NEON Microbial Productivity from Aquatic Samples

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I.   Version History

BMI_NEON epifluorProtocol_V5	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	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	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	Previous versions changes were not tracked

II. Objective

The purpose of this protocol 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 protocol will be used to determine microbial productivity from sites across the United States.

This protocol 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

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[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)
  - All glassware must be particle free
  - Wash glassware using Alconox, rinse in copious amounts of Milli-Q water and dry in oven prior to use

IV. Procedure

A. 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). 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 the 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 μL 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 -20 °C in slide box for long term storage after 24 hours.

6. All sample names, dates of filtration, person filtering, filtration volumes, and notes concerning filtration or staining should be recorded

B. 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.

C. 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

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. Count all additional cells not counted by the software.
    c. Record manually counted cells in the 'Additional' 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.

E. Determine Microbial Productivity

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

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

Where:

Cf = average number cells/mL
R = active area of filter/area of field counted
Fs = volume of initial sample filtered
  o For example, if 1 mL of sample is filtered, Fs=1, if 200 µL of sample is filtered, Fs = 0.2
  o Fs must consider any dilution made during sample processing for an accurate number to be calculated

Filter Size	Active Filter Area (cm2)	Area of Image Counted (cm2)	Objective Used	Conversion factor (R)***
	2.1	4.86E-05*	100X	43,209.88
25 mm	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.

This calculation is performed in the "MMYYYY" tab of the Sample Receipt_EpifluorProtocol_Tables spreadsheet

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. 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. Sample Receipt_EpifluorProtocol_Tables_- This workbook has been validated and password protected. Any future changes will be validated before the new version is implemented.

3. Technical and QA review – 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
- Work Instructions have been filled out completely, error corrections are done properly, correct reagents were used and within expiration date.
- 10% sample check of the Sample Receipt_EpifluorProtocol_Tables spreadsheet:
    a. Referenced cells are pulling data properly
    b. Dilution factors are correct
    c. PDE sample are all within criteria
    d. Formulas are correct