Virus and bacteria counts by epifluorescence microscopy with SYBR Green Version 2

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Abstract

The protocol provides an inexpensive, rapid (30 min) and reliable technique for obtaining counts of viruses and prokaryotes simultaneously. The method is from:
Patel A, Noble RT, Steele JA, Schwalbach MS, Hewson I, Fuhrman JA.Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. *Nat Protoc.* 2007;2(2):269-76.

Please see the full manuscript for more details.

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Guidelines

Overview:

Steps 1 - 8: Sample Collection
Steps 9 - 45: Slide preparation
Steps 46 - 55: Virus enumeration procedure

Materials (Reagents):

* SYBR Green I nucleic-acid gel stain, 10,000 X concentrate in anhydrous DMSO (500 µl; Molecular Probes-Invitrogen, cat. no. S-7563)

Caution: No human mutagenicity or toxicity data are available; however, as the chemical specifically binds to nucleic acids and is supplied in DMSO, it should be treated as a potentially mutagenic substance

* *p*-phenylenediamine dihydrochloride or 1,4-phenylenediamine dihydrochloride (Sigma, cat. no. P-1519)
Critical Do not use Sigma, cat. no. P-6001

Caution Toxic, avoid inhalation and contact with skin and eyes

* Glycerol (Sigma)

* PBS: 0.05 M Na2HPO4, 0.85% (wt/vol) NaCl (pH 7.5)

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* 0.02 µm filter-autoclaved MilliQ H2O

* 0.02 µm filtered seawater, formalin preserved, 2% (vol/vol) final concentration

* 0.02 µm filtered formalin (37–39% (wt/vol) saturated formaldehyde solution)

*Caution Toxic, avoid inhalation, ingestion or contact with skin, eyes or mucous membranes; dispense inside chemical fume hood if possible, or in an open-air environment with appropriate safety attire and eye protectio*n

* Ethanol (200 proof)

* Non-fluorescent immersion oil for microscopy (refractive index (nd) = 1.516; Olympus)

(Equipment):

* Epifluorescence microscope with filters for blue excitation and green emission with 100 × objective

* Microscope stage micrometer

* Glass 25 mm filter holder, 15 ml filter funnel with fritted glass base and clamp (Millipore, cat. no. XX10 025 00)

* 0.02 µm pore size, 25 mm diameter Anodisc Al203 filters (Whatman, cat. no. 6809-6002)

* 0.8 µm pore size, 25 mm diameter AA mixed-ester membrane filter (Millipore)

* Filter forceps (Millipore)

* Glass microscope slides, 25 × 75 mm, standard thickness, frosted at one end (VWR)

* Glass coverslips (25 mm × 25 mm, squares; VWR or Corning)

* 2.0 ml clean and sterilized micro-centrifuge tubes (VWR or Eppendorf)

* Pipettes suitable for 2–10 ml volumes (Rainin or Eppendorf)

* 5 ml and 10 ml clean, sterilized tips (VWR)

* 50 ml conical centrifuge tubes (polypropylene (PP); Falcon or VWR)

* Glass Erlenmeyer filter flask or multiple filter holder

* Polystyrene Petri dishes (VWR or Falcon)

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* Kimwipes (Kimberly-Clark)

* Vacuum source, suitable for at least 25 cm Hg vacuum (e.g., diaphragm pump; Gast, cat. no. DOA-P104-AA)

* Low-temperature dry-heat block with anodized aluminum heat block (VWR)

*1 l filter flask (polypropylene secondary filtrate waste reservoir or 'trap'; Cole-Parmer)

Equipment Setup:

Epifluorescence microscope We use a BX60 epifluorescence microscope (Olympus) with a 100 × UPlanApochromat objective, 150 W xenon lamp (Optiquip), UMWB blue excitation filter cube (wide band, excitation band pass (BP) 450–480 nm; dichroic mirror (DM) 500 nm; emission barrier filter (BA) 515) and 10 × focusing eyepiece with a 10 × 10 square grid reticle.

Glass coverslips Select the correct thickness to optically match the microscope objective you are using (e.g., our Olympus system uses #1½ or 0.17-mm thick cover glasses). The correct thickness is usually marked on the oil-immersion objective after a slash, or you can ask the lens manufacturer.

Filtration apparatus Connect the filter flask or filter manifold to the secondary 1 l filter flask ('trap'/waste reservoir) with appropriate diameter tubing. Connect the waste reservoir filter flask to the vacuum pump with tubing and hose clamps. At the vacuum pump end, we use a vacuum pump-protection filter to prevent liquids entering the vacuum pump, although this is optional. To set up the filter apparatus, first place the glass 25 mm filter holder in its silicone stopper (usually provided with the glass filter holder from Millipore) into the filter flask or a port in the multi-filter holder manifold. Once these are firmly in place, test whether you are getting good suction by turning on the vacuum pump and pipetting H_2O onto the fritted glass filter base. Use a vacuum of ≤ 25 cm Hg (~1/3 atm).

Glass 25 mm filter holder Note that the inside diameter of the filter funnel and the diameter of the porous fritted area of the glass base should be close to (or slightly larger than) the diameter of the porous part of the Anodisc filter (these filters have a non-permeable plastic outer ring); if the funnel or base is smaller, this might restrict the actual filtered area, which will need to be taken into account when calculating virus abundance (see Step 55).

Reagent Preparation:

10% (wt/vol) p-phenylenediamine

50% glycerol/50% PBS (1:1) (vol/vol) solution

1:400 SYBR Green I solution

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0.1% (vol/vol) p-phenylenediamine anti-fade mounting medium

Protocol

Sample Collection

Step 1.

Collect seawater samples (e.g., in 50 ml centrifuge tubes).

Sample Collection

Step 2.

Rinse each collection tube three times with sample water before filling.

Sample Collection

Step 3.

If planning to freeze samples, leave 10% empty space to allow expansion without cracking of the lid or tube at −80 °C or during liquid-nitrogen immersion (see Step 4).

NOTES

As stated above, we recommend using samples ≥50 ml to minimize the loss of counts over time.

Sample Collection

Step 4.

Fix samples with 0.02 µm filtered formalin (37–39% (wt/vol) formaldehyde solution) as soon as possible after collection to 2% (vol/vol) final concentration. Therefore, for 49 ml seawater sample add 1 ml formaldehyde.

Sample Collection

Step 5.

Use brief inversion of the sampling container to mix in the fixative, and then place it on ice.

DURATION

00:10:00 Additional info:

NOTES

As seawater samples fix quickly, samples are ready for slide preparation after 10 min on ice.

Sample Collection

Step 6.

If it is not possible to prepare slides within 4 h, samples should be placed at −80 °C for long-term storage

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after flash freezing in liquid nitrogen (see annotation).

NOTES

There are now significant data recommending flash freezing of aldehyde-fixed samples in liquid nitrogen as one of the best preservation methods for directly counting viruses[10, 14] and bacteria[13]. We suggest slides are made within hours or less of sample collection and preservation[14]. If you are unable to fix at the collection site, store samples on ice, preferably in large containers, and fix as quickly as possible.

Slide Preparation

Step 7.

For SYBR Green I working stock for four seawater samples analyzed in duplicate, you need to prepare eight filters.

NOTES

The following is an example of how much SYBR Green I reagent and *p*-phenylenediamine anti-fade mounting medium must be prepared for the staining of 10 Anodisc filters. This will allow you to enumerate bacteria and viruses in four water samples (see REAGENT SETUP in the full manuscript for details).

To be safe, make enough to cover ~20% failure rate, in case a filtration fails or a filter breaks.

Slide Preparation

Step 8.

Make the reagent in a clean, sterilized 2 ml micro-centrifuge tube.

NOTES

For example, 10 filters × 100 µl per filter = 1,000 µl. For 0.1% (vol/vol) *p*-phenylenediamine anti-fade mounting medium, for example, 10 filters × 30 µl per filter = 300 µl.

Slide Preparation

Step 9.

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

NOTES

Make the reagent in a clean, sterilized 2 ml micro-centrifuge tube.

For example, 10 filters × 100 µl per filter = 1,000 µl. For 0.1% (vol/vol) p-phenylenediamine anti-fade mounting medium, for example, 10 filters × 30 µl per filter = 300 µl.

Therefore, for 300 µl of 1:1 glycerol:PBS solution add 3 µl of 10% (wt/vol) stock p-phenylenediamine.

Slide Preparation

Step 10.

Make the reagent in a clean, sterilized 2 ml micro-centrifuge tube, keep it on ice and protect it from light (e.g., wrap in aluminum foil or cover the ice bucket).

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Slide Preparation

Step 11.

Label each glass slide (on the frosted end with a pencil or ultra-fine, oil-resistant laboratory marker pen).

NOTES

We recommend including the date of sample collection, date of slide preparation, depth and site, code of seawater sample origin, volume filtered and initials of slide preparer. You can mount both duplicate filters onto one microscope slide.

Slide Preparation

Step 12.

Prepare two or three sterile Petri dishes for the filter staining.

Slide Preparation

Step 13.

With a permanent marker pen, split the underside of the Petri dish into quadrants and label each successively 1, 2, 3 and 4, so that the numbers appear in the correct orientation when you flip the dish to look on the inside.

Slide Preparation

Step 14.

Repeat this for the next Petri dish, numbering its quadrants 5, 6, 7 and 8.

Slide Preparation

Step 15.

Finally, use numbers 9, 10, 11 and 12 for the last Petri dish.

Slide Preparation

Step 16.

Place the Petri dishes in a dark laboratory bench drawer or a dark box near where you intend to filter the samples.

NOTES

You will need frequent access to this drawer or box.

Slide Preparation

Step 17.

Before filtering any samples, prepare a control slide (following Steps 20–45) with 1 ml of the 0.02-µm filter-autoclaved MilliQ H2O used to make the SYBR Green I working dilution.

NOTES

This Anodisc will serve as the control filter to look for contamination, particularly in the water, the filter tower and the

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filter surface. We have discovered occasional batches or lots of 0.02 µm pore size, 25 mm diameter Anodisc filters to be covered with bacteria and viruses when they come from the manufacturer. Observe this filter under the microscope before proceeding with actual seawater samples.

Slide Preparation

Step 18.

Place seawater samples on ice after fixation to begin slide preparation.

Slide Preparation

Step 19.

If samples the samples have been kept at −80 °C, thaw them at room temperature (25 °C) and place on ice immediately after thawing. For near-surface seawater samples, filter 2 ml of the sample water. For much deeper water samples, 5–20 ml might be more appropriate.

NOTES

Adjust the sample filtration volume or dilute the sample based on the initial cell and virus densities observed under the microscope. We recommend volumes ≥2 ml, because at smaller volumes the meniscus becomes a significant portion of the sample and tends to concentrate cells near the rim. In a case where 2 ml is too much, dilute the sample with 0.02 µm filtered seawater preserved with 2% (vol/vol) formalin. Work through the complete slide-preparation procedure to ensure the volume of seawater being filtered is appropriate for enumeration. It is important to know the exact sample volume filtered (see steps 44-53).

Slide Preparation

Step 20.

Clean the 15-ml filter towers before adding sample by rinsing the insides with 0.02 µm filter-autoclaved MilliQ H2O, followed by ethanol (use a squirt bottle).

Slide Preparation

Step 21.

Wipe and dry the inside of each tower by gently pushing a small Kimwipe through it with clean forceps.

Slide Preparation

Step 22.

Mount the Anodisc filter onto the filter holder (immediately prior to sample filtration).

NOTES

This step should not be started until you are ready to filter the samples. All filters must be handled using clean forceps and only on the edge (i.e., the plastic rim of the Anodisc filter). Initially, wet a 0.8-µm pore size, 25 mm diameter AA mixed-ester membrane filter with filtered dH2O, and place it onto the center of the filter holder. The AA filter should look uniformly translucent (i.e., no blotches and no bubbles beneath). Draw this water through by turning on the vacuum pump briefly, and as soon as the water disappears turn the pump off, leaving the filter wet. Next, carefully place a 0.02-µm pore size, 25 mm diameter Anodisc filter on top of the AA filter. To do this, grasp the plastic rim of the Anodisc filter and place it on the AA filter with the same side facing upwards as in the manufacturer's packaging. The plastic rim must be oriented upwards (i.e., above the filter where they overlap).

Ensure the Anodisc filter sticks to the AA filter with no trace of bubbles or gaps in between; the AA filter must be wet (not just damp) before placing the Anodisc filter on top. If the AA filter is too dry and the Anodisc does not stick uniformly, re-wet the AA with a few drops of dH2O and, if necessary, briefly filter any puddled water through and try again. Note that

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the Anodisc filters are fragile and easily cracked. Handle them only with filter forceps and by the plastic rim. Discard any cracked filters.

Slide Preparation

Step 23.

Clamp the funnel and filter holder.

Slide Preparation

Step 24.

Gently place the clean glass 15 ml filter funnel on the filter holder until it is flush with the filter stage, then use the clamp provided with the filter apparatus to secure the two glass pieces sandwiching the two filters.

NOTES

Make sure the clamp is on correctly. An improperly clamped filter setup will cause the sample seawater to leak.

Slide Preparation

Step 25.

Using a 5-ml or 10-ml pipette, transfer the desired volume of sample water to the filter funnel and turn on the vacuum pump to 20 kPa or 20 cm Hg VAC (do not exceed 25 cm Hg VAC).

NOTES

See equipment setup in guidelines.

Slide Preparation

Step 26.

Due to the 0.02-µm pore-size filter, the water will go through relatively slowly, especially with coastal samples.

NOTES

Time each filtration from start to end; similar samples should filter at similar rates. A particularly slow rate indicates a problem, such as a vacuum leak or bubbles beneath the filter. A particularly fast rate indicates a cracked filter or leak around the funnel.

Slide Preparation

Step 27.

Shortly after the water has filtered through (watch from the top as it is about to finish, and then give it several seconds more), leave the vacuum pump on, and carefully remove the clamp and filter funnel.

Slide Preparation

Step 28.

With clean filter forceps, gently locate and grasp just the Anodisc filter, only by the plastic rim.

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Slide Preparation

Step 29.

As you lift to remove the filter away from the 0.8 µm AA, which should remain on the filter stage, you will hear and feel the release of the vacuum.

Slide Preparation

Step 30.

The same 0.8-µm AA filter can be used for multiple samples. If the AA filter is not damaged (i.e., the filter edge is not torn or creased) it can also be used on a future slide-preparation day.

Slide Preparation

Step 31.

At this point, while holding the Anodisc filter with the forceps, gently place it on a clean Kimwipe to blot the back of the Anodisc filter.

NOTES

The aluminum oxide Anodisc filter is brittle and any excessive pressure will cause it to crack or snap. A partially intact filter (cracked after the filtration is completed) can still be mounted, but will require extra care through the critical drying steps to follow.

Slide Preparation

Step 32.

Completely dry the filter by rubbing (option A), heating (option B) or blotting and leaving to dry (option C).

PROTOCOL

. Filter drying procedure

CONTACT: Jed Fuhrman

NOTES

The filter needs to be completely dried so that when it is held up to the light it appears uniform and nearly opaque, with an iridescent sheen. When an Anodisc filter is wet it will appear partially translucent, just like an oil stain on a white piece of paper. You might also notice the sparkle of the aluminum oxide (sapphire mineral) in the filter when it is moist.

(Option A): Drying the filter by rubbing with a Kimwipe

Step 32.1.

(A) Gently rub the filter back against a clean Kimwipe.

NOTES

Rub gently and on the correct side. Do not rub the top surface of the filter as this is sample side where the microorganisms are attached.

(Option B): Drying the filter using a heat block

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Step 32.2.
(B) After briefly blotting the back of the filter, place it on a clean glass microscope slide on top of the solid, flat side of an aluminum low-temperature dry-heat block set to 35–37 °C (be careful that the filters do not slide off). The heat block should be warm to the touch and no hotter than the recommended temperature.

NOTES

When using this accelerated drying method, leave the filter no longer than 5 min on the dry heat block. This method is recommended in regions of high humidity.

(Option C): Drying the filter by blotting and leaving in a dark drawer or box

Step 32.3.
(C) After briefly blotting the back of the filter with a clean Kimwipe, place it backside down on another new Kimwipe in a bench drawer in the dark (in humid climates, a darkened dessicator might be a good alternative).

(Option C): Drying the filter by blotting and leaving in a dark drawer or box

Step 32.4.
(C) Give the filter an opacity check after 3–4 min to determine whether it is dry. Place it back in the drawer if any patches of translucent area remain. If you are processing a number of samples, this method seems to work best as you can filter multiple samples and place filters in the drawer to dry in small batches (four to six). By staggering the slide preparation in this way, numerous samples can be efficiently processed.

Slide Preparation

Step 33.
While the filters are drying, use a pipette and tip to dispense a 100-µl droplet of the working concentration SYBR Green I reagent onto the middle of each quadrant of the Petri dishes labeled 1–8 (if you are making eight filters).

Slide Preparation

Step 34.
Once a set of four filters is dry, place the one that you filtered first onto the 10-µl SYBR reagent drop backside down in the quadrant labeled 1. The microorganisms on the topside of the filter will be stained by the reagent, which will easily pass through the filter from the underside to the top.

Slide Preparation

Step 35.
Repeat for successive filters, always keeping them in order of filtration so the next one will go onto the quadrant marked with a 2.

Slide Preparation

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Step 36.
Stain each filter for 18 min, keeping the staining filters in the dark in the closed drawer (or an equivalent dark area that is at room temperature, close by and easily accessible).

DURATION

00:18:00 Additional info:

NOTES

Do not under-stain the filters (15–20 min is an acceptable range), and ensure that the staining time is consistent for sets of samples for accurate enumeration. In our experience, over-staining by up to 30 min produces no noticeable effect.

Slide Preparation

Step 37.
After the staining period, carefully pick up the filter with clean forceps from the plastic rim and briefly blot the backside against a clean Kimwipe. The filter will have picked up a slight orange tint from the staining reagent.

Slide Preparation

Step 38.
Repeat the filter-drying procedure described in Step 32.

PROTOCOL

. Filter drying procedure

CONTACT: Jed Fuhrman

NOTES

We recommend that you use the dark-drawer drying method. Use the dry-heat-block drying method in geographic locations where high humidity is experienced. The filter must be dried in a dark place (e.g., a drawer, dark box or dry-heat block covered with a box), and must be completely dry before mounting. In humid climates, it can also help to use a dessicator.

(Option A): Drying the filter by rubbing with a Kimwipe

Step 38.1.
(A) Gently rub the filter back against a clean Kimwipe.

NOTES

Rub gently and on the correct side. Do not rub the top surface of the filter as this is sample side where the microorganisms are attached.

(Option B): Drying the filter using a heat block

Step 38.2.
(B) After briefly blotting the back of the filter, place it on a clean glass microscope slide on top of the solid, flat side of an aluminum low-temperature dry-heat block set to 35–37 °C (be careful that the filters do not slide off). The heat block should be warm to the touch and no hotter than the

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recommended temperature.

NOTES

When using this accelerated drying method, leave the filter no longer than 5 min on the dry heat block. This method is recommended in regions of high humidity.

(Option C): Drying the filter by blotting and leaving in a dark drawer or box

Step 38.3.

(C) After briefly blotting the back of the filter with a clean Kimwipe, place it backside down on another new Kimwipe in a bench drawer in the dark (in humid climates, a darkened dessicator might be a good alternative).

(Option C): Drying the filter by blotting and leaving in a dark drawer or box

Step 38.4.

(C) Give the filter an opacity check after 3–4 min to determine whether it is dry. Place it back in the drawer if any patches of translucent area remain. If you are processing a number of samples, this method seems to work best as you can filter multiple samples and place filters in the drawer to dry in small batches (four to six). By staggering the slide preparation in this way, numerous samples can be efficiently processed.

Slide Preparation

Step 39.

Place the dried Anodisc filter onto the labeled glass microscope slide (be careful that it does not slide off).

Slide Preparation

Step 40.

To mount the filter, use a pipette and tip to dispense 27–30 µl of 0.1% (vol/vol) *p*-phenylenediamine anti-fade mounting medium onto a 25 × 25 mm glass coverslip.

Slide Preparation

Step 41.

To facilitate positioning of the filter on the glass slide, it might be appropriate to dispense part (10 µl) of the mounting medium directly onto the glass slide where the filter is to be placed, and to dispense the remaining volume (20 µl) onto the coverslip.

Slide Preparation

Step 42.

Using forceps or gloved hands, pick up the coverslip, invert and place it on the Anodisc filter on the microscope slide. The coverslip should now be stuck to the filter.

NOTES

By gently pressing the coverslip against the microscope slide, the mounting medium should spread evenly by capillary action (including through the filter to the glass slide) and sandwich the filter. If carried out correctly, bubbles should

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naturally be eliminated through the sides as the mounting medium spreads. You can mount the duplicate filter for this sample on the same microscope slide. Two filters should easily occupy the length of a regular microscope slide. The slide is then ready for observation under the microscope.

Slide Preparation

Step 43.

For long-term storage, place the slides in a microscope slide box and store in a −20 °C freezer.

NOTES

If the slides are prepared correctly, staining might show little or no fading after several months, and might still be observed years later.

Virus enumeration procedure

Step 44.

To view the slide, use a 100 × fluorescence oil-immersion objective with immersion oil.

NOTES

The immersion oil should not have any background fluorescence under blue excitation.

Virus enumeration procedure

Step 45.

Place a drop of oil onto the center of a coverslip and carefully move the objective down to the drop of oil.

NOTES

Be careful not to mix different types of immersion oil, and clean off the microscope objective completely if a different immersion oil might have been used previously.

Virus enumeration procedure

Step 46.

SYBR Green I bound to dsDNA is excited with a maximum at 488 nm.

NOTES

A wide BP blue-excitation filter and long-pass (LP) green-emission filter will optimize observation of the cells and viruses.

Virus enumeration procedure

Step 47.

See (Figure 1) for a typical image.

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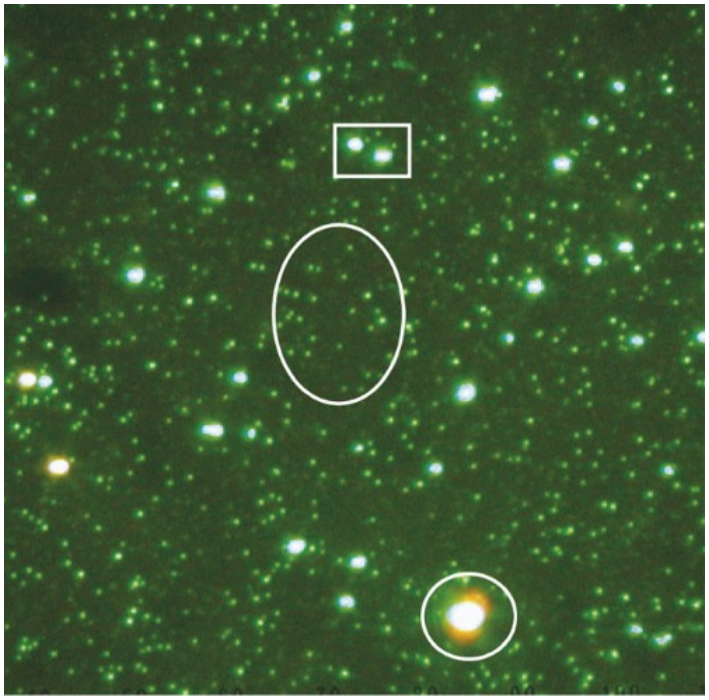

The rectangle indicates two prokaryotes, the ellipse indicates >25 virus-like particles and the circle indicates one protist.

NOTES

The viruses are the most numerous tiny dots, whereas the bacteria are larger and have defined shapes and edges.

Virus enumeration procedure

Step 48.

Look carefully at the slide to see whether all the viruses and bacteria are at nearly the same focal plane (i.e., that they come into focus at nearly the same point).

NOTES

To enumerate accurately, you will need to make micro-adjustments using the fine focus to ensure all viruses in a particular grid-reticle box are counted. However, if several viruses come into and out of focus as you move up off the filter, the viruses have come off the filter into the mountant and the slide cannot be used. We find this is often the case when it initially appears that viruses are less than five times as abundant as bacteria. In typical marine samples, the viruses are at least 10 times more abundant than the bacteria. All slides with viruses present at less than five times the bacterial abundance (or, in general, with a lower virus:bacteria ratio than similar corresponding samples in a set) should be carefully checked for the potential problem of virus detachment and suspension in the mountant. If such a problem is detected, discard the slide.

Virus enumeration procedure

Step 49.

Using an eyepiece with a 10 × 10 grid reticle (note in our setup the grid reticle is 94 µm × 94 µm = 8,836 μm^2), count enough of the 100 smaller boxes to produce a virus (or prokaryote) count between 30 and 40.

Virus enumeration procedure

Step 50.

Then, count this number of boxes in the grid for a total of 10 fields by randomly moving the stage to a new

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position around the entire filter; i.e., if 3 boxes resulted in a count of 34 for the first field, then count 3 boxes in each of 9 other areas of the filter (do not count different numbers of boxes in different fields of one filter).

Virus enumeration procedure

Step 51.

The total for 10 fields should be >200 viruses counted; therefore some fields may yield counts that are <30. But if the total count for a filter is <200, count extra fields.

Virus enumeration procedure

Step 52.

Do not look through the eyepiece when moving to a new field, as this might bias your next viewing position.

NOTES

Avoid counting in areas near the edge of the filter and around any air bubbles. Blank areas on the filter indicate a filtration problem.

Virus enumeration procedure

Step 53.

Calculate the number of viruses per ml of seawater as follows: → RSF × X × (100/n)/V. Here, X is the average number of viruses or prokaryotes counted per field, n is the number of boxes (of 100 total) counted within the 10 × 10 reticle grid per field, RSF is the grid-reticle scaling factor (representing the ratio of the filtered area to the 10 × 10 grid visible in the eyepiece) and V is the volume of seawater filtered (ml).

NOTES

The RSF value must be calculated for your microscope system using the following example: ~2.84 × 108 µm2 (the filterable area of the 0.02-µm pore-size Anodisc filter that has a diameter of 19 mm = 19,000 µm) divided by 8,836 µm2 (the area of the 10 × 10 square eyepiece grid reticle, 94 µm × 94 µm for our system; calculate the exact area of your grid reticle using a stage micrometer slide) = 32,141 (the RSF value for our microscope system).

If the filter funnel or fritted glass base is smaller that the filterable area of the 0.02-µm pore-size Anodisc filter, you must measure the actual area filtered by observing where the microorganisms on the filter stop (i.e., locate the tangents). This is done using the microscope stage markings in combination with a stage micrometer to make the measurements (most research microscope stages have 'mm' markings). Do not use the interior diameter of the filter funnel as a measurement, because the filtered area is often several mm larger.

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