Contrasting Life Strategies of Viruses that Infect Photo- and Heterotrophic Bacteria, as Revealed by Viral Tagging

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ABSTRACT Ocean viruses are ubiquitous and abundant and play important roles in global biogeochemical cycles by means of their mortality, horizontal gene transfer, and manipulation of host metabolism. However, the obstacles involved in linking viruses to their hosts in a high-throughput manner bottleneck our ability to understand virus-host interactions in complex communities. We have developed a method called viral tagging (VT), which combines mixtures of host cells and fluorescent viruses with flow cytometry. We investigated multiple viruses which infect each of two model marine bacteria that represent the slow-growing, photoautotrophic genus *Synechococcus* (*Cyanobacteria*) and the fast-growing, heterotrophic genus *Pseudoalteromonas* (*Gammaproteobacteria*). Overall, viral tagging results for viral infection were consistent with plaque and liquid infection assays for cyanobacterial myo-, podo- and siphoviruses and some (myo- and podoviruses) but not all (four siphoviruses) heterotrophic bacterial viruses. Virus-tagged *Pseudoalteromonas* organisms were proportional to the added viruses under varied infection conditions (virus-bacterium ratios), while no more than 50% of the *Synechococcus* organisms were virus tagged even at viral abundances that exceeded \((5 \times 10^5)\) that of their hosts. Further, we found that host growth phase minimally impacts the fraction of virus-tagged *Synechococcus* organisms while greatly affecting phage adsorption to *Pseudoalteromonas*. Together these findings suggest that at least two contrasting viral life strategies exist in the oceans and that they likely reflect adaptation to their host microbes. Looking forward to the point at which the virus-tagging signature is well understood (e.g., for *Synechococcus*), application to natural communities should begin to provide population genomic data at the proper scale for predictively modeling two of the most abundant biological entities on Earth.

IMPORTANCE Viral study suffers from an inability to link viruses to hosts *en masse*, and yet delineating “who infects whom” is fundamental to viral ecology and predictive modeling. This article describes viral tagging—a high-throughput method to investigate virus-host interactions by combining the fluorescent labeling of viruses for “tagging” host cells that can be analyzed and sorted using flow cytometry. Two cultivated hosts (the cyanobacterium *Synechococcus* and the gammaproteobacterium *Pseudoalteromonas*) and their viruses (podo-, myo-, and siphoviruses) were investigated to validate the method. These lab-based experiments indicate that for most virus-host pairings, VT (viral tagging) adsorption is equivalent to traditional infection by liquid and plaque assays, with the exceptions being confined to promiscuous adsorption by *Pseudoalteromonas* siphoviruses. These experiments also reveal variability in life strategies across these oceanic virus-host systems with respect to infection conditions and host growth status, which highlights the need for further model system characterization to break open this virus-host interaction “black box.”

The oceans cover more than 70% of the Earth’s surface and are dominated by complex and dynamic microbial communities, both in terms of biomass and metabolism (reviewed in reference 1). Phototrophic microorganisms harvest solar energy and produce the organic material that fuels nearly all processes in the ocean (2, 3), while their heterotrophic counterparts respire this fixed organic carbon. While global carbon cycling is fundamental to predictive modeling of climate change and represents one of the best-studied elemental cycling processes on Earth, our understanding of the balance between fixed and respired carbon on a global scale remains poorly understood (reviewed in reference 4).

With respect to carbon cycling, viruses have largely been ignored due to lack of data. This is in spite of the fact that, at least in the oceans, viruses are abundant \((\sim 10^8 \text{ to } 10^9 \text{ ml}^{-1} \text{ water})\), kill \(\sim 20\) to \(40\%\) of bacteria per day, and are responsible for large carbon fluxes in the oceans at 150 Gt year\(^{-1}\) (5). However, their carbon cycling impact is likely much larger. For example, cyanobacterial viruses (cyanophages) also contain photosynthesis genes, includ-
It is impossible to characterize millions of viruses per host (25), due to the difficulty in measuring viral abundance at the community level (26). Two decades of viral ecology research have focused on community predictions, modeling becomes of fundamental importance. With two decades of viral ecology research, we have developed viral tagging (VT) to document colocalized host and viral gene products, as long as genetic information is available for primer design for both virus and host (25). Second, Escherichia coli O157-specific immunomagnetic beads coated with fluorescently labeled viruses use the virus-host interaction to fluorescently label *E. coli* for detection using flow cytometry (27, 28), but this method currently requires high cell concentrations (10^5 cells ml^{-1}) for detection and the availability of immunomagnetic beads specific to individual host strains (27, 28). Third, fluorescently labeled viruses (FLVs) have been used as probes to tag their host cells for examination under the microscope (29-31), a method that is limited only by the fact that it is low throughput. Additionally, while FLVs and flow cytometry have been combined to increase throughput (32), labeling of nonhost cells has plagued these experiments even when a thymidine analog (EdU [5-ethyl-2′-deoxyuridine]) was used as an alternative virus-labeling agent (32).

Here we expand upon the above-described use of FLVs to tag host cells by (i) optimizing staining, (ii) incorporating flow cytometry to enable high-throughput detection of infected host cells (and sequencing in future work), and (iii) validating the methods by comparison against infection assays via controlled laboratory experiments. We then applied the optimized viral tagging (VT) method to two marine model virus-host systems—the slow-growing (doubling time ~ 24 h), photoautotrophic cyanobacterium *Synechococcus* and the fast-growing (doubling time ~ 1 h), heterotrophic alphaproteobacterium *Pseudoalteromonas* and their specific viruses—to investigate how they are affected by various infection and growth conditions. These two model systems make ideal candidates for this study, as they are widespread and ecologically important in the oceans (for example, see references 5, 19, and 33 to 37).

**RESULTS**

The objective of this study was to couple the use of FLVs for tagging their host cells (29-31) with high-throughput flow cytometry for rapid, culture-independent assessment of host-virus pairings initially in controlled, laboratory studies (this study) for eventual application in natural complex communities (future work). Here we developed and tested VT on multiple viral isolates from each of two well-studied marine virus-host model systems.

**Optimizing the viral tagging method.** To develop a robust method, we first optimized all three steps of the VT process: (i) viral staining, (ii) viral washing, and (iii) examining the mixture of bacteria and viruses using flow cytometry.

To optimize viral staining, we evaluated three stains (SYBR gold, SYBR green II, and SYBR Safe) and three previously described incubation conditions (80°C for 10 min, room temperature for 10 min, and 4°C overnight [38]) using the cyanophage Syn33 and the host strain *Synechococcus* WH7803 (see Fig. S1 in the supplemental material). SYBR gold at 80°C for 10 min (38) demonstrated the highest fluorescence intensity and least background noise (data not shown); thus, these conditions were chosen as the standard for the rest of the study. As in a previous study (32), we found that the SYBR gold-labeled cyanophages produced a number of plaques on host lawn agarose plates equivalent to that obtained with unlabeled phage (see Fig. S2 in the supplemental material).

We next optimized washing conditions to remove unincorporated dye molecules in the viral suspension that might stain host cellular DNA and result in an upward green fluorescence shift in...
and six heterophages (viruses that infect heterotrophic Pseudoalteromonas bacteria; one lytic myovirus, one lytic podovirus, and four siphoviruses) were challenged with 12 cyanobacterial and 12 Pseudoalteromonas strains in VT, plaque infection, and liquid infection assays. For the 264 possible bacterium-virus pairings (24 bacteria × 11 viruses), VT adsorption results matched infection results from plaque and liquid infection assays with the exception of bacterial pairings involving all four Pseudoalteromonas siphoviruses tested. Thus, when the VT signal was positive, so were the infection results, and vice versa, which suggests that for the bulk of the phage-host pairs under the conditions tested, adsorption equals infection. Infection assays indicated that the four exceptional Pseudoalteromonas siphoviruses four siphoviruses infected their specific known host strains, but their VT signals indicated that adsorption was more promiscuous and included both recognized and unrecognized host strains of Pseudoalteromonas. Monitoring adsorption kinetics with one of these Pseudoalteromonas siphoviruses on multiple host strains showed that it adsorbed to both host and nonhost strains (see Fig. S4 in the supplemental material), consistent with the VT signals observed.

Infection conditions and host cell physiology. We next explored how five hosts and their specific viruses responded to infection conditions that included various virus-to-bacterium ratios (VBR) and host growth phases. All host cells were acclimated through three inoculations and showed reproducible growth curves before use in the physiology experiments (Fig. 3A).

For the first set of experiments, a constant concentration (~10^6 cells ml^-1) of late-logarithmic-growth-phase cells was mixed with various fold numbers of their specific viruses (VBRs = 0.05, 0.1, 0.5, 1, 5, and 10) (Fig. 3B). We found that viruses adsorbed to heterotrophic Pseudoalteromonas cells and cyanobacteria.

Given an optimized VT method, we next sought to document whether a positive VT signal from diverse virus-host systems correlated with the results two existing gold standard infection assays (plaque and liquid infection assays). Five lytic cyanophages (two myo-, two podo-, and one siphovirus) and six heterophages (viruses that infect heterotrophic Pseudoalteromonas bacteria; one lytic myovirus, one lytic podovirus, and four siphoviruses) were challenged with 12 cyanobacterial and 12 Pseudoalteromonas strains in VT, plaque infection, and liquid infection assays (Fig. 2). For the 264 possible bacterium-virus pairings (24 bacteria × 11 viruses), VT adsorption results matched infection results from plaque and liquid infection assays with the exception of bacterial pairings involving all four Pseudoalteromonas siphoviruses tested. Thus, when the VT signal was positive, so were the infection results, and vice versa, which suggests that for the bulk of the phage-host pairs under the conditions tested, adsorption equals infection. Infection assays indicated that the four exceptional Pseudoalteromonas siphoviruses four siphoviruses infected their specific known host strains, but their VT signals indicated that adsorption was more promiscuous and included both recognized and unrecognized host strains of Pseudoalteromonas. Monitoring adsorption kinetics with one of these Pseudoalteromonas siphoviruses on multiple host strains showed that it adsorbed to both host and nonhost strains (see Fig. S4 in the supplemental material), consistent with the VT signals observed.

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rial cells differently. *Pseudoalteromonas* viruses adsorbed nearly proportionally to the amount added, until the cells were 100% virus tagged (VBRs > 2) after only 20 min of mixing. In contrast, the cyanophages never tagged more than ~40 to 50% of their host cells even at high VBRs (up to 10) and lengthened adsorption times (up to 60 and 120 min) (see Fig. S5 in the supplemental material).

In the second set of experiments, cells from three physiological growth phases (early log, late log, and stationary) were challenged with their specific viruses using a constant cell concentration of ~10^6 ml^-1 and VBRs of 1 and 10 for *Pseudoalteromonas*-virus pairs and *Synechococcus*-virus pairs, respectively (Fig. 3C). Again, we found contrasting results between these two virus-host systems; host cell growth phase was relatively constant in

**FIG 3** Percentage of tagged bacteria quantified using VT. Slow-growing photoautotrophic *Synechococcus* strains (WH7803 and WH6501) and fast-growing heterotrophic *Pseudoalteromonas* strains (H100, H71, and H105) were acclimatized through three inoculations (A; note that some cultivation times are in hours and some are in days). These strains were infected by their specific viruses under different infection conditions (B; VBR = 0.05, 0.1, 0.5, 1, 5, or 10) or host growth physiologies (C; logarithmic-, late-logarithmic-, and stationary-phase samples were taken for all three; phases are indicated in the growth curves in panel A; the VBR was 10 for *Synechococcus*-virus pairs and 1 for *Pseudoalteromonas*-virus pairs). In experiment B, percentages of tagged cells were documented at the time point after FLVs were inoculated for 20 min at various VBR, while in experiment C, percentages of tagged cells were documented along the inoculation time of FLVs. Each test was done in triplicate (error bars show standard deviations) at cell concentrations of 10^6 cells ml^-1 and various VBR and a virus-host mixture time of 20 min in the first experiment but at a VBR of 1 or 10 in the host growth physiology experiments.
cyanobacterium-virus pairs but not in *Pseudoalteromonas*-virus pairs, where the percentage of tagged cells was lower in both early log and stationary phases than in the late log phase.

**DISCUSSION**

Here we built on previous studies that specifically label bacterial host cells (including *Cyanobacteria* and *E. coli* in complex microbial communities using FLVs [30, 31, 39]) by coupling flow cytometry analyses to FLV washing conditions, which enabled specific, high-throughput linkage between viruses and the microbes to which they adsorb.

**For ocean viruses, does adsorption equal infection?** For most of the viruses tested (all cyanophages and myo- and podovirus heterophages), VT signals were equivalent to infection assay results, suggesting that adsorption of these ocean viruses might always lead to infection. However, for all four heterosiphoviruses this was not the case. We envision two possible mechanistic explanations for this. First, these viruses may adsorb more promiscuously to nonhost cells. There is, however, some specificity to these interactions, as heterosiphoviruses bound only to *Pseudoalteromonas* strains, not to cyanobacteria (Fig. 2). Some viruses bind irreversibly to a single receptor (e.g., podovirus N4 to NfrA [40]), while other viruses reversibly bind primary receptors that then trigger structural rearrangements of the virus to irreversibly bind a second receptor (e.g., myovirus T4 [41]). Perhaps the *Pseudoalteromonas* siphovirus-positive VT signals capture the reversible adsorption step. Alternatively, adsorption may be specific but not result in lytic infection under our plaque or liquid infection assay conditions. Given that siphoviruses are commonly temperate (i.e., capable of entering the lysogenic or prophage state), though at least one of these lacks a readily identifiable integrase gene (PAS-HS4 [42]), they may form nonlytic infections with some or all of the hosts to which they adsorb. While prophage states present a complex VT signal, such cryptic temperate phage infections remain one of the most challenging aspects of phage biology to study in spite of their documented importance in the wild (e.g., 40 to 60% of marine bacteria contain inducible prophage [43]). If follow-up work demonstrates that these siphoviruses indeed form lysogenic infections, we posit that VT coupled to traditional growth assays may aid in highlighting potential lysogenic infections that are undetectable by conventional infection assays. If such studies suggest the opposite, then one must cautiously interpret the relationship of adsorption and infection for these more challenging virus-host groups.

**Impact of infection conditions on viral tagging.** Modeling suggests that fast-growing bacteria may exist in low abundances in the wild due to intensified viral predation (e.g., 44). Indeed, both mesocosms experiments and observations of ocean viral abundances support these results. In mesocosms, reduced viral abundance correlates with rare bacterial lineages becoming dominant (45), while broad ocean surveys show that viral abundances are commonly negatively correlated with (presumably fast-growing) heterotrophic bacteria and positively correlated with slow-growing cyanobacteria (for example, see references 19, 22, 23, and 46 to 48).

Mechanistically, one could imagine fast growers outcompeting co-occurring microbes for nutrients using a high-affinity nutrient transporter that doubles as a viral receptor. Indeed, marine microbial genomes and metagenomes suggest increased transporter diversity among fast-growing relative to slower-growing bacteria (34, 49-51), and new naming conventions have been proposed, such as “opportunitroph” and “passive oligotroph” (51) and “oligotroph” and “copiotroph” (50). Relevant to the host strains examined here, 18 marine cyanobacterial strains (7 *Synechococcus* and 11 *Prochlorococcus*) and the only *Pseudoalteromonas* strain (*Pseudoalteromonas atlantica* T6c) analyzed in this way are defined as oligotrophs and copiotrophs, respectively (50). In the viral literature (for example, see the review in reference 36), it is posited that such fast-growing microbes are *r*-selected species (52), with high metabolic rates and rapid response times but low natural abundances due to increased top-down pressures from grazers and viruses. In contrast, slower-growing, *K*-selected passive oligotrophs are thought to be more resistant to viral and grazing predation, which was empirically measured for cyanobacteria and their viruses decades ago (48). While they are based upon limited data, and clearly not the focus of this study, our observations here that all *Pseudoalteromonas* cells and only a portion of cyanobacterial cells could be virus tagged are consistent with copiotrophs’ susceptibility and oligotrophs’ resistance to their co-occurring viruses.

**Impact of host cell physiology on viral tagging.** Our observation that the number of *Pseudoalteromonas*-virus pairs, assessed as the percentage of tagged cells, is lower in both early log and stationary phases than late log phase is consistent with previous research with other heterotrophic virus-host systems (53). In these systems (*E. coli* and *Pseudomonas*), increases in viral adsorption with host growth rate is thought to be due to a change in the abundance and structure of the cell surface receptor sites. Perhaps similar mechanisms underpin our observations.

In contrast, host growth phase did not impact the cyanobacterium-virus interaction in one of the virus-host systems studied and only minimally impacted it in the other. Two surveys of wild populations in marine systems reported constant viral adsorption rates irrespective of the physiological status of cells (30, 33), which was later interpreted as a “bet-hedging strategy” (54). Such bet-hedging implies that some viruses may attach to host cells regardless of the host’s physiological state, to maximize the probability of the viral population’s producing progeny under starved or challenging conditions when few host cells are proximal. If such a bet-hedging strategy exists and implicates genetic variation in the pool of viral progeny, then perhaps that may explain why not all cyanobacterial cells could be virus tagged. In the oceans, where cyanobacterial cells are abundant, the fraction that are hosts for any particular virus is likely to be very low, with contact rate estimates, guided by likely imperfect culture-dependent strain-specific phage titer estimates, suggesting that every virus-host contact leads to infection (5, 47, 48, 55). Perhaps the overall cyanophage population engages in bet hedging, given the relatively low abundances of cyanobacteria that serve as host cells in the open ocean environments.

Alternatively, two methodological issues may have resulted in the observed patterns among cyanobacterial virus-host systems. First, we may have failed to accurately recognize the later stages of cyanobacterial host cell growth. We used fluorescence as a proxy for cyanobacterial biomass, which is susceptible to changes in chlorophyll per cell over different growth phases in batch culture (56). While subsequent comparison of fluorescence-based and cell count-based growth curves suggests that we did not assay stationary-phase cells in our VT experiments (see Fig. S6 in the supplemental material), it remains a robust and intriguing obser-
viation that one virus-host pairing showed that early-log-phase cells are virus tagged differently than late-log-phase cells, while the other did not (Fig. 3C). Second, our studies were conducted with host cells grown under continuous light, as opposed to natural, diurnal varying light levels or even on-off light-dark cycles. Light quality impacts adsorption of myovirus S-PM2 to diurnal varying light levels or even on-off light-dark cycles. Light other did not (Fig. 3C). Second, our studies were conducted with cells are virus tagged differently than late-log-phase cells, while the observation that one virus-host pairing showed that early-log-phase

table 1 summary information about the cyanobacterial and pseudoalteromonas strains and the viruses used in this study

| Virus       | Original name | Isolation host | TEM morphology classification | Use in this study | Reference(s) |
|-------------|---------------|----------------|-------------------------------|-------------------|--------------|
| S-SM1       | Synechococcus WH6501 |                 | Myoviridae                    | 1, 3              | 10, 73, 74,  |
| Syn33       | Synechococcus WH7803 |                 | Myoviridae                    | 1, 3              | 48, 75,     |
| Syn3        | Synechococcus WH8012 |                 | Podoviridae                   | 1                 | 48          |
| P-SSP7      | Prochlorococcus MED4 |                 | Podoviridae                   | 1                 | 9, 14, 59,  |
| P-SS8       | Prochlorococcus MIT9313 |               | Siphoviridae                  | 1                 | 77          |
| PSA-HM3     | Pseudoalteromonas H71 |               | Siphoviridae                  | 1, 3              | 76          |
| PSA-HP1     | Pseudoalteromonas H100 |               | Myoviridae                    | 1, 3              | 76          |
| PSA-HS4 (H105/1) | Pseudoalteromonas H105 |             | Podoviridae                   | 1                 | 76          |
| PSA-HS2     | Pseudoalteromonas 13-15 |            | Siphoviridae                  | 1, 2              | 76          |
| PSA-HS6     | Pseudoalteromonas 11-68 |            | Siphoviridae                  | 1, 2              | 76          |
| PSA-HS5     | Pseudoalteromonas H103 |            | Siphoviridae                  | 1                 | 76          |

a viruses used for host range test; 2, viruses used for adsorption kinetics; 3, viruses used for VBR and host growth phases tests.

**MATERIALS AND METHODS**

**Culturing conditions.** Bacteria and viruses used in this study are listed in Table 1. Details on the culture conditions used, see Text S1 in the supplemental material.

**Phage enumeration.** Viral particles were enumerated as previously described (72). In all samples, at least 500 viral or bacterial particles were counted to get accurate numbers for estimating VBR.

**Host range experiments.** Phage isolates were screened (in triplicate) for their ability to grow on 12 cyanobacterial and 12 Pseudoalteromonas hosts using three different methods. First, liquid infection host range analyses were performed in a volume of 300 μl (VBR = 10; concentrations determined by SYBR gold) in 96-well microtiter plates and analyzed using an Appliskan plate reader as described in the “Culturing conditions” section in Text S1 in the supplemental material. Second, plaque assays at a VBR of 10 were used to confirm the observed host range under liquid infection experimental conditions. Host cell suspensions immobilized in agarose (for Prochlorococcus and Synechococcus hosts) or agar (for Pseudoalteromonas) were incubated under host growth conditions either overnight (Pseudoalteromonas) or 3 weeks (Prochlorococcus and Synechococcus). Third, the VT assay was conducted with each phage-host pair as described below but at a VBR of 10 and bacterial concentrations of 10^6 ml^{-1}.

**Adsorption kinetics experiments.** Viruses were mixed with bacteria in late logarithmic phase at concentrations of ~10^6 cells ml^{-1} and a VBR of 0.1 to optimize adsorption of all viruses. Subsamples were taken at 0, 5, 10, and 30 min after virus addition and immediately filtered (0.2 μm) to remove bacteria, and the filtrate was used in plaque assays to determine the number of free viruses in the medium at each time point. The percentage of free viruses was calculated from the decline in free viruses at each time point relative to the number at time zero.

**VT experimental details.** Viral lysates were purified using a cesium chloride step gradient (2 ml of 1.65 g ml^{-1}, 3 ml of 1.4 g ml^{-1}, 3 ml of 1.3 g ml^{-1}, and 1 ml of 1.2 g ml^{-1} in 0.02-μm-filtered and autoclaved SNAX medium). Gradients were ultracentrifuged in a Beckman LM-80M with an SW28 rotor at 24,000 rpm for 4 h at 4°C. The resulting purified virus fraction was dialyzed into modified TM storage buffer (600 mM NaCl, 200 mM Tris, 10 mM MgCl2, 1 mM DTT) and stored at 4°C for 3 weeks and then at -80°C.
100 mM MgCl$_2$, 100 mM Tris-HCl; pH 7.5). Purified, dialyzed viral samples were used for optimizing staining and washing procedures, and all stains, reagents, and buffers were filter sterilized using 0.2-µm-pore-size Atoptin disposable syringe filters (catalog no. 09-926; Whatman Inc.).

See Results for a description of the optimization of the VT stain and washing conditions. To maximize viral recovery from washing steps, two treatments were required: (i) pretreating ultracentrifugal devices (10-kDa cutoff; Nanosep, catalog no. 29300-608; Pall, New York, NY) with 0.5 ml of 0.2-µm-filter-sterilized 1% BSA (catalog no. E531-1.5ML; Bioexpress, Kayville, UT) in phosphate-buffered saline (PBS) incubated for 1 h at room temperature; and (ii) posttreating the ultrafiltration devices by adding back 50 µl TE buffer and sonicating (VWR Signature ultrasonic cleaner; BI500A-DTH) for 3 min using settings of 50 W at 42 kHz.

Stained and washed viruses were mixed with bacteria at the concentrations and ratios desired for flow cytometer analysis as the second component of the VT assay. All VT experiments were done with a negative control, which was prepared identically to the stained and washed virus samples except without viruses; this controlled for the appearance of cells that were false-positive for virus tagging as a result of free dye.

**Flow cytometer analyses.** Bacterial and viral samples were examined using an iCyt Reflection flow cytometer equipped with a 200-mW 488-nm air-cooled solid-state laser. Fluorescence was detected using a 520/40 band pass filter with an amplified photomultiplier tube. Events were detected using a forward scatter trigger, and data were collected in logarithmic mode and then analyzed with WinList 6.0 software (Verity Software House). Fluorescent polystyrene Flow Check microspheres (1 µm yellow-green beads; catalog no. 23517-10; Polysciences Inc., Warrington, PA) were used as an internal standard.

In separate experiments, single cells were imaged and documented using an ImageStreamX imaging flow cytometer with two lasers (488 nm and 980 nm-pore-size H9262 m-filter-sterilized 1% BSA (catalog no. E531-1.5ML; Bioexpress, Kayville, UT) in phosphate-buffered saline (PBS) incubated for 1 h at room temperature; and (ii) posttreating the ultrafiltration devices by adding back 50 µl TE buffer and sonicating (VWR Signature ultrasonic cleaner; BI500A-DTH) for 3 min using settings of 50 W at 42 kHz.

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## SUPPLEMENTAL MATERIAL

Text S1, DOCX file, 0.1 MB.
Figure S1, DOCX file, 0.1 MB.
Figure S2, DOCX file, 0.1 MB.
Figure S3, DOCX file, 0.1 MB.
Figure S4, DOCX file, 0.1 MB.
Text S1, DOCX file, 0.1 MB.

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