

NEON Microbial Productivity from Aquatic Samples

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I. Version History

Document Number	Effective Dates	Revision
BMI_NEON epifluorProtocol_V7.1	N/A	Temperature ranges for cold storage units were changed to the accepted range of the equipment in use. Measured and added the temperature of the drying oven. Added the sample list name from which analysts can find the filtration volume/dilution of samples to be tested. Changed water used for sample filtration to Milli-Q. Added more description on using ImageJ for computer counts and manual counts. Update the Section G – Quality Review to better reflect actual practice.
BMI_NEON epifluorProtocol_V7	10/18/2021	Added Section A – Sample Receipt Added Section C – Post-Filtration Procedure
BMI_NEON epifluorProtocol_V6	12/15/2021	The Quality Review Section, IV-G, has been updated to include Long Term QC. The Data Delivery Guidelines, IV-H has been added to the SOP. Appendix A added to the SOP The appendix pulls in Detection Limit Criteria and recommended sample volumes and dilution guidance from the SOW.
BMI_NEON epifluorProtocol_V5	08/17/2021	The Quality Review Section, IV-F, has been updated to further define the quality review process of work instructions and the Sample Receipt_EpifluorProtocol_Tables spreadsheet. As per recommendations from the AY20 Cell Counts Audit, Prolong Diamond P36970 (10mL vial) and Prolong Diamond with DAPI (10mL vial) were added to the Materials & Equipment Log as alternative products. Removed verbiage mentioning the shiny side of the filter from the Sample Filtration and

		Staining. The filters used do not have a shiny side.
BMI_NEON epifluorProtocol_V4	11/12/2019	Previously the percent difference in enumeration (PDE) consisted of monthly analysis of a quality control image by technical staff reporting data. The current version of the SOP describes a PDE procedure where each month 10% of samples (one image per selected sample) will be counted by two technical staff using automated assisted image analysis to verify the Image Analysis process.
BMI_NEON epifluorProtocol_V3	08/08/2017	Changes incorporated into version 3 include: Formatting changes; addition of the protocol name and version number to page 1; a Quality section that includes PDE determination and calculation; and updating the footer to include date approved, protocol name/number.
BMI_NEON epifluorProtocol_V1-2	N/A	Previous versions changes were not tracked

II. Objective

The purpose of this Standard Operating Procedure (SOP) is to enumerate microbial cells in water from various domains of NEON within the United States using propidium iodide (PI) staining, black filter filtration, and epifluorescence microscopy. Data from this SOP will be used to determine microbial productivity from sites across the United States.

This SOP is an on-slide staining technique adapted from Boulos et al.¹; modifications include change of fluorophore and concentration, mounting media and volume of sample filtered.

III. Materials and Equipment

Material	Manufacturer	Catalog #
Propidium Iodide (PI)	Molecular Probes	P21493
25mm Black polycarbonate membranes	Sterlitech	1270059

Prolong Diamond	Invitrogen	P36961 5x2ml aliquot or P36971 (with Dapi) 10 ml vial P36970 10 ml vial
Coverslips	Premier Brand	SC-92222-1
Epifluorescence microscope	Zeiss Axio Imager A.2	N/A

¹ Boulos et al. J. Microbial Methods 37(1):77-86 (1999)

- Immersion Oil
- Microscope Slides
- Milli-Q water (particle-free or 0.22 µm filtered water)
- 30 µM PI working stock
- Fume Cabinet
- Vacuum manifold and pump
- Oven (to dry glassware)
- 25 mm glass vacuum filter holders (including fritted glass funnel and base, aluminum clamps and stopper)

IV. Sample Analysis

A. Sample Receipt

1. The Sample Receipt_EpifluorProtocol_Tables spreadsheet used for sample receipt and data analysis will be version controlled.
2. After sample receipt, samples will be stored at 2-8 °C Samples received by Friday of the scheduled shipping week will be analyzed within 4 weeks and results reported within 6 weeks.
3. Up to 1 field blank sample per site per year will be received, analyzed, and reported with the sample data. Samples will have the string “blank” in the sample identifier.
4. Further Sample handling instructions can be found in the NEON AMC Sample Handling SOP.

B. Sample Filtration and Staining

1. Allow working staining solution (30 µm PI) and Prolong Diamond Antifade Mountant media to reach room temperature. **The stain and ProLong are light sensitive** and should be warmed in the dark (or covered completely with foil).

2. Label microscope slides as follows: name of sample, date of filtration, analyst initials
3. Soak black filters in MilliQ water for 1-5 minutes prior to use.
4. Place filters on the filter manifold. Add 3-5mL of MilliQ water to each filter to both ensure filters stay moist, as well as to check for any leaks in the filter apparatus.
5. *Critical Step:* Filter sample
 - a. If sample volume is <15 mL, add 15 mL of MilliQ water to the filter headfirst.
 - b. Add appropriate volume of each sample (or dilution) (see Sample_List_Imaging_Info work instruction for volume and dilution information). This should be recorded in the study records and Sample Receipt_EpifluorProtocol_Tables spreadsheet under the “Sample Processing” tab and is needed to calculate microbial productivity. Turn on vacuum and slowly filter sample at low vacuum until all liquid has been pulled through the filter. **Filter to near-dryness, but do not dry the filter – this will result in poor images.**
6. Stain Sample

Note: Steps 6b-6g must occur in the dark. Make sure to work in a dark fume hood or dark area on the bench.

 - a. Gently remove filter from the filter manifold with forceps and lay the filter face up on a microscope slide.
 - b. Place slide in a dark humid box (sealable box lined with damp paper towels/kimwipes.
 - c. Gently pipette 130 μ L of 30 μ M PI working solution over the surface of the filter (filter area should be completely covered with staining solution). Place cover on humid box. Record volume of PI solution used.
 - d. Allow stain to sit on the filter for 10 minutes +/- 1 minutes in the dark.
 - e. Stop staining by placing the slide on a dry kimwipe that has been folded over itself a few times. The slide should be placed on the kimwipe at a slight angle, just enough for the solution to soak gently into the kimwipe.
 - f. Immediately place 45 μ L of ProLong Diamond Antifade Mountant onto the center of the filter and slowly place a coverslip over the mountant media to avoid trapping air bubbles. Do not slide the coverslip relative to the slide or press the coverslip to remove air bubbles.
 - g. Allow ProLong to cure for 24 hr. at room temperature (RT) in the dark. Slides should be stored at -30° to -15 °C in slide box for long term storage after 24 hours.
7. All sample names, dates of filtration, person filtering, filtration volumes, and notes concerning filtration or staining should be recorded on the Filtration_Staining_NEON_AMC work instructions and in the Sample Receipt_EpifluorProtocol_Tables spreadsheet.

C. Post Filtration Procedures

1. Manifold Cleaning

- a. After each batch of samples has been filtered, close the stopcocks, fill the ports in each manifold with DI water, turn on pump and allow water to be pulled through.
 - b. After the last batch of samples for the month has been filtered, clean filtration manifolds by running concentrated bleach, 70% isopropanol and Milli-Q water through the ports.
 1. Close the stopcocks.
 2. Fill the ports in each manifold with concentrated bleach.
 3. Turn on pump and allow bleach to be pulled through.
 4. Repeat steps 1 – 3 with 70% isopropanol followed by Milli-Q water.
2. Glassware
- All glassware must be particle free. Clean filtration units by:
- Wash glassware using hot water and Alconox Rinse thoroughly with Milli-Q water.
 - Wrap filtration units in foil and dry in an oven ($85 \pm 5^\circ\text{C}$) for at least 1 hour.

D. Capture Image from Stained Slides

1. Observe slides within 5-7 days.
2. Take images on microscope using a 40X or 100X (preferred) objective lens and save in appropriate data file folder. Image fields should be randomly selected from across the filter. Take at least 15 images per slide. Name images such that they can be tracked back to the specific slide they are acquired from.

Note:

- Counting will be from recorded images, not through the oculars. Analysis of samples is based on the images, so it is imperative to take images in which the number of in-focus cells is maximized.
 - When taking images, use the fine focus adjustment to maintain focus – but make certain that all images are taken from the same focal plane.
 - Images should be of organisms on the filter and not those in between the filter and the coverslip.
 - Avoid collecting image fields off the edge of the filter and around any air bubbles. If a sample has many blank areas or air bubbles, it may indicate a filtration problem and should be noted.
3. Record objective, exposure, number of images taken and any comments in the “Image Info” tab of the Sample Receipt_EpifluorProtocol_Tables spreadsheet.

E. Assisted Automated Image Analysis using ImageJ

Note: Bacteria densities will range, but generally should be between 5-250 cells per field.

1. Open images using ImageJ software software.
2. Examine the focus of each image per set.

- Select images that are in focus and can be easily adjusted using contrast and brightness.
3. In ImageJ, adjust the parameters of each image for consistent background by selecting: “Image” → “Adjust” → “Brightness and Contrast”
 4. Select a contrast range and annotate it on the analysis paperwork.
 - a. Have multiple images from the same set opened simultaneously.
 - b. Check the setting and ensure that the background staining is eliminated uniformly from each image to be counted.
 Note: Contrast is changed based upon the image taken.
 5. In toolbar, select “Image” → “Adjust” → “Color Threshold”
 6. Adjust the Brightness setting and annotate the value on the analysis paperwork (Adjusting the brightness setting maximizes the number of cells that ImageJ will recognize).
 - a. Observe each image and adjust each set consistently.
 - b. Do not adjust the Hue setting. This will only change intensity of the color and not the threshold.
 7. Once all appropriate adjustments are complete, select “Analyze” → “Analyze Particles”
 - a. Type in the range 20-2000 as the particle size range to be counted. This is a blanket approach to limit the number of objects identified as microbial cells to those between 20 and 2000 pixels with no relationship to magnification.
 - b. Select “Show Overlay Masks,” “Summary,” and “Display Results”.
 - c. The number of counted particles will appear in the “Summary” tab and individual areas are tabulated in “Display Results”.

Required if an image has a scale bar: When looking at the image overlay, be sure check the scale bar and omit it from the analysis. This is necessary as the image scalebar is counted as an object when performing the analysis.

8. Record all particles that the software counts and manually count any particles that the software has missed. Record ImageJ counts in the ‘Computer’ column of the “Data Table” tab in the Sample Receipt_EpifluorProtocol_Tables spreadsheet.
 - a. In the Threshold Color window, click the Original button to remove the overlays that were not counted by ImageJ.
 - b. Move overlay masks using the computer mouse to determine if there are multiple cells under any of the masks or if mask are covering debris. Move overlays covering debris to cells not identified by ImageJ.
 - c. Record cells identified by ImageJ in the ‘Computer’ counts column of the “Data Table” tab in the Sample Receipt_EpifluorProtocol_Tables spreadsheet. If the number of cells identified by the software is higher than the actual number of cells,

subtract the number of ‘extra’ cells counted from the computer counts by entering a negative number in the ‘Manual’ counts column.

- d. Count all additional cells not counted by the software. Record manually counted cells in the ‘Manual’ column of the “Data Table” tab in the Sample Receipt_EpifluorProtocol_Tables spreadsheet.

9. Repeat steps 3 through 8 for all images for each slide across all domains analyzed.

F. Determine Microbial Abundance

1. Calculate Microbial Cell counts using data captured in step 4 and the following formula:

$$Cf = \frac{(\text{Average number of cells/field}) \times R}{Fs}$$

Where:

Cf = average number cells/mL

R = active area of filter/area of field counted

Fs = volume of initial sample filtered

- For example, if 1 mL of sample is filtered, Fs=1, if 200 µL of sample is filtered, Fs = 0.2
- Fs must consider any dilution made during sample processing for an accurate number to be calculated.

Filter Size	Active Filter Area (cm ²)	Area of Image Counted (cm ²)	Objective Used	Conversion factor (R) ^{***}
25 mm	2.1	4.86E-05*	100X	43,209.88
	2.1	8.60E-05**	40X	24,420.51

* Images are 80.56 µm x 60.36 µm using the 100X objective

** Images are 107.13 µm x 80.27 µm using the 40X objective

*** R factor calculated for a specific Zeiss Axiovision A2 Epifluorescence Microscope and color AxioCam MRc5 camera and calibrated Axiovision software. R factor must be calculated for each microscope and camera used for image capture and analysis.

2. Samples with cell counts below the limit of detection see Appendix A) will be referenced in the data return as:
 - LX** = less than limit of detection, no measurable counts observed on slide
 - LR** = less than limit of detection, <100 counts per slide used for calculation

3. The cell count calculation is performed in the “MMMMYYYY” tab of the Sample Receipt_EpifluorProtocol_Tables spreadsheet. Samples with microbial abundance below the limit of detection will be flagged in the “MMMMYYYY” tab.

F. Use of Sample Receipt_EpifluorProtocol_Tables spreadsheet

1. Sample information is cut and pasted into the spreadsheet from the sample manifest
2. A barcode scanner is used to log the samples in during sample receipt. Refer to SOP BMI_NEON_Aquatic samples for full sample receipt instructions.
3. The ReadMe tab should be followed as directions for populating the spreadsheet
4. Spreadsheet data will go through both technical and QA review

G. Quality Review

1. Batch QC- Percent Difference in Enumeration - To verify the image analysis procedure, 10% of samples each month will be analyzed by two technical staff and the PDE will be calculated for each sample. The PDE is the percent difference of the cell counts from each analyst.

a. 10% of the samples each month will be chosen for quality review (one image per sample selected for the PDE check(s)).

b. Two technical staff each will analyze the images chosen for quality review using the previously described analysis method (Section C, steps 3 through 9). Each technician will be assigned Analyst 1 and 2 prior to doing analysis.

c. The PDEs for each sample will be calculated using data captured in previous step via the following formula:

$$PDE = \left(\frac{|Count1 - Count2|}{Count1 + Count2} \right) * 100$$

Where:

Count 1 = Analyst 1 cell counts

Count 2 = Analyst 2 cell counts

d. If the PDE is <5% (0.05), image counts by the staff member are accepted.

e. If PDE is outside of the acceptable range, the two staff originally performing the PDE check will repeat the count(s). If the PDE continues to be outside of the acceptable range, a third staff member will perform the count(s) and be compared to Analyst 1. Any issues concerning the PDE will be discussed with the Task Lead (TL) before any work continues. The TL will determine if the issues with the PDE check warrant a recount of samples analyzed in the same set as the problem PDE.

2. Long Term QC Reporting - This report will include the minimum, maximum, and average of all PDE percentages returned over the course of a year. These data will be reported using Attachment 2d_NEON cell count long-term QC datasheet_20211110 and uploaded to the NEON Data Portal annually by the end of January for the months in the previous calendar year and any time throughout the year if there is a change to Contractor's SOPs that involves equipment, standard solutions, or calculations.

3. Sample Receipt_EpifluorProtocol_Tables_ - This workbook has been validated and password protected. Minor changes to the workbook, not involving changes to calculations, will be submitted to IT for updates. The TL and QA Lead will verify the changes before the new version is implemented.. A data calculation verification package is required for any change that is made to a calculation embedded in the workbook. This package will be submitted to NEON for review and approval before the updated version is implemented

4. Technical– Reviews will be conducted on the monthly ingest, Sample Receipt_EpifluorProtocol_Tables and raw data. QA will use Pilgrim to record their review in Pilgrim as an audit.

Technical review includes the following:

- Sample receipt information in the Sample Receipt_EpifluorProtocol_Tables spreadsheet matches the receipt form.
- Sample Receipt_EpifluorProtocol_Tables spreadsheet:
 - a. Referenced cells are pulling data properly.
 - b. Dilution factors are correct.
 - c. PDE samples are all within criteria.
 - d. .

QA review includes the following:

- Sample receipt information in the Sample Receipt_EpifluorProtocol_Tables spreadsheet matches the receipt form.
- Work Instructions have been filled out completely, error corrections are done properly, correct reagents were used and within expiration date.
- Sample Receipt_EpifluorProtocol_Tables spreadsheet:
 - e. Verify that all samples in the sample receipt manifests are included in the monthly digest.
 - f. All hand transferred information matches the raw data worksheets.
 - g. PDE samples are all within criteria.
 - h. Each sample has 10 images counted
 - i. Data Table Tab Column S – Verify that Column S cells do not contain #REF.

H. Data Delivery Guidelines

Completed data ingest spreadsheets will be uploaded directly to the NEON data portal following guidance in Attachment 2a, field descriptions in Attachment 2b. Samples received by Friday of the scheduled shipping week will have results reported within 6 weeks of that Friday. The templates listed in Table below must be used.

Data Return to the NEON Data Portal Data Ingest Template	Table name for type of data to upload	Type of Data Return	Frequency
Receipt form from shipping email Attachment 2c	scs_shipmentVerification_in	Used to report condition of samples upon receipt	Each sample shipment
	amc_cellCounts_in	Used for individual sample results and associated batch QC	Each monthly analytical batch
Attachment 2d	amc_cellCountLabSummary_in	Long-term summary of method and quality information	Annually in January for the previous calendar year, plus any time throughout the year if there is a change to lab SOPS as noted in Section 2.3.

Appendix A

Sample Volumes

Initial recommended sample volumes for 25 mm filters based on historical results for the site types and collection season are listed in Table 3; however, the Contractor should consider not only the information in Table 3, but also any additional results for the sample sites obtained throughout the sampling year, and any input from the field on expected sample conditions that could affect cell count.

Table 3: Recommended Initial Sample Volumes for 25 mm Filters Based on Expected Productivity

Site Type	Summer	Sample Volume	Winter	Sample Volume
Stream	Low	10-15 mL	Low	15-20 mL
Lake	High	5-10 mL	Low	15-20 mL
River	High	3-5 mL	High	5-10 mL

Sample Dilutions

If a sample requires dilution (e.g., too numerous to count, for example >300 cells per image in automated analysis), the sample will be diluted, filtered, and the diluted sample analyzed either in the month of sample receipt or the following month, provided sufficient sample is available. If possible, field information that will inform if a dilution will be required (e.g., algal blooms, high leaf litter, etc.) will be provided when samples are shipped so that dilutions can be carried out in the initial analysis for efficiency. Sites that typically require dilution: ARIK, MAYF, PRLA, PRPO, SYCA.

Detection Limits

Detection limit must be equal to or lower than the detection limit specified in Table 2. These limits are based on specific sample volumes and filter sizes as described in Table 2. The standard filter size used for sample processing will be 25 mm. Samples with counted cell densities below the limit of detection will be referenced in the data return through a dedicated quality flag field (QAQC status field) using the following codes:

LX = less than limit of detection, no measurable counts observed on slide

LR = less than limit of detection, <100 counts per slide used for calculation.

Required Detection Limits Sample Volume	Limit of Detection (cells/mL)
25 mm filter	
1 mL	$3.4 \times 10^4 \pm 1 \times 10^4$
5 mL	$6.8 \times 10^3 \pm 3 \times 10^3$
10 mL	$3.4 \times 10^3 \pm 1 \times 10^3$