Protocol for SYBR Counts of Cyanophages and Bacteria

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Abstract

Purpose: to determine the concentration of DNA-containing particles in a given sample; useful in finding cell counts or phage titers.

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Guidelines

Materials:

- 0.02 µm pore size, 25 mm diameter Anodisc filters (Whatman inorganic Al_2O_3)

- 0.8 µm pore size, 25 mm diameter AA Millipore mixed-ester membrane filters

- Glass 25 mm diameter filter holder w/ 15 mL funnel, Millipore-type

- 10% SYBR I solution from Molecular Probes, diluted with 0.02 µm filtered deionized water. Make sure to wrap the stock in aluminum foil and to keep it in the dark.
As time passed the SYBR stock should degrade, acquiring a more reddish color while frozen and a a paler orange color after being thawed. This can initially be addressed by doubling the amount of SYBR used, but it is a sign that the stock should soon be replaced.

- Antifade mounting solution: 50% glycerol, 50% PBS (120 mM salt, 10 mM sodium phosphate, pH 7.5), 0.1% phenylenediamine (Sigma cat#P6001).
Original protocol says to add the phenylenediamine from a 10% stock; however the maximum solubility of the free base is only 4.1 g/mL (the 10% concentration can be prepared by adding HCl to make the chloride salt). To make the stock, dissolve the powder in filtered deionized water by vortexing and adding HCl as needed; wrap in aluminum foil and stare at -20°C. Stock is light and temperature sensitive, and will go darker with time, corresponding to a decline in quality. A fresh free base solution is pink-orange while the chloride salt is purple. Use of antifade made with poor (e.g. brown or black) phenylenediamine may result in slides with particles that appear faint or seem to fade with time; with a brown stock this may be corrected by using a higher (e.g. 5x) concentration of phenylenediamine.
An alternative (which we have been recently doing) is making a 1% stock of the free base dissolved in 50% PBS, 50% glycerol. A fresh stock of this should be pinkish in color. The advantage of using this is several-fold: first, adding concentrated acid is no longer necessary; second, because the stock is more dilute it should be used up faster, so that it will need to be remade more often and thus remain fresher; third because it is 50% glycerol it does not freeze at -20 thus saving thawing time.

- Sample, autoclaved seawater, Petri dish, pipettes and tips, dessicator, kimwipes, syringe, and 0.02 µm 25 mm Anotop filters.

Source: from the original protocol (July 2001), adapted to working with cyanophages.
Noble, T.T., and J.A. Fuhrman. 1998. "Use of SYBR Green I for Rapid Epifluorescence Counts of Marine Viruses and Bacteria." Aquatic Microbial Ecology. 14: 113-118.

Protocol

Step 1.
Make dilutions of the sample(s). Record volume of samples used. Custom has been to use 20 µl of phage stocks or 200 µl of cells diluted to 2 ml with autoclaved seawater. If the final slides appear too sparse or dense then the volume of sample used can be adjusted accordingly.

NOTES
James Thornton Jr 11 Aug 2015
Slides made from 0.2 µm filtered, autoclaved seawater (the base of our medium) alone appear to contain significant amounts of "cell-like" and "podovirus-like" particles, which may hinder the counting of actual viruses. (In a slide made from an actual sample, if the slide was made well the contaminant particles should appear relatively faint and can easily be ignored; however if here were problems such as a bad antifade then the actual particles may appear similarly faint and the contaminants may increase the count by as much as a magnitude.) Filtering the seawater twice with a 0.2 µm filter seems to get rid of this problem.

Step 2.
Remove the 10% SYBR and phenylenediamine from the freezer. Keep the stocks away from light (such as in a drawer) while they are thawing.

Step 3.
Connect the glass flask to the vacuum pump. Attach the filter holder so that the grout is flat and level with the table.

Step 4.
Place a 0.8 µm filter on top of the grout. Make sure it is completely flat and centered, with no air bubbles underneath (pre-wetting the grout helps). This filter can be used many times so long as it stays intact and flat.

Step 5.
Place the 0.02 µm filter on top of the 0.8 µm filter Again, make sure it is flat and centered with no air bubbles underneath. Turning on the vacuum for a brief while can help achieve this.

Step 6.
Clamp the funnel on top of the filters. Add the sample and turn on the pump (pressure should be 20 kPa or 7 mmHg). After the last liquid passes through the funnel and clamp should be removed with the vacuum still on.

Step 7.
Turn off the vacuum; remove 0.02 µm filter. Keep track of which side of the filter is the top. Blot out any seawater on the bottom or the top plastic rim with a kimwipe.

Step 8.
It is very important to make sure the filters are completely dry before continuing. It is a good idea to rub the back of the filter with a kimwipe and then stick it in a dessicator (we used a makeshift box with some drying rocks) for a few minutes.

Step 9.
Prepare a 100 µL drop of SYBR, made fresh from 2.5 µL 10% stock + 97.5 µL 0.02 µm filtered deionized water, on the bottom of a plastic Petri dish.

Step 10.
Lay the 0.02 Anodisc filters sample side up on the drops of the SYBR staining solution for 15 minutes in a dark drawer or box.

DURATION
00:15:00

Step 11.
While waiting it may be a good time to prepare the antifade solution.

Step 12.
Dry completely as in steps 7-8. Do not touch the top of the filter.

NOTES
James Thornton Jr 11 Aug 2015
If there is a film or drop of liquid on top of the filter then it was either not prepared properly or defective, and may need to be redone; keep this in mind if the cells/phages don't look good under the microscope.

Step 13.
Place the filter sample-side up on a glass slide. Place 30 µL of antifade on a cover slip; then invert slip and place on top of the filter. Appy pressure to ensure that the antifade fills the space underneath the square.

Step 14.
View with blue excitation.

NOTES
James Thornton Jr 11 Aug 2015
If the viruses are on more than one focal plane or appear to move, the slide must be redone-- even if they still appear countable you won't get an accurate number. If they appear to be fading rapidly they probably weren't dried properly or the antifade was not good; in the latter case it may be a good idea to use more phenylenediamine or to make a new stock. A well done slide gives a SYBR value with a ± 20% error or less.

Step 15.
Examine at least ten fields in the microscope (We usually examine 20). Count at least 200 viruses or bacteria total for ten fields (400 for 20 fields). Field size may be full (counting all particles in all 100 small squares) or smaller (e.g. 5 small squares), depending on the virus/bacteria concentration.

NOTES
James Thornton Jr 11 Aug 2015
To calibrate: Viruses should appear as the smallest green particles. A Synechococcus cell appears both green and orange (which soon fades to green), a Prochlorococcus cell boh red and green (which fades to green) and hetertrophic bacteria appear only green.

Step 16.
Find the average number of particles per quadrant (25 small squares). Multiply this by a scaling factor of 1.26×10^5 to get the total number of particles on the filter. Divide this by the volume used (e.g. 20 µL) to get the titer in particles per ml.