Enumeration of virus particles in aquatic or sediment samples by epifluorescence microscopy

Curtis A. Suttle and Jed A. Fuhrman

Abstract

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Guidelines

Authors: Curtis A. Suttle[1] and Jed A. Fuhrman[2]
[1]Departments of Earth & Ocean Sciences, Microbiology & Immunology, and Botany, University of British Columbia, Vancouver, BC, Canada
[2]Marine Environmental Biology Section, University of Southern California, Los Angeles CA, USA

MATERIALS

Equipment

•Epifluorescence microscope equipped with the following:
•≥100 W Hg-vapor or 150 W xenon lamp (xenon lamps are richer in the blue spectrum and have longer lifetimes and lower lifetime cost)
•100x fluorescence objective (phase contrast objectives typically reduce brightness)
•blue excitation filter (wide bandpass preferred for maximum brightness, e.g., 450-□480 or 460-500 nm; excitation peaks 497, 495, and 491 nm for SYBR Green 1, SYBR Gold, and Yo-Pro-1, respectively)
•dichroic mirror (beam splitter), typically 500 or 510 nm
•long-pass sharp cutoff filter (typically 515 nm; emission peaks 520, 537, and 509 nm for SYBR Green 1, SYBR Gold, and Yo-Pro-1, respectively; a long-pass filter allows maximum brightness)
•ocular reticule divided into 100 grid squares
•stage micrometer
•Filtration unit, to hold 25-mm-diameter filters
•Vacuum pump
•Vacuum flask
•Pipettes suitable for dispensing 1 µL to 2 mL

•Event counter
•Filter forceps
•Sonicating bath (sediment samples only)
•Squeeze bottle (or similar) containing 0.2-µm filtered MilliQ water that will be used to wet the underlay filter.

Reagents and solutions
•(SYBR only) SYBR Green or SYBR Gold nucleic-acid gel stain, 10,000x concentrate in anhydrous DMSO (Invitrogen)
•(Yo-Pro only) Yo-Pro-1, 1 mM stock solution in a 1:4 solution of dimethyl sulfoxide and water (Invitrogen)
•(SYBR only) Antifade solution: p-phenylenediamine dihydrochloride or 1,4-phenylenediamine dihydrochloride (se Sigma P-1519 and not P-6001 [Patel et al. 2007] and store in a tightly capped container away from light)
•Spectrophotometric-grade glycerol
•Phosphate-buffered saline (PBS): 0.05 M Na_2HPO_4, 0.85% NaCl (wt/vol), pH 7.5)
•(Sediment samples only) Pyrophosphate (10 mM)
•0.02-µm filter autoclaved MilliQ H2O
•Tris EDTA (TE) buffer, pH 8 (for acidic samples)
•(Yo-Pro only) Aqueous NaCl solution (0.3% wt/vol)
•(SYBR only) 25% EM-grade glutaraldehyde, kept at 4°C
•Ethanol
•DF- or FF-grade immersion oil (refractive index 1/4, 1.516; Olympus).

Cautions: Nucleic acid stains, phenylenediamine dihydrochloride and aldehyde-based fixatives are toxic; great care should be taken to avoid inhalation or contact with skin.

Disposable supplies
•Anodisc Al_2O_3 filters, 0.02-µm pore size, 25 mm diameter, with support ring (Whatman)
Note: At the time of writing, GE Healthcare, which recently purchased Whatman, is considering discontinuing production of the Anodisc membrane. Currently, there are no other membranes known that are suitable for EFM counts of virus particles. For polycarbonate filters, the background fluorescence is too high (even if stained black) and the porosity is too low to filter an adequate volume of water.
•Nitrocellulose membrane filters, 0.45-µm pore size, 25 mm diameter
•Precleaned glass microscope slides, 25 x 75 mm
•Glass coverslips (25 x 25 mm) of proper thickness (each microscope objective has an optimal coverslip thickness--the cover glass is part of the optics; for example, an Olympus infinity-corrected 100x UVPlanApo is imprinted "∞/0.17," which indicates a 0.17-mm-thick cover glass, also known in the US as #1 1/2)
•2.0 mL sterile polypropylene microcentrifuge tubes
•2.0 mL screw-cap cryovials
•10 µL, 200 µL, and 1 mL sterilized pipette tips
•Polypropylene centrifuge tubes (15 or 50 mL)
•Petri plates
•Kimwipes (Kimberly-Clark) or other lint-free paper wipes
•9-cm-diameter paper filters (Whatman #1)
•Additional supplies for long-term storage of samples for SYBR staining:
    •Liquid nitrogen

•Nylon stockings

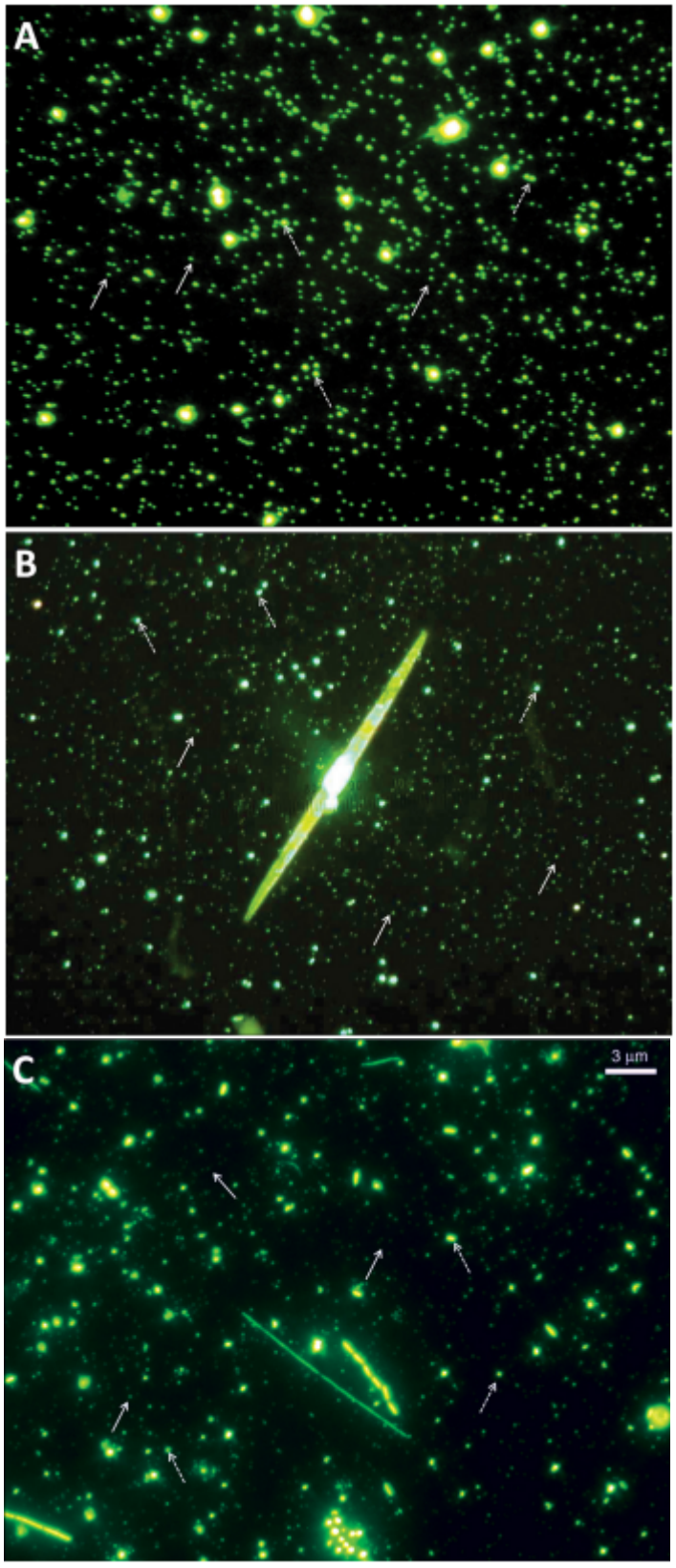

Fig. 1. Epifluorescence micrographs of natural water samples. Viruses are indicated by solid arrows and bacteria by broken arrows.
(A), Yo-Pro 1–stained slide of a water sample from the Arctic Ocean (courtesy of J. P. Payet).
(B), SYBR Green I–stained water sample from the coastal waters of Southern California. The large object in the center is a pennate diatom.

(C) SYBR Gold–stained sample of a water sample from the Chesapeake Bay (courtesy of F. Chen and K. Wang). The slides emphasize the differences that occur between samples and different staining protocols.

Protocol

Preparation of Reagents

Step 1.
Prepare reagents.

PROTOCOL

. Preparation of reagents

CONTACT: Xu Zhong

NOTES

Xu Zhong 02 Sep 2015
Reagents must be made in freshly prepared deionized 0.02-µm filtered water to prevent virus particles being introduced into the samples and causing high blanks.

Stock stain solution

Step 1.1.
For SYBR stains, make a secondary stock by diluting the concentrated dye supplied by the manufacturer 10-fold with 0.02-µm filtered deionized water (dH_2O) and dispense into polypropylene screw-cap microcentrifuge tubes.

NOTES

Xu Zhong 02 Sep 2015
Stains should always be handled in low light to prevent photodegradation. Because the stains are sensitive to repeated freezing and thawing, the stains should be aliquoted as stock solutions in small volumes.

Stock stain solution

Step 1.2.
For Yo-Pro-1, dilute to 50 µM in an aqueous solution of 2 mM NaCN to prevent any microbial growth during the 48-h staining period.

NOTES

Xu Zhong 02 Sep 2015
Because the fluorescence of the dyes is very pH sensitive, it can be helpful to dilute the stain in TE buffer (pH 8) if processing strongly acid or basic samples (Chan, pers. comm.).

Stock stain solution

Step 1.3.
The diluted stains should be stored at –20°C, and ideally should be used within a week.

NOTES

Xu Zhong 02 Sep 2015

The dye should be checked before use to make sure that it has not precipitated or adsorbed to the walls of the storage tube. Adsorption of the stain to the tube walls is minimized when stored in polypropylene.

Stock stain solution

Step 1.4.

Each filter requires 2 µL SYBR stain; hence, 40 µL dispensed into each tube provides enough stain for 20 filters.

Stock stain solution

Step 1.5.

For Yo-Pro, freeze 800-µL aliquots for enough dye to stain 10 filters.

Glycerol/PBS solution

Step 1.6.

Prepare a solution of 50% glycerol and 50% PBS. The solution should be shaken or vortexed to ensure complete mixing, 0.02 µm filtered, and stored as a liquid at –20°C.

NOTES

Xu Zhong 02 Sep 2015

Alternatively, for long-term storage, 990 µL of the filtered mixture can be dispensed into microfuge tubes and frozen until ready for use.

Antifade (SYBR only)

Step 1.7.

Prepare a 10% stock solution of the antifade reagent by diluting 1 g phenylenediamine (PDA) in 10 mL of 0.02-µm filtered autoclaved dH_2O.

NOTES

Xu Zhong 02 Sep 2015

The PDA should go completely into solution, producing a colorless liquid If the stock solution is tea-colored or darker, it has oxidized and should not be used.

Antifade (SYBR only)

Step 1.8.

Dispense 500-µL aliquots of the working solution into microcentrifuge tubes and store at –20°C to minimize freezing and thawing.

NOTES

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The frozen reagent should be white; if it has a brownish tint it should not be used. It is possible to use other antifade reagents such as 0.5% (wt/vol) ascorbic acid in 50% (vol/vol) glycerol/PBS or SlowFade (Invitrogen), but they may provide less protection against fading (Noble and Fuhrman 1998). In contrast, DABCO (1,4-diazabicyclo[2.2.2]octane) in TE/glycerol is reported to be a superior antifade to PDA (Ortmann, pers. comm.).

Antifade (SYBR only)

Step 1.9.

Immediately before preparing the slides, make a 0.1% working solution of the antifade by adding the 10% phenylenediamine solution to the glycerol-PBS mixture.

Antifade (SYBR only)

Step 1.10.

Estimate 50 µL of reagent per slide.

Sample Collection and Preparation

Step 2.
Collect and prepare sample according to sample type.

PROTOCOL

. Sample collection and preparation

CONTACT: Xu Zhong

Aqueous samples

Step 2.1.
Collect the samples in sterile containers that are rinsed three times with the sample water.

NOTES

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Polypropylene centrifuge tubes or bottles work well as sample containers.

Aqueous samples

Step 2.2.
The range of viral abundances suitable for enumeration is 10^5 to 10^7 mL^{-1}; hence, dilution may be necessary for very productive natural samples or cultures.

Aqueous samples

Step 2.3.
If necessary, dilute the sample with 0.02-µm filtered water, ideally prepared from the same or very similar water from which the sample was obtained.

Sediment samples

Step 2.4.
Undisturbed sediment samples should be collected with a piston corer.

NOTES

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The sediment–water interface can be sampled with a wide-bore serological pipette to minimize disruption to the sediment surface.

Sediment samples

Step 2.5.
Samples from deeper within the core can be obtained by carefully pushing the core up from the bottom of the core barrel and slicing the sediment at the desired depth.

NOTES

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The sediment should be sampled from the center of the core, leaving a well-defined area of sediment around the periphery to ensure the sample is not contaminated with sediment smeared along the side of the core barrel.

Sediment samples

Step 2.6.
Remove a 0.5-cm^3 subsample of sediment from the center of the core slice and transfer it into 4 mL of 0.02-µm filtered seawater and 1.0 mL pyrophosphate (10 mM final concentration).

Sediment samples

Step 2.7.

Sonicate the mixture for 3 min.

DURATION
00:03:00

NOTES
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(Ortmann and Suttle 2009)

Sediment samples

Step 2.8.
Centrifuge at 800g for 1 min.

DURATION
00:01:00

NOTES
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(Ortmann and Suttle 2009)

Sediment samples

Step 2.9.
The supernatant can then be diluted and the slides prepared as outlined.

Sediment samples

Step 2.10.
For different types of sediments and soils, the amount of pyrophosphate and length of sonication may need to be tested and optimized.

NOTES
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Potassium citrate has been reported to be superior to sodium pyrophosphate for extracting phage from soils (Williamson et al. 2003) and may be an alternative for aquatic sediments, as well.

Preservation of samples (SYBR only)

Step 2.11.
For preservation of samples (SYBR only), see guidelines.

Filtration and staining of sample

Step 3.
Prepare slide labels with critical information such as the date, sample location, and volume filtered to keep track of the samples once they have been filtered and stained.

NOTES
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The labels should be affixed when each slide is prepared.

Filtration and staining of sample

Step 4.
For every set of four samples to be stained, use a permanent pen to mark the bottom of a plastic Petri plate into four labeled sections.

Filtration and staining of sample

Step 5.
(SYBR only) For each filter that is to be prepared, add a 78- µL drop of 0.02-µm filtered dH_2O on each section of the marked Petri plates.

NOTES

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Note that the efficacy of the stain is pH dependent; hence, if samples are from an acidic environment, diluting the stain in pH 8 TE buffer rather than dH_2O has been found to result in more stable fluorescence (Chan, pers. comm.).

Filtration and staining of sample

Step 6.

(SYBR only) Thaw a 40-µL vial of the stock SYBR solution and add 2 µL stock solution to each drop (78 µL) of sterile dH_2O or buffer. Mix the stain by gently pipetting up and down.

Filtration and staining of sample

Step 7.

(SYBR only) Place the Petri plates in the dark so that the stain is not bleached.

Filtration and staining of sample

Step 8.

(SYBR only) Prepare the antifade solution in a clean, sterilized 2-mL microcentrifuge tube.

NOTES

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About 40 µL of antifade solution will be required for each filter.

Filtration and staining of sample

Step 9.

(SYBR only) Dilute the 10% (wt/vol) stock of p-phenylenediamine 1:100 using glycerol/PBS as the diluent.

NOTES

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For example, add 1 µL stock p-phenylenediamine per 99 µL of 1:1 glycerol:PBS solution.

Filtration and staining of sample

Step 10.

(SYBR only) Keep the solution on ice and protect it from light.

Filtration and staining of sample

Step 11.

(Yo-Pro only) For each filter that is to be prepared, add a 80-µL drop of thawed Yo-Pro working solution on each section of the marked Petri plates.

Filtration and staining of sample

Step 12.

(Yo-Pro only) Place a 9-cm-diameter filter paper soaked with 3 mL aqueous NaCl solution (0.3% wt/vol) in the lid of the Petri plates to prevent evaporation of the stain.

Filtration and staining of sample

Step 13.

Connect a filtration unit for 25-mm filters to a vacuum source, ensuring the vacuum is ≤13 kPa.

NOTES

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A stronger vacuum will likely crack the filter.

Filtration and staining of sample

Step 14.

Place a 0.45-µm nitrocellulose backing filter on each filter support, and overlay it with a thin layer of

dH_2O.

NOTES

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If the filter does not wet evenly (if there are areas or spots that remain white), replace the filter with another one. Backing filters can be reused if they are smooth, are wet evenly with water, and have no holes.

Filtration and staining of sample

Step 15.

Carefully pick up a 0.02-µm Anodisc filter by its plastic ring and lay it over the wet backing filter, with the plastic ring facing upward.

NOTES

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The Anodiscs are ceramic and break easily; make sure it is not cracked and that air is not trapped between the filters. If necessary, pull excess water through the filters using the vacuum, but make sure the filters remain wet.

Filtration and staining of sample

Step 16.

(SYBR only) If the sample has been preserved and frozen as described, thaw it in a 37°C water bath.

Filtration and staining of sample

Step 17.

(SYBR only) For a sample that has just been collected, fix it with 0.5% glutaraldehyde for 15–30 min at 4°C before preparing slides.

Filtration and staining of sample

Step 18.

(SYBR only) In addition, prepare duplicate control samples by fixing 1 mL of the 0.02-µm filtered water that was used to dilute the SYBR stain.

NOTES

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For some samples, fixation may improve the fluorescence of the particles and make counting easier. Also, some virus particles are prone to breakage if not fixed (Chan, pers. comm.).

Filtration and staining of sample

Step 19.

(Yo-Pro only) Because divalent cations interfere with the binding of the stain, seawater samples should be diluted to <7 psu with 0.02-µm filtered dH_2O before filtration.

Filtration and staining of sample

Step 20.

It is a good idea to make test slides (including a control with no sample added) to be sure an appropriate volume is filtered, that the procedure is working, and that the filters and reagents do not have viruses on or in them (some batches of Anodiscs have been covered with bacteria and viruses).

Filtration and staining of sample

Step 21.

For most lake and coastal seawater samples, which have viral abundances of 10^7 mL^{-1}, 0.8–1.0 mL sample is added to the surface of the Anodisc filter while the vacuum is off.

NOTES

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A filtration tower is not needed, as surface tension will hold the water on the surface of the filter.
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Make sure the entire volume is within the plastic ring, or the sample will be pulled under the edge of the filter.

Filtration and staining of sample

Step 22.
Turn on the vacuum and suck the sample through the filter.

Filtration and staining of sample

Step 23.
For oligotrophic or very deep ocean samples, it may be necessary to filter 4 mL or more.

NOTES
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If the volume to be filtered is slightly greater than 1 mL, the additional volume can be added while the sample is filtering, being careful to ensure the entire filter surface is continuously covered with liquid during filtration.
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For larger volumes, a sterile filtration tower can be used; if the inside diameter of the tower is less than the filter diameter inside the plastic ring, it must be taken into consideration when calculating viral abundance.

Filtration and staining of sample

Step 24.
Filter towers must be cleaned between samples. Rinse the towers with 0.02-µm filtered dH_2O followed by ethanol. Dry with lint-free paper (e.g., Kimwipe).

Filtration and staining of sample

Step 25.
Once the sample is filtered, remove the Anodisc with the vacuum still on.

NOTES
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There should not be any liquid on the surface of the filter. Touch only the plastic ring, so as not to crack the membrane. To assist in lifting the filter, a 10-µL pipette tip can be cut at a slight angle and slid under the filter edge. It also helps to place the Anodisc filter about a millimeter off center on the 0.45 underfilter, to provide an edge you can grasp with forceps.

Filtration and staining of sample

Step 26.
Allow the filter to air-dry (typically a minute or less), until the surface is visibly dry.

Filtration and staining of sample

Step 27.
Place the Anodisc, sample side up, on a drop of stain in the Petri dish.

Filtration and staining of sample

Step 28.
(SYBR only) Allow the filter to stain for 15 min in the dark.

DURATION
00:15:00

Filtration and staining of sample

Step 29.
(Yo-Pro only) Allow the filter to stain for 48 h in the dark, at room temperature.

DURATION
48:00:00

Filtration and staining of sample

Step 30.
Add a drop of dH_2O on the backing filter, lay the stained Anodisc on top, and use the vacuum to remove any remaining fluid.

NOTES
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Do not use the filter if there is visible water on top of the Anodisc when it is done staining, as it is likely cracked.

Filtration and staining of sample

Step 31.
(Yo-Pro samples and samples with high background fluorescence) Some samples (e.g., sediments, vent fluid, and humic waters) may require the filters to be rinsed to reduce background fluorescence. If so, while the vacuum is still on and the filter is damp, rinse the filter twice with 1 mL of 0.02-µm filtered dH_2O.

NOTES
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For some samples, such as those with high humic content, or from vent environments, samples can be rinsed with TE buffer to reduce background fluorescence (Chan and Winget, pers. comm.).

Filtration and staining of sample

Step 32.
Remove the Anodisc while the vacuum is on.

Filtration and staining of sample

Step 33.
Place the Anodisc, sample-side up, on a 9-cm filter paper or Kimwipe in the dark, and allow the filter to dry until it appears opaque.

NOTES
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The filter paper can be placed inside the lid of a Petri plate, and a foil-lined Petri plate bottom can be used as a lid. It usually takes about 5 min for the filter to dry, but it can be longer when humidity is high. The process can be accelerated by laying the filter on a glass slide that is heated to 35–37°C on a heating block, often very helpful when humidity is high.

Filtration and staining of sample

Step 34.
(SYBR only) Place 12–15 µL antifade solution on a labeled glass slide and lay the dry Anodisc on top.

Filtration and staining of sample

Step 35.
(SYBR only) Add 20 µL antifade on top of the Anodisc and cover with a coverslip.

NOTES
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If the slides are to be frozen, add a little more antifade to compensate for sublimation.

Filtration and staining of sample

Step 36.
(Yo-Pro only) Place 12–15 µL spectrophotometric-grade glycerol on a labeled glass slide and lay the

dry Anodisc on top.

Filtration and staining of sample

Step 37.

(Yo-Pro only) Add 20 µL glycerol on top of the Anodisc and cover with a coverslip.

Filtration and staining of sample

Step 38.

Remove any air bubbles that are trapped under the coverslip by gently pressing on the surface.

Filtration and staining of sample

Step 39.

The slides can be counted immediately or stored frozen at –20°C for at least 4 months with no decrease in estimates of viral abundance.

NOTES

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Slides can be individually wrapped in a Kimwipe and placed in small batches in foil packets before freezing. This allows a few slides to be thawed at a time. Once thawed, the slides should be counted immediately.

Determining abundance

Step 40.

Count the viruses at 1000× magnification using a 100× oilemersion objective.

NOTES

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Make sure that the area of the filter covered by the 10 × 10 ocular reticule has been determined using a stage micrometer.

Determining abundance

Step 41.

Begin by checking the test filters to ensure that the reagents or filters were not contaminated and the filtered volumes were appropriate.

NOTES

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Viral and bacterial particles will appear green (SYBR Green and Yo-Pro) or yellow (SYBR Gold) when excited with blue light (see Fig. 1 in guidelines).

Determining abundance

Step 42.

Check each slide before counting to make sure that the filter is evenly stained and that the viruses are on a single plane of focus and not suspended in the mounting medium and are evenly distributed across the filter.

Determining abundance

Step 43.

Using the ocular reticule, select an appropriate number of grid squares so that there are 10–100 stained viruses in each field.

NOTES

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Viruses can generally be distinguished from cells by their staining characteristics. Viruses appear as bright pinpricks of light, whereas cells generally have discernable size (see Fig. 1 in guidelines).

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If the 95% confidence intervals (see step 48) overlap for three transects of 20 random fields containing at least 200 viruses, it indicates that 20 fields is adequate to compensate for the variation in viral abundance among fields.

Determining abundance

Step 44.

Estimate the abundance of viruses by counting at least 20 random fields. Keep a tally of the number of particles in each field so that the variation in abundance of particles among fields can be determined.

NOTES

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These data can be used to determine whether the distribution of particles across the filter is random. (The data should follow a Poisson distribution where the mean equals variance, although 20 fields should resemble a normal distribution.)

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Particles touching two edges of the grid (e.g., left side and top) should be counted, whereas particles touching the other two edges (e.g., right side and bottom) should not be counted.

Determining abundance

Step 45.

For each sample, record the number of particles counted in each field, the number of fields counted, the area of the field, and the volume of sample filtered.

Determining abundance

Step 46.

The abundance of viruses mL^{-1} (V_t) in the sample = $V_c \div F_c \times A_t \div A_f \div S$, where V_c = total number of viruses counted, F_c = total number of fields counted, A_t = surface area of the filter (μm^2) (see note below), A_f = area of each field (μm^2), and S = volume of sample filtered (mL).

NOTES

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Note that for a 25- mm Anodisc filter, the diameter of the filter inside the plastic ring is 19 mm, which corresponds to an area of 283,528,737 μm^2. If a filtration tower is used that has a diameter less than that of the surface of the filter, it will be necessary to correct for the smaller filtration surface area. This is most accurately done by using the microscope to determine the maximum width of the filter across which virus particles can be observed [Patel et al. 2007].

Determining abundance

Step 47.

The total number of particles counted will determine the size of the 95% confidence intervals on the estimates of viral abundance. By assuming a Poisson distribution, the 95% confidence intervals can be estimated using the following equations (Suttle 1993):

$$\text{Upper } 95\% = V_c + 1.96 \times \sqrt{(V_c + 1.5)} + 2.42$$

$$\text{Lower } 95\% = V_c - 1.96 \times \sqrt{(V_c + 0.5)} + 1.42$$

Warnings

Nucleic acid stains, phenylenediamine dihydrochloride and aldehyde-based fixatives are toxic; great care should be taken to avoid inhalation or contact with skin.