Counting viruses using polycarbonate Track Etch™ membrane filters as an alternative to Anodisc™ membrane filters.

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SUMMARY

An alternative epifluorescence microscopy (EFM) technique for virus enumeration has been developed using polycarbonate Track Etch (PCTE) filter membranes in place of aluminum oxide Anodisc membranes. The established EFM technique, first developed by Hennes and Suttle in 1995 and later expanded upon by Noble and Fuhrman in 1998, uses supported 20 nm pore-size Anodisc filter membranes to determine virus abundance in natural environments. Increased price and sporadic availability of Anodisc filters stimulated the evaluation of alternative filters for use in the procedure. The feasibility of using 30 nm pore-size PCTE filters for virus enumeration was assessed using the Anodisc filter procedure as a control. Although virion particle counts are slightly less precise using PCTE filters, they offer a substitute for Anodiscs while requiring only minor adjustments to the established protocol. Per slide, the PCTE costs approximately ten times less to prepare than the Anodisc-based method.

ABBREVIATED PROCEDURE:

1. Place a 0.2 µm pore support filter on the fritted glass base of a filtration unit and dampen with 200 µL of virus-free water.
2. Mount a PCTE filter on top of the damp support filter.
3. Clamp the funnel to the fritted base.
4. Stain the PCTE filter in place by drawing 1 mL of 4 µg/mL Sudan black B (in 50% ethanol) through the filter.
5. Filter at least 0.5 mL of virus-containing solution through the stained PCTE filter.
6. Release vacuum and stain viruses *in situ* using 0.5 mL of a 1:400 SYBR-Gold solution prepared using SM or TE buffer adjusted to pH 8.0.
7. Allow viruses to stain for 10 minutes in the dark, without vacuum.
8. After 10 minutes, re-apply vacuum to draw the SYBR Gold solution through the filter.
9. Rinse SYBR-Gold stained filter with 1 mL of SM or TE buffer by drawing the wash solution through the PCTE filter.
10. Place a 5 µL drop of 1X antifade solution onto the surface of a clean slide, and an additional 5 µL on a cover slip.
11. Carefully remove the PCTE filter from the support filter with forceps and briefly air dry.
12. Mount the PCTE filter on the slide beneath the coverslip, saturating the PCTE filter with antifade.
13. View the PCTE slide by epifluorescent microscopy within a few hours of preparation.

BACKGROUND

Quantifying virus abundance in environmental samples is the cornerstone of virus ecology research. The discovery that most environments are teeming with viruses and bacteriophages has dramatically transformed our understanding of microbial ecosystems (Weinbauer 2004; Wommack and Colwell 2000). Viruses and bacteriophages are now known to play a major role in microbial ecosystem nutrient cycling (Wilhelm and Suttle 1999), host population control (Wilhelm et al. 2002) and global biogeochemical processes (Danovaro et al. 2008; Rodriguez-Brito et al. 2010; Suttle 2005; Wommack and Colwell 2000). Virion particle enumeration is typically performed utilizing epifluorescent microscopy (EFM), which facilitates detection of virus-size particles containing DNA or RNA, often referred to as "virus-like particles" (VLPs), retained on the surface of supported 20 nm pore Anodisc filters (Noble and Fuhrman 1998; Patel et al. 2007; Suttle and Fuhrman 2010). VLPs are stained with a nucleic acid-specific fluorescent dye such as SYBR Green, SYBR Gold or Yo-Pro (Invitrogen) (Hennes and Suttle 1995), and the brightly fluorescent VLPs are then counted to estimate the number of viruses contained in the original sample.

Counting VLPs by Anodisc-based EFM is quick, reliable, and amenable to field settings. Originally developed for aquatic samples, the protocol can be adapted for viruses derived from sediments (Danovaro et al. 2008; Suttle and Fuhrman 2010), soil (Williamson et al. 2005), sewage (Wu and Liu 2009), and virus cultures (Hennes and Suttle 1995). It is often preferable to prepare virus EFM slides at the time of sample collection as preservation of virus-containing liquid samples often requires fixation with glutaraldehyde, flash freezing in liquid nitrogen, and storage thereafter at -70ºC (Wen et al. 2004). Once the Anodisc filter slides are prepared for microscopy, however, they may be stored long-term at -20ºC without significant deterioration of fluorescence intensity (Wen et al. 2004).

GE Healthcare became the sole manufacturer of supported Anodisc filters after acquiring Whatman Plc in 2008. Since then, increased cost and sporadic availability of supported 20 nm pore Anodisc filter membranes has necessitated the exploration of alternative methods for virus enumeration. Flow cytometry is one such alternative (Brussaard 2004), however, lack of widespread access to flow cytometers makes the simple and versatile filter-based EFM technique the preferred method of the viral ecologist.

Polycarbonate Nucleopore membrane filter discs (Whatman / GE Healthcare) are commonly used for microbial EFM (Daley and Hobbie 1975; Hobbie et al. 1977; Seo et al. 2010). Polycarbonate filters with pore sizes greater than 200 nm are pre-stained black to reduce autofluorescence of the polycarbonate material, however, polycarbonate filters with pores small enough to retain virus-sized particles (10 and 30 nm) are not available pre-stained and must be stained by the user. Irgalan Black, also known as Acid black 107, is typically used to stain polycarbonate membranes (Budinoff et al. 2011; Hobbie et al. 1977). However, Sudan black B (Sigma-Aldrich) has also been shown to adequately stain polycarbonate materials to reduce background fluorescence (Kepkay et al. 1990; Zimmermann et al. 1978). Results presented herein demonstrate that background fluorescence of 30 nm pore polycarbonate Track Etch (PCTE) filter membranes (Sterlitech) can be adequately reduced using Sudan black B to yield VLP counts comparable to Anodisc filters.

MATERIALS

Epifluorescent microscopy materials:

Anodisc™ sample slides were prepared according to the protocols outlined in Patel et al. (2007) and Suttle and Fuhrman (2010). All materials required for the PCTE-based method are the same as those listed in the Patel et al. or Suttle and Fuhrman protocols except for the PCTE filters and the Sudan black B stain. Polycarbonate Track Etch™ (PCTE) 25mm diameter filter discs with 30 nm pores were obtained from Sterlitech Corporation (SKU: PCT00325100). Hydrophilic 220 nm pore size, 25 mm Durapore™ PVDF membranes, used in this procedure as support filters, are manufactured by Millipore (cat. # GVWP 02500). Use of this specific support filter is not required, however a comparable support filter is necessary to provide a smooth surface for the PCTE filter. Sudan black B dye (Sigma-Aldrich cat.# 199664) is first dissolved in 100% ethanol to produce a 1,000X stock solution [4 mg/mL], which is diluted in 50% ethanol, prepared using distilled virus-free water, resulting in a 1X Sudan black B concentration of 4µg/mL (Zimmerman et al. 1978). The Sudan black B solutions may be stored long-term at room temperature, and should be centrifuged or filtered to remove undissolved dye particles before use.

PCTE membrane filters with 10 nm pores (Sterlitech SKU: PCT00125100) are not recommended for general use due to the difficulty of handling and ~10X decrease in flow rate when filtering. However, 10 nm pore filters, which require a Polonium-210 Static Eliminator device (Sterlitech SKU: STATIC), were used to assess the effect of filter pore size on environmental sample counting precision.

DETAILED METHOD

Procedure:

1. A 200 nm pore, 25mm diameter PVDF support filter is first placed onto a fritted glass base to provide an even surface for the diaphanous PCTE filter. The PVDF filter is saturated with 200 µL of virus-free water after being placed on the fritted glass base. Slight vacuum suction may be applied to absorb water drops into the PVDF support filter.
2. The PCTE filter is then carefully mounted on top of the damp support filter. Placing the PCTE filter on the support filter with a slight overlap is preferable, if possible, so that it is easier to remove the PCTE filter after the process is finished.
3. The funnel is then mounted on the fritted base and clamped in place to secure the support and PCTE filters.
4. The PCTE filter is then stained with Sudan black B while held in place between the fritted base and funnel. To stain the PCTE filter, 1 mL of 4 µg/mL Sudan black B solution is added to the funnel and is drawn through the filter at a vacuum pressure of -70 kPa. Suction is applied until the PCTE filter appears dry. The flow rate through 30 nm pore PCTE filters is approximately 0.1 mL/min, at -70 kPa using a 25mm diameter funnel.
5. The virus solution is then added to the funnel and drawn through the stained PCTE filter at -70 kPa. It is preferable to filter a volume of at least 0.5 mL of virus solution as smaller volumes may not uniformly cover the membrane surface during filtration.
6. When the virus particles have been extracted from the suspension, vacuum is released and 0.5 mL of a 1:400 SYBR Gold solution is added to the funnel. The SYBR Gold solution is prepared using SM buffer (100mM NaCl, 10mM $MgCl_2$ and 50mM Tris-Base, adjusted to pH 8.0) or TE Buffer (10mM Tris-Base, 1mM EDTA, adjusted to pH 8.0) to maintain a pH between 7.5 and 8.3, as pH outside this range adversely affects fluorescence of the SYBR Gold stain (Invitrogen).

7. The PCTE filter, now with viruses on the surface, is allowed to stain for 10 minutes in the dark, without vacuum. After 10 minutes, vacuum is re-applied to draw the SYBR Gold solution through the filter.
8. One mL of SM or TE buffer is then drawn through the filter to rinse away excess stain and to remove viruses that may have stuck to the walls of the funnel during staining. Vacuum is maintained until the wash solution has completely passed through the PCTE filter.
9. While the filter wash process is underway, a 5 µL drop of 1X antifade (Patel et al. 2007) is placed on the surface of a clean microscope slide, and an additional 5 µL drop is placed on a cover slip.
10. When the wash is complete, vacuum is released and the PCTE filter is carefully removed from the support filter with forceps and waved very gently in the air a few times to dry.
11. The PCTE filter is then placed face-up on the 5 µL drop of antifade on the surface of the slide. The cover slip, with the 5 µL drop of antifade, is placed drop-side down on top of the PCTE filter. The cover slip is gently pressed down to remove air bubbles, to smooth small wrinkles in the filter, and to saturate the filter with antifade solution. Using more than 5 µL of antifade will significantly increase wrinkling of the PCTE filter on the glass slide. As 30 nm pore PCTE filters are exceedingly thin, small wrinkles will shift the focal plane when the slide is being viewed under the microscope, making counting difficult. It is therefore recommended that the PCTE filter is pressed as flat as possible underneath the cover slip before viewing.
12. The PCTE slide should be viewed by EFM within a few hours after preparation as fading occurs even when stored at -20ºC.

VLP enumeration:

Anodisc and PCTE membrane slides prepared for the preparation of this protocol were viewed using an Olympus BX60 epifluorescence microscope under blue excitation (488nm) and images were captured using QCapture Pro v.6.0 software. The image area of the captured field of view is first calculated using a stage micrometer, and the number of VLPs per mL is extrapolated based on the ratio of image area to filter area. For example, the length and width of the rectangular field of view (FOV) image captured at 1000X under oil immersion is calculated to be 88.5 x 66.2 microns, yielding a total area of 5858.7 microns^2. The diameter of the filter through which the virus solution passes is 15.5 mm, yielding an area of 1.887 x 10^8 microns^2. The scaling factor (SF), 3.2207 x 10^4, is obtained by dividing the filter area by the FOV image area. To calculate VLPs/mL, the equation (N_{FOV} x SF x DF) ÷ V is used, such that N_{FOV} is the average number of VLPs per field of view, DF is the dilution factor and V is the volume filtered.

Virus samples used in the assessment of the protocol

Two laboratory grown virus stocks and two environmental samples representing natural virus communities were used to assess PCTE filters for virus enumeration by EFM. PRD1 phage (obtained from the Felix d'Herelle Reference Center for Bacterial Viruses, HER number 23), with an average capsid diameter of 62 nm (Olsen et al. 1974) were propagated using host *Samonella typhimurium* LT2 (provided by Leonard Mindich). Briefly, a starter culture of *S. typhimurium* was grown in LB media at 37ºC with shaking until in exponential growth phase (OD600 ≈ 0.3). The culture was then infected with a 1/100 volume of PRD1 stock and incubated for another 2 hours until complete lysis of the host culture had occurred. Cellular debris was removed using centrifugation (1500 x g for 10 minutes at 4ºC) and the supernatant containing PRD1 particles was filtered through a Minisart 200 nm pore SFCA syringe filter (Sartorius-Stedim Biotech), yielding a sterile PRD1 stock. Production of PRD1 particles was verified by plaque assay (Bamford et al. 1995)

Bacteriophage T4 were prepared using a method developed by Elizabeth Kutter (Carlson 2005). Briefly, bacteriophage T4 are mixed with *E. coli B* in a thin layer of agarose containing growth medium to produce "lacy" or confluent plaques. Five mL of sterile phage buffer (100 mM Tris/HCl and 50 mM $MgCl_2$ at pH 7.5) is pipetted onto the surface of the plaques and the plate is gently agitated for 15 to 30 minutes to bring the T4 phage into suspension. The phage suspension is then carefully collected with a sterile pipette. Bacteriophage T4 has a 115 x 85 nm prolate capsid (Baschong et al. 1988), and a 100 nm length tail structure (Derosier and Klug 1968).

One of the environmental samples used in this analysis was collected from the Willamette River in downtown Portland, Oregon in April, 2011. The aqueous sample was immediately preserved in 2.5% glutaraldehye (v/v, final concentration) and stored at 4ºC within 20 minutes of sample collection. The following day the sample was filtered through a 450 nm SFCA syringe filter to remove large microorganisms and debris prior to use in the virus enumeration procedure.

Pore water from Boiling Springs Lake sediment (Lassen Volcanic National Park, CA) was collected in August, 2010 and was used as the second natural virus community sample. Boiling Spring Lake (BSL) has a pH of 2.5 and temperatures ranging between 52ºC and 95ºC, and is hence referred to herein as a "hot spring". The sediment sample collected for virus enumeration was obtained from the perimeter of BSL at a depth of approximately 0.5 meters below the water's surface (Diemer and Stedman 2012). Pore water was collected by sediment centrifugation at 21,000 x g for 10 minutes at room temperature. Virus-containing pore water was then passed through a 200 nm SFCA syringe filter. Viruses that remained adhered to the clay particulates in the sediment after the pore water was removed were released by resuspending the sediment in pH 9.0 SM phage buffer (100mM NaCl, 10mM $MgCl_2$ and 50mM Tris-Base). The SM buffer supernatant containing virus particles was collected by centrifugation and pre-filtration (as described above for pore water) prior to deposition onto PCTE and Anodisc filter membranes.

Bacteriophage ϕX174 utilized in EFM detection limit experiments was obtained from Carolina Biological Supply Company (Cat. # 124425).

FIGURES AND FIGURE LEGENDS

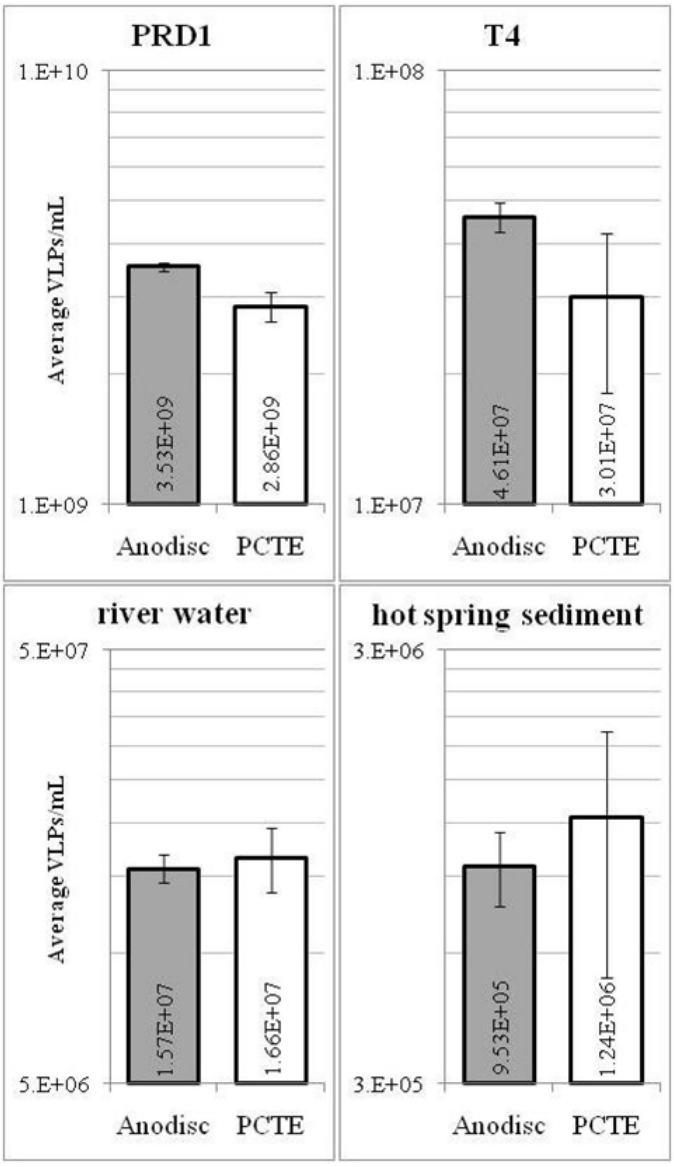

Figure 1: Comparison of VLP counts with 30 nm PCTE and 20 nm Anodisc filters. Viruses and VLPs were counted in approximately ten fields per filter membrane and averaged to calculate VLPs/mL. Average VLPs/mL are indicated within columns. Error bars denote standard deviation. PRD1 and river water samples were counted on three different filters, whereas T4 and hot spring sediment samples were counted on one filter of each type.

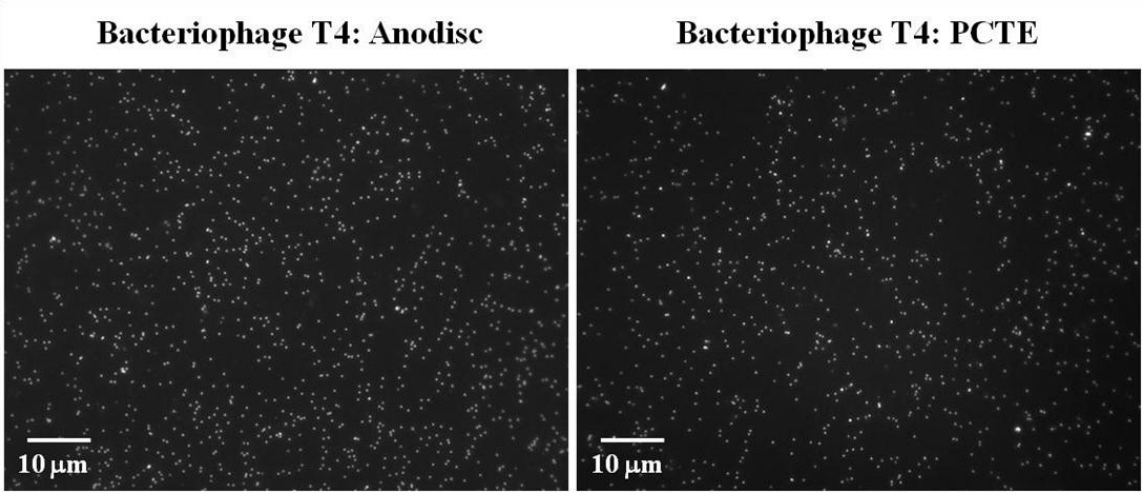

Figure 2: Representative EFM images of bacteriophage T4 on Anodisc (Left) and PCTE filters (Right), captured with an Olympus BX60 epifluorescence microscope under blue excitation (488nm) and imaged using QCapture Pro v.6.0.

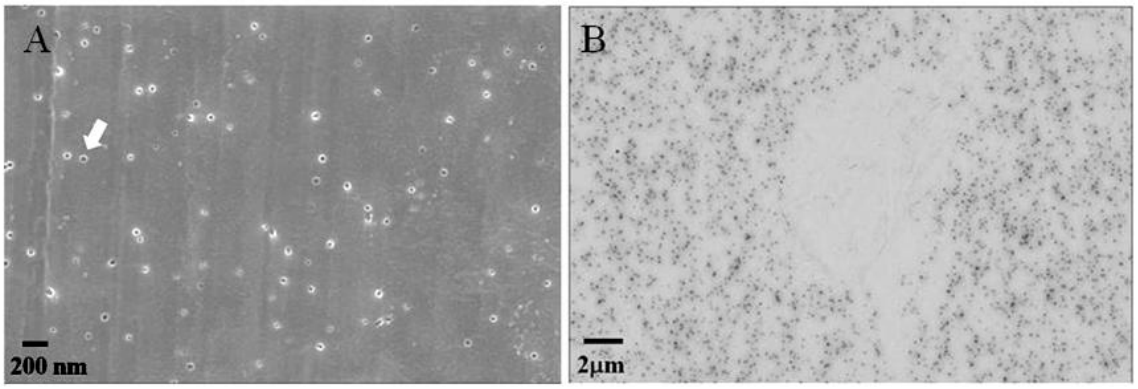

Figure 3: Scanning electron micrographs of a Sudan black B-stained, 30 nm pore PCTE filter. Image (A) illustrates the distribution of pores within the polycarbonate membrane. The arrow indicates a pore measured to be approximately 50 nm in diameter. A reverse-color image (B) shows a region of the PCTE filter devoid of pores. Images were acquired with a Zeiss Sigma VP FEG SEM at 5kV using an in-lens detector.

ASSESSMENT

Laboratory grown PRD1 and bacteriophage T4 virus controls were counted along with river water and hot spring sediment samples to test whether PCTE filter membranes can be used as a substitute for Anodisc filters. The PCTE filters, prepared according to the adapted procedure described above, were compared to Anodisc filters prepared using the established method described by Patel et al. (2007). Assays on both filter types were prepared contemporaneously using the same virus stocks and staining reagents. Filters of each type were prepared in triplicate, with the exception of the hot spring and T4 samples, for which one slide was prepared per filter type. Roughly ten fields of view per filter were counted utilizing the ImageJ software (Collins 2007) automatic counting feature, or counted manually when image contrast was low.

The 30 nm pore-size PCTE filter membranes yield VLP counts that are comparable to counts obtained using 20 nm pore-size supported Anodisc filters. VLP counts for T4 and PRD1 monocultures were slightly lower on PCTE than Anodiscs, whereas VLP counts for both environmental samples were the same. VLP counts were consistently less precise using PCTE, however [Figure 1]. Background fluorescence of the Sudan black B-stained PCTE membrane is minimal and similar to Anodiscs [Figure 2].

To determine whether loss of VLPs smaller than 30 nm in diameter (when using 30 nm pore PCTE filters) adversely affects counting precision, PCTE filters with 10 nm pores were tested using river water samples. However, even when using a Polonium-210 static eliminator to reduce static charge, difficulties encountered in handling the 10 nm pore PCTE filters resulted in poor reproducibility of the experiment, and direct comparisons between the 10 and 30 nm pore PCTE filters could not be made.

It is generally assumed, however, that EFM is not sensitive enough to detect small RNA and single-stranded (ss) DNA viruses because of their minimal nucleic acid content (Suttle and Fuhrman 2010). Recent data confirms that in most cases the fluorescence of small ssDNA viruses lies below the lower limit of detection of EFM even when using SYBR Gold (Holmfeldt et al. 2011). To determine whether the difference in pore size between PCTE and Anodisc filters might adversely affect the precision of VLP counts when using PCTE filters, both 30 nm pore PCTE and 20 nm pore Anodisc filters were used to count virions of the ssDNA ϕX174bacteriophage. With a diameter of approximately 30 nm (Hafenstein and Fane 2002), ϕX174 should be retained by 20 nm pore Anodiscs, but pass through the 30 nm pores of the PCTE filter. However, no ϕX174virions were visible on either PCTE or Anodisc filters by EFM. Thus, the loss of 20 to 30 nm diameter VLPs is not expected to adversely affect the precision of VLP enumeration of environmental samples [see Figure 1].

Differences between the nominal and actual pore sizes in polycarbonate membranes have been reported (Budinoff et al. 2011). This was also investigated as a possible source of the discrepancies observed between filter types. Sudan black B-stained and unstained 30 nm pore PCTE filters were examined by scanning electron microscopy, and pore sizes substantially larger than the nominal value were detected in both stained and unstained filters, however, the vast majority were in the 30nm range. This examination also revealed relatively large regions devoid of pores, and that the pores, overall, are not distributed uniformly within the membrane [Figure 3].

However, in light of the observation that the T4 and PRD1 monoculture counts are lower on PCTE than Anodisc, whereas the environmental samples are equivalent on both filter types [Figure 1], pore size variability does not explain the difference in precision between the two filter types.

DISCUSSION

The PCTE-based method described herein costs roughly ten times less than the Anodisc method per filter, with comparable fluorescence intensity of VLPs and very low membrane autofluorescence [Figure 2]. Although counts obtained using Anodiscs are more precise [Figure 1], PCTE filters offer a potential substitute for quantitative analysis of viruses in laboratory and environmental samples.

VLP enumeration using 30 and 15 nm pore Nucleopore filters stained with Acid black 107 has been reported, although the number of countable VLPs was found to be roughly an order of magnitude lower than what was achieved using Anodisc filters (Budinoff et al. 2011). Discrepancies between the Nucleopore (Budinoff et al. 2011) and the PCTE results reported here may be due to the use of dissimilar protocols. The PCTE results presented herein demonstrate that while VLP counts are comparable between Sudan black B-stained PCTE and Anodisc filters, the PCTE filter counts are less precise. Several properties of the PCTE membrane filters were investigated to account for this observed variability (see Assessment).

Anodisc filters, overall, appear to have a more uniform distribution of VLPs on their surface, whereas empty voids and regions of VLP pooling are commonly observed on PCTE filters (not shown). Moreover, the lack of membrane rigidity and absence of an annular support ring makes it difficult to sufficiently flatten the PCTE filter on the glass slide to achieve a uniform focal plane that spans the entire field of view during epifluorescence microscopy, and VLPs located outside of the focal plane may go uncounted. VLP pooling appears to be the result of surface deformations in the PVDF base filter, and small wrinkles which form in the PCTE membrane during handling. Irregular VLP distribution on the surface of PCTE filters appears to be the salient factor affecting the variability of VLP counts, not pore size. The manufacture of pre-stained PCTE filters that are composed of a more rigid material, or membranes supported by an annular ring might remedy these issues.

The precision of enumeration may be improved by simply increasing the number of fields counted when calculating the average VLP density when using PCTE membranes. Higher precision was obtained from PRD1 and river water samples, which were calculated using three PCTE slides each, whereas lower precision was observed in T4 and hot spring sediment samples that were calculated using one PCTE slide each. Counting three PCTE slides, or roughly 30 fields of view per slide, resulted in a level of precision equivalent to what was achieved by counting 10 fields of one Anodisc slide [Figure 1].

COMMENTS AND RECCOMENDATIONS

The time required to prepare one mounted PCTE slide is roughly equivalent to that required to prepare one mounted Anodisc slide. PCTE slides, however, must be prepared one at a time, whereas several Anodiscs may be stained simultaneously prior to mounting on the glass slide. As bulk processing of PCTE filters is not possible, it will require more time to prepare multiple PCTE slides than is required for an equivalent number of Anodiscs.

One key advantage of the PCTE-based method is that the materials and reagents required are the same as those listed in the established Patel et al. (2007) or Suttle and Fuhrman (2010) protocols, except for the PCTE filters

and the Sudan black B stain. As the two filter types are interchangeable, established laboratory protocols may be easily adapted to include the PCTE filter procedure using the same set of equipment and staining reagents.

SYBR Gold-stained VLPs on Sudan black B-treated PCTE filters have also been observed to diminish in fluorescence intensity more rapidly than on Anodiscs, even with storage at -20ºC and saturation with anti-fade reagent. It is therefore recommended that PCTE slides be viewed within four hours after preparation; however, users of this method should determine the fluorescence longevity for each sample type and fixation process utilized in the procedure.

PCTE filters are also quite thin and fragile, making them more difficult to handle during processing, and less amenable to preparation in the field. If it is not possible to view slides soon after they are prepared, or if conditions in the field are unsuitable, use of Anodiscs is preferable. Overall, however, it is considerably less expensive to use PCTE filters to establish appropriate dilutions of virus containing samples, or to achieve a semi-quantitative assessment of VLP concentrations in a test sample before using the more expensive Anodisc.

Those interested in working with 10nm pore sized PCTE membranes are encouraged to contact the authors for a more detailed account of preparative procedures as working with the 10nm is much more difficult than the 30nm PCTE membranes.

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