but only add the solution to the odd-number columns using 8 tips.

Sample plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	0	50	0	50	0	50	0	50	0	50	0
B	50	0	50	0	50	0	50	0	50	0	50	0
C	50	0	50	0	50	0	50	0	50	0	50	0
D	50	0	50	0	50	0	50	0	50	0	50	0
E	50	0	50	0	50	0	50	0	50	0	50	0
F	50	0	50	0	50	0	50	0	50	0	50	0
G	50	0	50	0	50	0	50	0	50	0	50	0
H	50	0	50	0	50	0	50	0	50	0	50	0

Volume of 0.5% sodium ascorbate solution (µL)

Cover all plates with foil to protect them from dust and light.

PART 4: DILUTE CALIBRATOR AND ADD TO CALIBRATOR PLATE

4.1 PREPARE 5-METHYL-THF CALIBRATOR WORKING SOLUTIONS

- Prepare calibrator working solutions about 15 min before use.
    - The calibrator may not be stable if prepared too early before use (>30 min).
    - The preparation is done on a different bench to prevent folate contamination.

- Thaw 1 vial of frozen calibrator stock (5-methyl-THF, 1 µmol/L) for about 15 min at room temperature protected from light and mix well.

- To prepare a 1:5 diluted stock solution (200 nmol/L), add 400 µL of 0.5% sodium ascorbate to a 2-mL cryovial; add 100 µL of calibrator stock solution and mix well.

- Add approximately 45 mL of 0.5% sodium ascorbate each to two 50-mL grade A volumetric flasks labeled as Level I and Level II.

- Prepare working solution I: transfer 50 µL of 1:5 diluted stock solution to the Level I flask (1:1000 dilution; 200 pmol/L) and make up to 50 mL volume as detailed below:
    - Using a plastic transfer pipette that has a fine tip, make up with sodium ascorbate to the 50-mL volume mark.
    - Be sure that the bottom of the fluid meniscus touches the calibration line of the volumetric flask at eye level. This is a very critical step to achieve accurate results.

- Prepare working solution II: transfer 250 µL of 1:5 diluted stock solution to the Level II flask (1:200 dilution; 1 nmol/L) and make up to 50 mL volume as detailed:
    - Using a plastic transfer pipette that has a fine tip, make up with sodium ascorbate to the 50-mL volume mark.
    - Be sure that the bottom of the fluid meniscus touches the calibration line of the volumetric flask at eye level. This is a very critical step to achieve accurate results.

- Slowly invert the volumetric flasks 20 times to thoroughly mix the calibrator working solutions.

- Move back to the sample preparation area. Change gloves and bench cover after diluting the calibrator to prevent folate contamination.

4.2 ADD LEVEL I AND LEVEL II WORKING SOLUTIONS TO CALIBRATOR PLATE

- Pour the Level I and Level II working solutions into two labeled basins.

- Add calibrator working solutions to plate #1 using the smaller 12-channel electronic pipette (20-300 µL) with the single pipetting function and 8 tips. Transfer the appropriate volumes of the working solutions to plate #1 as shown here.

Calibrator plate

Add level I and II working solution

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	25	50	75	100	30	40	50	60	80	100
B	0	0	25	50	75	100	30	40	50	60	80	100
C	0	0	25	50	75	100	30	40	50	60	80	100
D	0	0	25	50	75	100	30	40	50	60	80	100
E	0	0	25	50	75	100	30	40	50	60	80	100
F	0	0	25	50	75	100	30	40	50	60	80	100
G	0	0	25	50	75	100	30	40	50	60	80	100
H	0	0	25	50	75	100	30	40	50	60	80	100

Level I working solution (µL) Level II working solution (µL)

Calibrator concentration in well (nmol/L)											
Blank	0.00	0.05	0.10	0.15	0.20	0.30	0.40	0.50	0.60	0.80	1.00

- Gently hand-seal the calibrator plate with a sealing membrane and set it aside as well as the basins containing the working solutions.

- Change gloves to prevent accidental folate contamination of the sample plates in the next step.

***NOTE:** The calibration curve is made up of 11 different concentration points. The total volume is 100 µL in each well (0.5% sodium ascorbate + level I or level II working calibrator solution).*

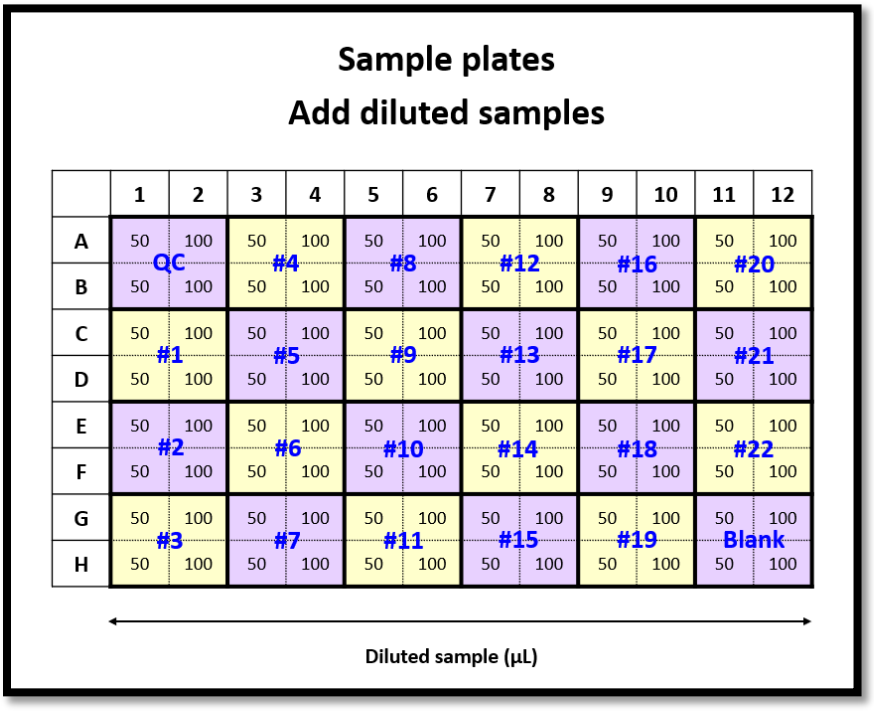
PART 5: ADD DILUTED SAMPLES TO SAMPLE PLATES

- To transfer an aliquot of the diluted samples to the sample plates, use a single-channel electronic pipette (20-300 µL) with the repetitive pipetting function and volume set to 50 µL.

- It is very important that you thoroughly vortex-mix each diluted sample prior to taking an aliquot.

- Add diluted sample into 4 wells each, 2 x 50 µL and 2 x 100 µL (pipette 50 µL twice to get 100 µL).
    - Add 50 µL of diluted sample to 2 wells in an odd-number column
    - Add 100 µL of diluted sample to 2 wells in an even-number column
    - Total sample volume (0.5% sodium ascorbate + diluted sample) is 100 µL in each well

- The full plate layout is shown here.



- After finishing with one plate, gently hand-seal the plate with a sealing membrane.

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PART 6: SEAL AND INCUBATE PLATES AND SET-UP SOFTWARE FOR THE RUN

6.1 SEAL PLATES USING A HEAT SEALER

- Pre-set the heat sealer to 130°C.
- Apply manual sealing to the plates using a flat object, press the sealing membrane down to remove residual air.
- Once the temperature is stable, place a piece of aluminum foil on top of the sealing membrane to protect the samples from overheating.
- Place the plate on the holder, and apply heat for 5 sec.
- Remove the foil membrane.
- Immediately place an ice pack on top of the plate for a few seconds to cool down the membrane.
- Apply additional manual sealing to prevent leakage and thus improve precision: using a flat object, apply pressure to the surface of the sealing membrane, then carefully seal all edges and corners.

6.2 SEAL PLATES MANUALLY

- If a heat sealer is not available, use the flat side of a plastic object that is not sharp and doesn't tear up the sealing membrane to apply pressure to the surface of the plate by running the object firmly in all directions. Pay special attention to thoroughly seal all edges and corners by tracing with the object firmly around the curved corners of the wells on each of the 4 sides.

6.3 INCUBATE PLATES FOR 42 HOURS AT 37°C

- Once properly sealed, the plates are ready for incubation.
- Stack the plates and sandwich them in between two empty plates.
- Make sure the temperature in the incubator is within ±2°C of 37°C and that the fan works to distribute the heat evenly.
- Place the plates in the center of the incubator.
- Keep the plates incubated for approximately 42 hours.
- Remember to time the start and end of this process carefully so you aren't forced to remove the plates in the middle of the night. Record start and end time in the assay run sheet.

NOTE: ***Each lot of microorganism inoculum may display slightly different growth characteristics and thus may need fewer or more hours of incubation for optimal growth. The folate assay kit provides information on the optimum incubation time for the microorganism.***

6.4 INSTRUMENT SOFTWARE REQUIREMENTS

- There are different microplate readers available.

- The reader you use must allow you to:

  1) Use a wavelength of 590 nm

  2) Set up an experiment with multiple plates, because you'll use the calibration curve from plate #1 to calculate results from the sample plates; and

  3) Choose the correct curve fit to calculate results, which is the polynomial 3rd degree curve.

- At the CDC, we use the Bio-Tek PowerWave reader with the Gen5 software to collect data. Set-up of experiment templates with this software is described in the following pages.

- For more flexibility with the size of your run–either fewer or more plates–you can generate different experiment templates according to the number of plates used in the run.

6.5 SET UP RUN IN INSTRUMENT SOFTWARE

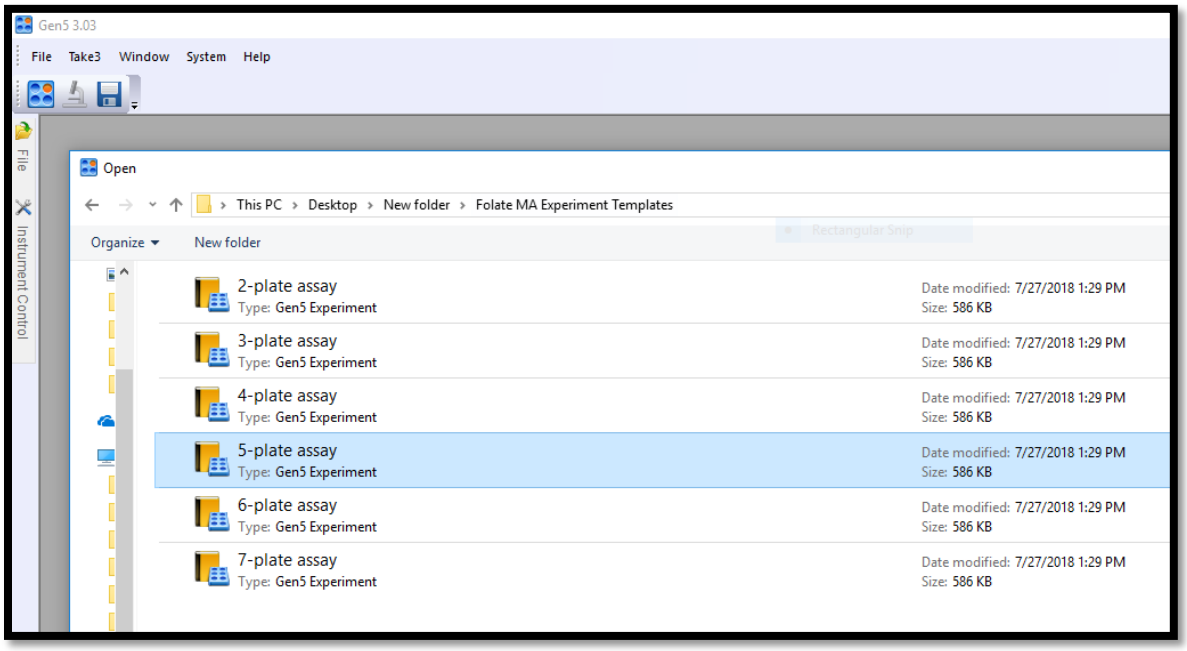
1. Scan the sample ID's for the unknown samples into Notepad in the same sequence as they were diluted. Return the sample vials to the freezer.

2. Setup experiment templates in Gen5 software.

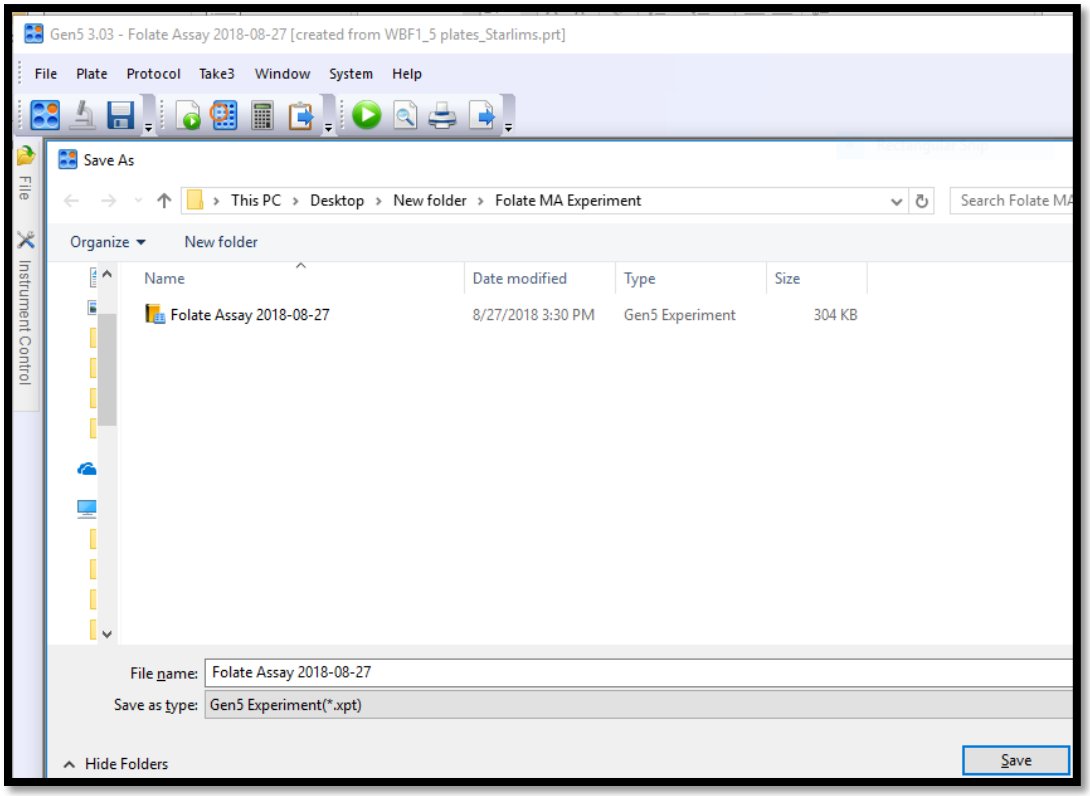
The Bio-Tek PowerWave microplate reader operated by Gen5 software is used to read plates. The reading parameters are shown below. Experiment templates for different run sizes (2-7 plates) can be created.

Gen5 Parameter	Setting
Reading Type	End Point
Wave Length	590 nm
Shaking Intensity	0
Shaking Duration	0
Temperature control	No
Data Interpolate	M590
X Axis	Lin
Y Axis	Lin
Curve Fit	Polynomial Regression, Degree 3

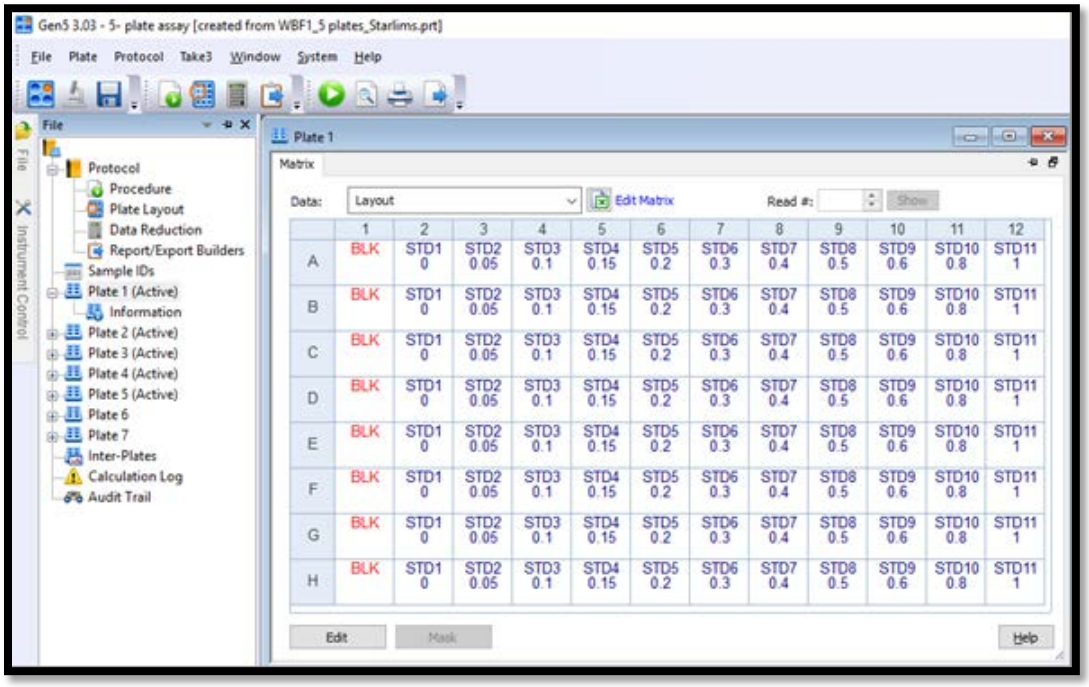
3. Open an experiment template according to the number of plates in the current run.



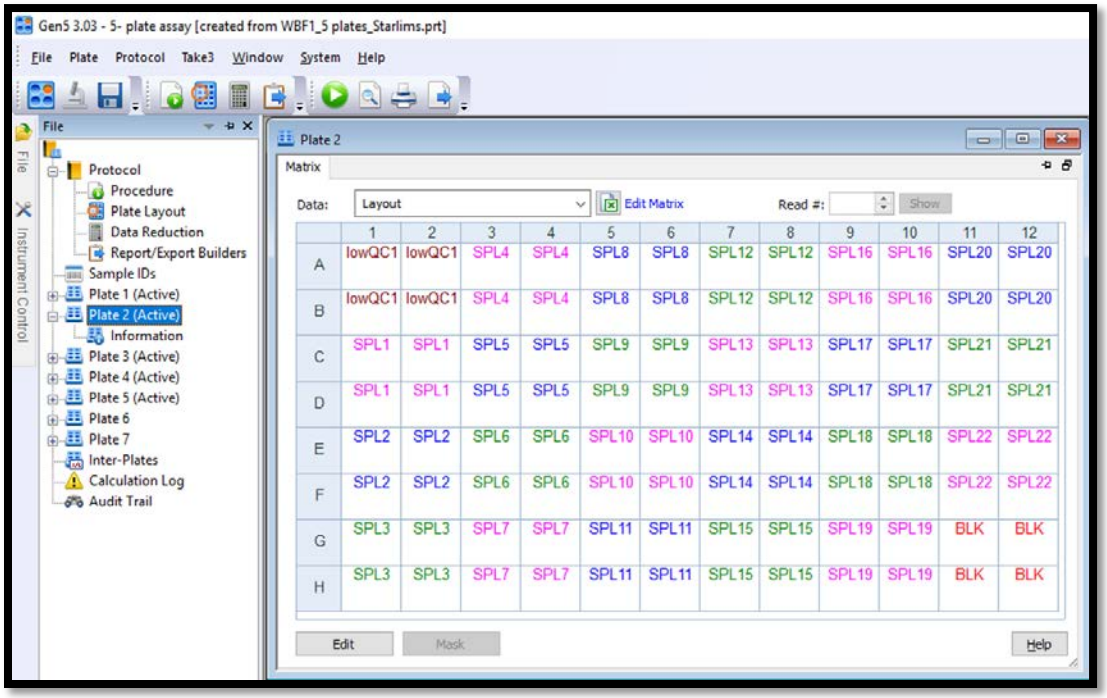
4. Save the experiment template with a new name, such as today's date.



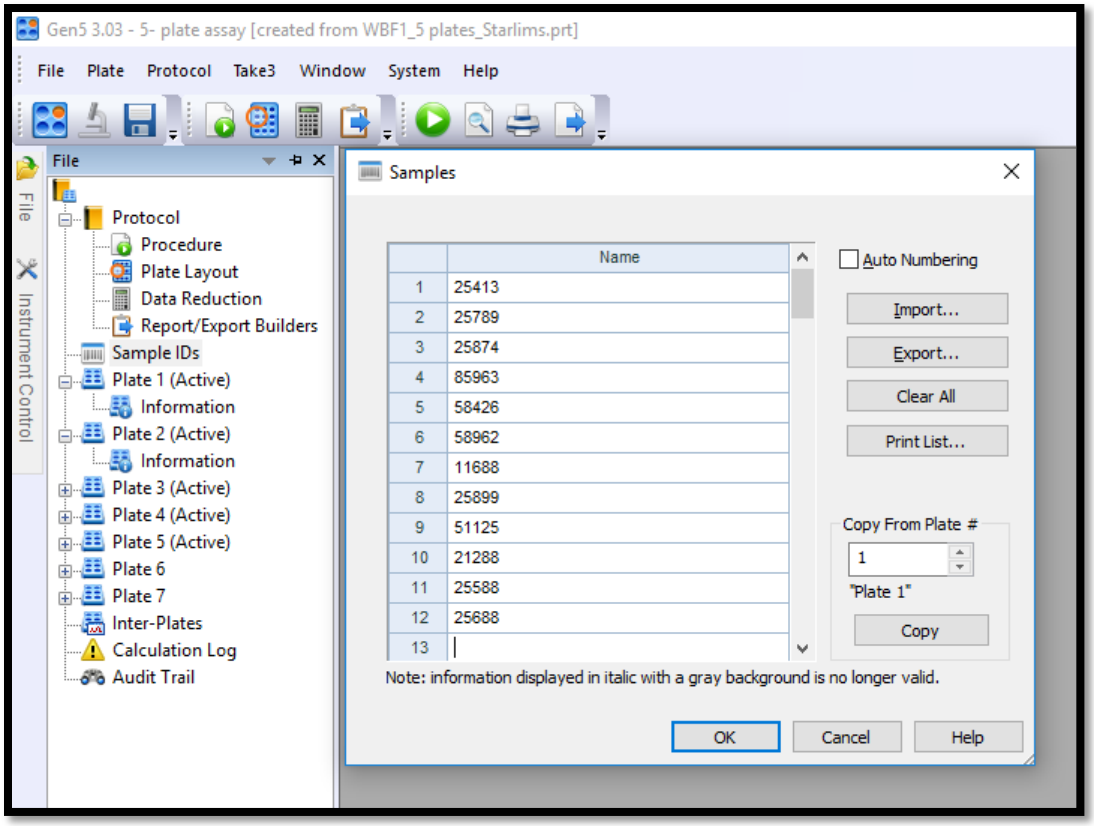
5. Check the layout for the calibrator plate and make changes as necessary.



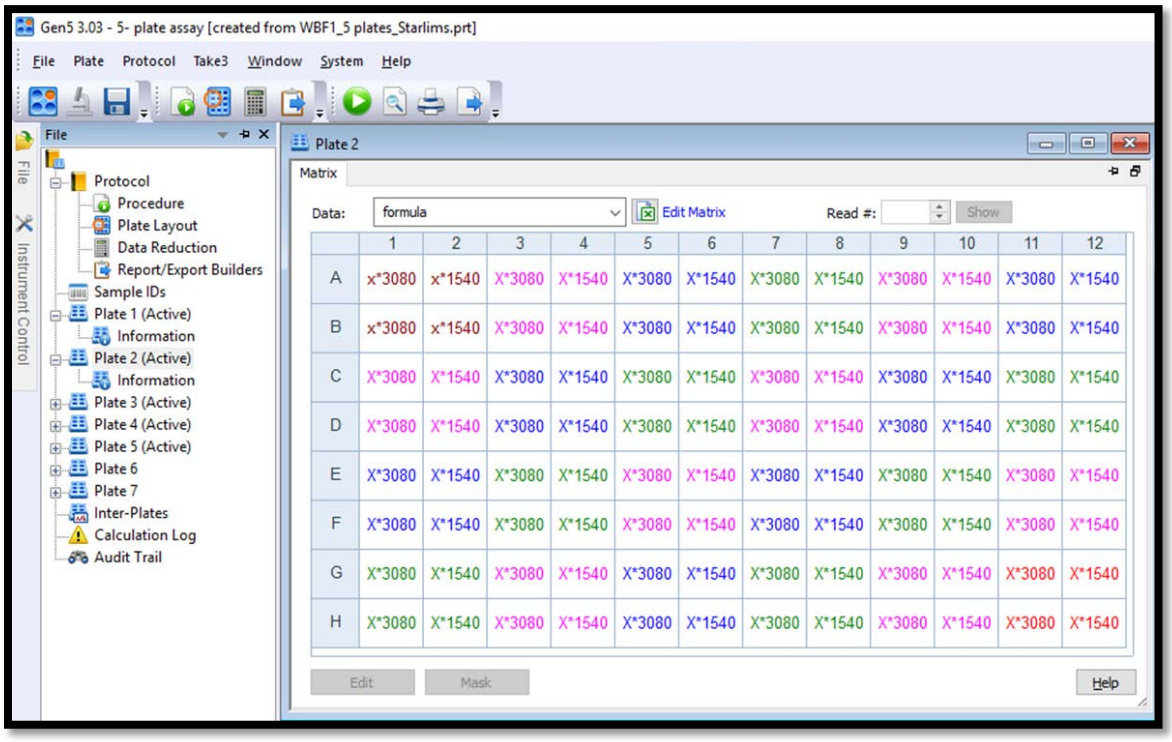
6. Check the layout for the sample plates and make changes as necessary.



7. Upload the sample ID file from Notepad into this newly created experiment.



8. **Make sure correct sample dilution factors are indicated; edit the dilution factors if irregular dilutions are used**.



9. **Conduct clean-up and disposal as described in Part 9 and as required by your local safety regulations. This is the last step that needs to be completed on day 1 of the folate microbiologic assay.**

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PART 7: READ PLATES

- On day 3, take plates out of the incubator after ~42 hours incubation.

- Turn on Bio-Tek PowerWave (or other) microplate reader 30 min before use.

- Let the plates cool down to room temperature while rotating them on a 360 degree rotator. The cooling and mixing take at least 30 minutes.

- Open the experiment created 2 days ago in the Gen5 software when the experiment was setup.

- Remove one plate at a time from the orbital rotator and read the plates one-by-one in exactly the same way:
    - Strictly control the time from stopping the mixing to reading the plate to 1, maximum 1.5 min. For example, if you read the first plate at 1:25 min, read all other plates at ~1:25 min (±10 sec).
    - Wipe the bottom of the plate with a tissue to remove any particles or dust.
    - Gently open the sealing membrane to prevent liquid spilling and place the plate into the plate reader.
    - Gently fan the air above the plate to remove micro-bubbles.
    - Do not remove more than one plate from the orbital rotator and do not remove the sealing membrane from more than one plate at a time.
    - If plates sit longer on the bench prior to reading the OD, uneven settlement of the organism can lead to inaccurate results.

- After reading each plate, save the results.

- Generate a PowerExport report containing information regarding the assay file name, reading time, microorganism lot #, calibrator lot #, medium lot #, reader #, analyst, and calibration curve equation, sample IDs, plate #, well #, OD at 590 nm, raw concentration, dilution factor, raw concentration x dilution, count, mean folate concentration, SD, CV and any notes.

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PART 8: REVIEW RESULTS

8.1 DATA REVIEW

- Visually check the calibrator plate for leaking, dark color, or abnormal volume; review the data for the calibrator plate in the software for potential contamination or outlier ODs that are largely different from the average of 8 replicates. If outliers are confirmed as random occurrences, "mask" the wells in the software so that they are excluded from the calibration curve. The calibration curve represents the following concentrations: 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 nmol/L.

- Visually check the sample plates for leaking, dark color, or abnormal volume; review the data for the sample plates in the software for potential contamination or outlier ODs. If outliers are confirmed as random occurrences, "mask" the wells. The criteria for replicate results are as follows:
    - If the CV of the 4 replicates is ≤15%, report the result.
    - If the CV of the 4 replicates is >15%, evaluate whether deleting one replicate that appears to be an outlier (i.e., 6, 8, 8, 9 – 6 appears to be an outlier) reduces the CV to ≤10%; if it does, use the mean of the remaining 3 replicates for reporting; if the CV of the remaining 3 replicates is >10%, keep all 4 replicates, and repeat the sample in a future run.
    - If there is no clear outlier (i.e., 6, 8, 8, 10), leave all 4 replicates in. Do not report the result and repeat the sample in a future run.
    - Do not report results from less than 3 replicates. Repeat the sample in a future run.

- Results outside the calibration curve range need to be repeated. The raw concentration of the calibration curve is from 0.05–1 nmol/L. If the raw concentration of a sample is <0.05 nmol/L (lowest calibrator), the Gen5 software marks the result with a symbol; the sample needs to be repeated with less dilution in a future run. If the raw concentration of a sample is >1 nmol/L, the Gen5 software does not calculate a result; the sample needs to be repeated with higher dilution in a future run.

8.2 CALCULATE RED BLOOD CELL FOLATE CONCENTRATION

To be able to calculate red blood cell (RBC) folate results, you need the results for whole blood lysate folate, serum folate, and hematocrit (see traditional formula). This calculation provides the most accurate RBC folate result and thus a good reflection of body stores.

Traditional formula:

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

"Fill values" can be used if serum folate and/or hematocrit results are not available, however this can only be done for a small portion of samples in a study, otherwise the population data may no longer be valid. "Fill values" should not be used when individual RBC folate results are interpreted.

- If the serum folate result is not available, a fill value of 18 nmol/L or the median serum folate concentration of the population can be used.
- If the Hct result is not available, a fill value of 40% or the median Hct value for the population can be used.

Some laboratories use a simplified formula that does not correct for the serum folate contribution, but this approach slightly overestimates the RBC folate concentration:

Simple formula:

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

PART 9: CLEAN UP AND DISPOSAL

BE SURE TO DISPOSE IN ACCORDANCE WITH YOUR LOCAL SAFETY REGULATIONS

Day 1 (experiment set-up)

- Add bleach to the leftover growth medium containing the microorganism for a final concentration of approximately 10% and let it sit for 30 min to inactivate the microorganism. After that time, the growth medium turns brown and can be discarded into the drain.

- Place disposable gloves and plastic, glass, and paper items (pipette tips, vials, glass tubes, gloves, microplates, disposable bench liners etc.) that may have come in contact with serum or blood into a biohazard autoclave bag and keep the bags in appropriate containers until they are autoclaved and properly disposed.

- Discard leftover 0.5% sodium ascorbate solution into the drain.

- Wash beakers with deionized water.

- Wipe all work surfaces with 10% bleach solution after the daily work is completed.

Day 3 (after reading plates)

- Discard the plates into a biohazard autoclave bag after reading is completed and the plates have been visually inspected.

- Wipe work surfaces with 10% bleach solution.

NOTE: This assay is very sensitive at detecting trace levels of folate and therefore you need to work carefully to prevent folate contamination.

- ***Use a separate bench to prepare your calibrator working solutions.***
- ***If possible, use separate sinks to clean up your reusable glassware and to discard and clean up your left-over calibrator solutions.***
- ***Also remember to regularly decontaminate the bench surface and pipettes used for sample preparation.***

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PART 10. TIPS AND REFERENCES FOR THE FOLATE MICROBIOLOGIC ASSAY

10.1 ADVANCES IN AND ADVANTAGES OF THE FOLATE MICROBIOLOGIC ASSAY

- **Historically considered the "gold standard" measurement for serum and RBC total folate**
    - Measures multiple forms of folate species with vitamin activity
    - Does not measure folate species that lack vitamin activity

- **Improvements in efficiency and robustness allow reliable use in high-throughput routine settings**
    - Development of chloramphenicol-resistant strain of *L. rhamnosus*
    - Ability to cryopreserve the inoculum
    - Miniaturization of the assay to 96-well plate format

- **Very sensitive; requires only a small volume of sample (~50 µL)**
    - Appropriate for samples collected from fingerstick
    - Validated for dried blood spot samples

- **Suited for low resource settings**
    - Required instrumentation is simple and relatively inexpensive
    - Required laboratory technique is simple
    - Calibration and long-term performance can be controlled in-house
    - Results in general agreement with higher order LC-MS/MS methods

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Adapted from Bailey et al., 2015; see original document for additional details.

10.2 KEYS TO OPTIMIZING PERFORMANCE OF THE FOLATE MICROBIOLOGIC ASSAY

- **Use a liquid handler to dilute and dispense samples and reagents**
  - Improves precision
  - Reduces manual labor
  - Increases efficiency
- **Select an appropriate calibrator**
  - 5-methyl-THF constitutes the largest portion of total folate in both serum and whole blood samples
  - 5-methyl-THF recommended calibrator for improved accuracy and comparison of results between laboratories (Pfeiffer et al., 2011)
- **Handle calibrator with great care**
  - 5-methyl-THF is sensitive to oxidation – limit exposure to air and room temperature
  - If preparing 5-methyl-THF stock solutions in-house, prepare with great care (use antioxidants, purge solutions with nitrogen)
  - Store stock solutions in small single-use aliquots at -70°C
- **Prepare ready-to-use reagents**
  - Prepare reagents to be added to the medium in advance to reduce daily workload and chance of contamination
  - Ready-to-use reagents can be stored at -70°C for up to 1 year and easily added to the medium at the time of preparation
- **Generate a "sensitive" *L. rhamnosus* inoculum**
  - Helps ensure good assay sensitivity (growth response per unit of folate)
  - Stop the growth of the organism during the log growth phase and freeze multiple inoculum samples at -70°C for subsequent sample analysis
  - Ideally, create each new inoculum from an original culture (freeze-dried organism); avoid creating more than two subcultures from the original culture
  - Test the new inoculum lot prior to using it for sample analysis
  - Use a negative control (medium with no folate) to ensure that no microorganism is growing in the absence of folate

- **Use appropriate sample dilution**
    - Required dilution varies with the folate concentrations in serum and whole blood samples
    - Samples from a population exposed to folic acid fortification require greater dilution than samples from a deficient population
- **Prevent contamination**
    - To avoid cross-contamination, use separate work areas and a separate set of pipettes for the preparation of calibrator stock solutions and the preparation of reagents and analysis of samples
    - Use disposable supplies when possible
    - Use durable supplies for dedicated purposes and thoroughly clean them after each use to avoid cross-contamination
- **Follow pre-established sample and run QC criteria**
    - Ensures objective data review and allows for automated (criteria based) acceptance, rejection and repeat analysis determination
    - Proposed criteria:
        - For sample with 4 replicates (2 dilutions): accept result if n = 4 and CV ≤15% or n = 3 and CV ≤10%; reject result if n ≤2 and repeat sample
        - If sample result is outside of calibration range, repeat sample at higher or lower dilutions
        - If QC included in the run is outside of pre-established limits, repeat entire run
- **Conduct regular calibration verification and instrument checks**
    - Verify accuracy of pipettes and automatic liquid handler
    - Ensure proper calibration of the microplate reader
    - Monitor temperature of the incubator, freezer and refrigerator

Adapted from Bailey et al., 2015; see original document for additional details.

10.3 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 517–574.

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

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APPENDIX A. SUPPLY AND EQUIPMENT LISTS FOR FOLATE MICROBIOLOGIC ASSAY

Prices are in USD and represent non-contracted price if purchased in the US. Updated 10/01/2018.

Manufacturer information provided as example only; it does not represent an endorsement of specific manufacturers; alternative manufacturers can be used as long as quality is comparable. Numbers indicated in the right hand column of the tables below indicate that further information on some items can be found at the indicated websites, which are listed on page 51.

Folate Microbiologic Assay -- Essential (tier I) chemicals, supplies, and equipment

Chemicals	Unit price	Units needed	Total price	Comment	#
Folic acid casei medium (Himedia M543, 100 g). No alternative available as of 2018.	$130	3	$390	One bottle sufficient for 9 runs with 5-6 plates/run (~700 samples) or 12 runs with 2-4 plates/run; **purchase 3 bottles to set up assay and have some extra for a small study**	1.
L-Sodium ascorbate, 99% purity (Ex: Fisher Item # AC352685000, 500 g). Alternate brand can be used.	$113	1	$113	One bottle lasts for several years; need only 2.5 g/run	2.
Tween-80 (Ex: Fisher Item # T054625G, 25 g). Brand can be substituted.	$16	1	$16	One bottle lasts for several years; need only 60 uL for 200 mL medium	2.
Ethyl alcohol, 100%, USP (Ex: Fisher Item# 07-678-005, 500 mL). Alternate brand can be used.	$43	1	$43	Used to clean pipettes	2.
Bleach or alternate disinfectant				Added to leftover medium to deactivate non-pathogenic bacteria	
Disinfectant hand soap - can be purchased from household store				Used to wash hands after lab work	
SUM	**$302**		**$562**		
Expendable Supplies	**Unit price**	**Units needed**	**Total price**	**Comment**	**#**
Pipette tips					
Eppendorf tips, 2-200 uL (Eppendorf 022491938, 960/10 boxes)	$107	2	$214	One pack sufficient for ~700 samples; used for pipetting 60 uL Tween-80 and 15 uL sample; if no dedicated pipettes for calibrator, pipette 100 uL of calibrator stock and 50 uL of diluted calibrator; **purchase 2 packs to set up assay and have some extra for a small study**	2.
Eppendorf tips, 100-1000 uL (Eppendorf 022491954, 960/10 boxes)	$116	1	$116	One pack sufficient for ~2,000 samples; used for dispensing 700 uL microorganism; if no dedicated pipettes for calibrator, pipette 400 uL of sodium ascorbate to generate diluted calibrator and 250 uL of diluted calibrator	2.
Eppendorf North America COMBITIPS ADVANCED 25 mL PCR, dispensing range: 0.25-5 mL (Eppendorf 0030089472, 100/case)	$171	1	$171	One case sufficient for ~2,000 samples; used for dispensing sodium ascorbate into glass tubes for sample dilution; need to buy adapter separately	2.
Eppendorf adapter for COMBITIPS ADVANCED pipet tips (Item # 13-683-727)	$26	1	$26	One adapter is sufficient; can be autoclaved and re-used. Case of 7 available for $128.07	2.

Rainin tips for 20-300 uL electronic single and for 12-channel pipette (Rainin GPS-L300, Item # 30389303, 768/pack)	$56	3	$167	One pack sufficient for ~700 samples; used for transferring sodium ascorbate and diluted sample into plate; if lab doesn't have Integra 12-channel pipette to add medium to plate, this pipette is used and 12 extra tips/run are used; **purchase 3 packs to set up assay and have some extra for a small study**	3.
Well plates and accessories					
96-Well microplate, non-treated, clear, without lid, non-sterile (Ex: Fisher Item # 12-565-226, 60/pack). Alternative brand can be used.	$184	2	$369	One pack sufficient for ~10 runs (700 samples) or for 20 small runs to set up assay; **purchase 2 packs to set up assay and have some extra for a small study**	2.
ThermalSeal 2 Films for PCR plate, TS2-100, 100/pk (Thomas Scientific # 1204C20). Not recommended to substitute with other brand as this provides best sealing based on CDC testing.	$85	2	$171	One pack sufficient for 20 runs at 5 plates/run (1400 samples); **purchase 2 packs to set up assay and have some extra for a moderate-size study**	4.
Disposable pipette basins for 12-channel pipette (Ex: Fisher Item # 13-681-500, 100/pack)	$78	2	$156	One pack is sufficient for ~20 runs; **purchase 2 packs to set up assay and have some extra for a moderate-size study**	2.
Disposable culture tubes, 6 mL capacity, glass, round bottom, non-sterile (Ex: Fisher Item #14-958-10B, 1000/case). Alternative brand can be used.	$120	2	$240	One case sufficient for ~10 runs (~700 samples) or 20 small runs to set up assay; used for sample dilution; **purchase 2 cases to set up assay and have some extra for a small study**	2.
Glass tube safety caps, need to fit disposable culture tube (Ex: Fisher Item #15-182-220, 1000/pack). Alternative brand can be used.	$121	1	$121	One pack sufficient to set up assay and for ~20 runs (~700 samples).	2.
Medium size weighing boat (Ex: Fisher Item # 13-735-743, 50/pack). Alternative brand can be used.	$31	2	$62	One pack sufficient for ~20 runs; **purchase 2 packs to set up assay and have some extra for a small study**	2.
Cryovials (Ex: Fisher Item # S39021, 500/pk). Alternative brand and/or alternative item (e.g., microcentrifuge tube) can be used.	$78	1	$78	Used for diluted calibrator stock; one pack sufficient for ~500 runs	2.
Common lab supplies					
Biohazard bags (small for bench-top holder; Ex.: Fisher Item # 01-826-1, pack of 200)	$114	1	$114	To collect small daily biohazard waste that gets transferred to larger container at end of day; one pack sufficient for ~200 runs	2.
Gloves, safety glasses, lab coats - need to be available in laboratory				Part of personal protective equipment (PPE)	
Paper towels - can be purchased from household store				To wipe down lab bench, clean durable supplies and equipment, etc.	
Aluminum foil - can be purchased from household store				To cover plates and protect from dust and light	
SUM	**$1,287**		**$2,004**		
Equipment and Durable Supplies	**Unit price**	**Units needed**	**Total price**	**Note**	**#**
Equipment					
Microplate reader, able to read at 590 nm wavelength, multi-plate reading function (Ex: BioTek Epoch2, including Gen5 software, installation and training)	$13,260	1	$13,260	Replacement for earlier PowerWave 340 model; covers wavelength 200-900 nm, but slightly slower in reading (8 sec/plate)	5.
Computer for microplate reader	$1,000	1	$1,000	Need spreadsheet calculation software (ex. Microsoft Excel) for data analysis	

Printer for microplate reader	$200	1	$200		
37°C Incubator, no need of CO2 or humidity (Ex: Fisher item #50125590H, temperature uniformity of ±1.2°C). Alternative brand can be used.	$808	1	$808	Temperature uniformity should be around ±1 or 2°C	2.
Rotator Genie compact rotator with standard platform (USA Scientific Company # 7400-2100)	$879	1	$879	Help to improve assay imprecision by reducing variability from plate reading	6.
Vortexer (Ex: Vortex-Genie2, Scientific Industries No. SI-0236 Model G560). Alternative brand can be used.	$366	1	$366	To mix samples, calibrator	7.
Stirring hotplate (Ex: Fisher Item # 14-490-202). Alternative brand can be used.	$396	1	$396	Listed item is for 120 V	2.
Balance with weighing precision of at least 0.1 gm (Ex: Fisher Item # 01-918-320). Alternative brand can be used.	$445	1	$445	To weigh sodium ascorbate and growth medium; the old price is for Mettler Toledo precision balance with 0.1 mg precision	2.
Access to deionized water - generally available in laboratories					
Pipettes					
Eppendorf adjustable pipette (Item # ES-100, 10-100 uL)	$382	1	$382	Add Tween-80 (60 uL) to medium; transfer sample from cryovial to glass tube (15 uL); if no dedicated pipettes for calibrator, pipette 100 uL of calibrator stock, and 50 uL of diluted calibrator	8.
Eppendorf adjustable pipette (Item # ES-1000, 100-1000 uL)	$382	1	$382	Add microorganism (700 uL) to medium and pipette sodium ascorbate (400 uL) to generate diluted calibrator; if no dedicated pipettes for calibrator, pipette 250 uL of diluted calibrator	8.
Eppendorf electronic repeater E3 (Eppendorf # 4987000118, 1 uL to 50 mL, Fisher # 13-683-550, with charging adapter)	$1,135	1	$1,135	Add sodium ascorbate to glass tubes for sample dilution; greatly speeds up process	2.
Rainin 12-channel electronic pipettor E12 20-300XLS+ (Rainin Item #: 17013801, 20-300 uL)	$1,844	1	$1,844	Add different volumes of sodium ascorbate and calibrator working solutions to plate	3.
Rainin single-channel electronic pipettor E4-300XLS (Rainin E4 XLS, 20-300 uL, LTS, Item # 17014488)	$922	1	$922	Add diluted sample to plate; one aspiration of 300 uL allows to fill all 4 wells: 50, 50, 2x50, and 2x50 uL	3.
Durable common lab supplies					
Grade 'A' 50-mL volumetric flask (Ex: Fisher Item # 10-200B). Alternative brand can be used.	$72	4	$287.84	Min of 2 needed; sold individually or as pack of 12 for $610; **purchase at least 4 as this is used daily and may break**	2.
500-mL glass beakers (Ex: Fisher Item # 02-540M, pack of 6). Alternative brand can be used.	$61	1	$61	Min of 2 needed; pack of 6 is good to have	2.
Stirring bars (Ex: Fisher Item # 14-513-57SIX, pack of 6). Alternative brand can be used.	$34	1	$34	Min of 2 needed; pack of 6 is good to have	2.
Weighing spatula (Ex: Fisher Item # 14-357Q, pack of 6). Alternative brand can be used.	$27	1	$27	Min of 2 needed; pack of 6 is good to have	2.
Rack for culture tubes (Ex: Fisher item # 14-809-60). Alternative brand can be used.	$48	4	$193	Min of 4 needed; 12x6 is commonly available. Pack of 8 for $237.40	2.
Rack for sample vials (for 2-mL cryovials)				Min of 4 needed; best choice is 12x4 rack. Does not appear to be commercially available with 12 spaces per row.	2.

Thermometer for 37°C incubator; incubator may already have a thermometer (Ex: Fisher Item # 13-202-486)	$56	1	$56	Common in lab	2.
Biohazards waste bag holder (small bench-top) (Ex: Fisher Item # H13193-1000). Alternative brand can be used.	$35	1	$35	Common in lab	2.
Gold fluorescent lights (yellow lights)				Light exposure has to be kept to a minimum	2.
SUM	**$22,352**		**$22,713**		

Folate Microbiologic Assay - Helpful (tier II) chemicals, supplies, and equipment

Chemicals	Unit price	Units needed	Total price	Note	#
Sodium azide, 99% purity (Ex: Fisher Item # AC190380050, 5 g)	$13	1	$13	Only used for hemoglobin color correction if whole blood lysate samples are diluted 1:40 due to low folate concentration	2.
SUM	**$13**		**$13**		
Expendable Supplies	**Unit price**	**Units needed**	**Total price**	**Note (amounts sufficient for ~700 samples)**	**#**
Pipette tips					
Integra tips for 1,250 uL 12-channel pipette (Item # 4442, 5 inserts of 96 tips, 480 tips/pack)	$31	1	$31	One pack sufficient for ~40 runs; used for dispensing 200 uL medium into plates	9.
Mains Adapter for Integra 12-channel VIAFLO II electronic pipette, Integra #4200	$45	1	$45	Adapter for Integra 12-channel pipette E12-300XLS	9.
Eppendorf North America COMBITIPS ADVANCED 5.0 mL, 100/case (Fisher Item # 13-683-705)	$136	1	$136	Used to add sodium ascorbate to glass tubes for sample dilution if smaller volume is needed due to lower dilution	2.
SUM	**$212**		**$212**		
Equipment and Durable Supplies	**Unit price**	**Units needed**	**Total price**	**Note**	**#**
Equipment					
Microplate Heat Sealer, ALPS 50 V (Thermo # AB1443A)	$4,841	1	$4,841	Makes plate sealing much easier and avoids having to apply a lot of manual pressure	2.
Bar code scanner (Ex: Brady. Fisher Item # 19-104-464). Alternative brand can be used.	$387	1	$387	Makes documenting sample IDs much easier and avoids human error	2.
Freezer, -80°C. No specific brand needed.	$8,000	1	$8,000	For storing samples, QC, folate microbiologic assay kit	
Refrigerator. No specific brand needed.	$1,000	1	$1,000	For storing reagents and intermittently storing samples	
Pipettes					
Integra 12-channel VIAFLO II electronic pipettes 50-1250 uL (Integra Bioscience # 4634)	$1,195	1	$1,195	Transfer 200 uL of medium into plate; allows dispensing 4 rows at a time; but this step could be done	9.

				with 12-channel Rainin pipette 1 row at a time	
Eppendorf fixed volume pipette 50 uL (Item # ES-50F)	$263	1	$263	Dedicated for CAL: Add diluted calibrator to volumetric flask for level I working solution	8.
Eppendorf Fixed volume pipette 100 uL (Item # ES-100F)	$263	1	$263	Dedicated for CAL: Add calibrator stock to cryovial for 1:5 dilution	8.
Eppendorf Fixed volume pipette 250 uL (Item # ES-250F)	$263	1	$263	Dedicated for CAL: Add diluted calibrator to volumetric flask for level II working solution	8.
SUM	**$16,212**		**$16,212**		

The number indicated in this column refers to the website link for additional product information:

1. **www.himedialabs.com**
2. **https://www.fishersci.com/us/en/home.html**
3. **https://www.shoprainin.com/**
4. **https://www.thomassci.com/Laboratory-Supplies/Sealing-Films/_/THERMALSEAL?q=TS2-100**
5. **https://www.biotek.com/**
6. **https://www.usascientific.com/**
7. **https://www.scientificindustries.com/**
8. **https://www.pipette.com/**
9. **https://shop.integra-biosciences.com/**

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APPENDIX B. REVIEW OF PLATE LAYOUTS

Plate 1 – Calibrator plate

Add growth medium (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200	200	200	200	200	200	200	200	200	200	200	200
B	200	200	200	200	200	200	200	200	200	200	200	200
C	200	200	200	200	200	200	200	200	200	200	200	200
D	200	200	200	200	200	200	200	200	200	200	200	200
E	200	200	200	200	200	200	200	200	200	200	200	200
F	200	200	200	200	200	200	200	200	200	200	200	200
G	200	200	200	200	200	200	200	200	200	200	200	200
H	200	200	200	200	200	200	200	200	200	200	200	200

Add sodium ascorbate (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	75	50	25	0	70	60	50	40	20	0
B	100	100	75	50	25	0	70	60	50	40	20	0
C	100	100	75	50	25	0	70	60	50	40	20	0
D	100	100	75	50	25	0	70	60	50	40	20	0
E	100	100	75	50	25	0	70	60	50	40	20	0
F	100	100	75	50	25	0	70	60	50	40	20	0
G	100	100	75	50	25	0	70	60	50	40	20	0
H	100	100	75	50	25	0	70	60	50	40	20	0

Add calibrator working solutions (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	25	50	75	100	30	40	50	60	80	100
B	0	0	25	50	75	100	30	40	50	60	80	100
C	0	0	25	50	75	100	30	40	50	60	80	100
D	0	0	25	50	75	100	30	40	50	60	80	100
E	0	0	25	50	75	100	30	40	50	60	80	100
F	0	0	25	50	75	100	30	40	50	60	80	100
G	0	0	25	50	75	100	30	40	50	60	80	100
H	0	0	25	50	75	100	30	40	50	60	80	100

µL of level I working solution — µL of level II working solution

Calibrator concentration in well (nmol/L)											
Blank	0.00	0.05	0.10	0.15	0.20	0.30	0.40	0.50	0.60	0.80	1.00

Plate 2 - 5 – Sample plates

Add growth medium (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200	200	200	200	200	200	200	200	200	200	200	200
B	200	200	200	200	200	200	200	200	200	200	200	200
C	200	200	200	200	200	200	200	200	200	200	200	200
D	200	200	200	200	200	200	200	200	200	200	200	200
E	200	200	200	200	200	200	200	200	200	200	200	200
F	200	200	200	200	200	200	200	200	200	200	200	200
G	200	200	200	200	200	200	200	200	200	200	200	200
H	200	200	200	200	200	200	200	200	200	200	200	200

Add sodium ascorbate (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	0	50	0	50	0	50	0	50	0	50	0
B	50	0	50	0	50	0	50	0	50	0	50	0
C	50	0	50	0	50	0	50	0	50	0	50	0
D	50	0	50	0	50	0	50	0	50	0	50	0
E	50	0	50	0	50	0	50	0	50	0	50	0
F	50	0	50	0	50	0	50	0	50	0	50	0
G	50	0	50	0	50	0	50	0	50	0	50	0
H	50	0	50	0	50	0	50	0	50	0	50	0

Add diluted samples (µL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	100	50	100	50	100	50	100	50	100	50	100
B	50	100	50	100	50	100	50	100	50	100	50	100
C	50	100	50	100	50	100	50	100	50	100	50	100
D	50	100	50	100	50	100	50	100	50	100	50	100
E	50	100	50	100	50	100	50	100	50	100	50	100
F	50	100	50	100	50	100	50	100	50	100	50	100
G	50	100	50	100	50	100	50	100	50	100	50	100
H	50	100	50	100	50	100	50	100	50	100	50	100

Plate 2 – sample plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	QC low 1		#4		#8		#12		#16		#20	
B												
C	#1		#5		#9		#13		#17		#21	
D												
E	#2		#6		#10		#14		#18		#22	
F												
G	#3		#7		#11		#15		#19		Blank	
H												

Plate 3 – sample plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	QC high1		#26		#30		#34		#38		#42	
B												
C	#23		#27		#31		#35		#39		#43	
D												
E	#24		#28		#32		#36		#40		#44	
F												
G	#25		#29		#33		#37		#41		Blank	
H												

Plate 4 – sample plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	QC low 2		#48		#52		#56		#60		#64	
B												
C	#45		#49		#53		#57		#61		#65	
D												
E	#46		#50		#54		#58		#62		#66	
F												
G	#47		#51		#55		#59		#63		Blank	
H												

Plate 5 – sample plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	QC high 2		#70		#74		#78		#82		#86	
B												
C	#67		#71		#75		#79		#83		#87	
D												
E	#68		#72		#76		#80		#84		#88	
F												
G	#69		#73		#77		#81		#85		Blank	
H												