

FLV tracer assays

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Abstract

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Guidelines

Note: It is recommended that rates of bacterial production be measured simultaneous to all time points for virus measurements. There are two reasons for this: (1) if measured simultaneously, the researcher can estimate virus production throughout the experiment, rather than relying simply on bacterial production estimates from time zero; and (2) bottle effects are common in small-volume experiments such as these. Measurements of bacterial production throughout the experiment will help the researcher to identify times when bacterial production is heightened (or reduced) due to bottle effects.

Calculation of virus production and removal rates

Production and removal rates are calculated from the equations of Glibert et al. (1982) and Fuhrman (1987). The decay constant, k , is calculated as

$$k = \frac{\ln\left(\frac{R_0}{R_t}\right)}{t}$$

where t is the incubation time and R_0 and R_t are the ratios of labeled to unlabeled viruses at time 0 and time t , respectively. The first two time points in this experiment were t_0 and t_1 . For example, R_0 is FLV_0 , divided by the number of total number of virus particles (stained and unstained), C_0 , at time 0.

The mean specific activity, \bar{R} , is then calculated as

$$\bar{R} = \left(\frac{R_0}{k \times t}\right) \times (1 - e^{-kt})$$

The viral decay or removal rate, D_v , is calculated as

$$D_v = \frac{(FLV_0 - FLV_t)}{(\bar{R} \times t)}$$

where FLV_0 and FLV_t are the concentrations of FLV at t_0 and at time t , respectively.

The viral production rate, P_v , is calculated as

$$P_v = \frac{\ln\left(\frac{R_0}{R_t}\right)}{\ln\left(\frac{C_0}{C_t}\right) \times t} \times (C_0 - C_t)$$

where C_0 and C_t are the concentrations of virus particles at t_0 and time t , respectively.

If the virus abundance does not change over time, then the removal rate is equal to the production rate (and the equation is not used). For each experiment, initial rates (using the first two time points, t_0 and t_1) and overall rates (using the entire time course) of production and decay are calculated. Initial rates of decay/production are closest to in situ rates, as all of the experiments can be started at dusk and held under ambient natural conditions. Overall rates represent decay/production under natural conditions for ~12 h, but samples held in the dark the following morning should not be exposed to natural sunlight.

Estimates of viral-induced bacterial mortality can be calculated using overall rates of virus production, mean viral abundance, mean bacterial abundance and growth rates, and either an empirically measured or estimated burst size. Briefly, virus production rates are divided by the estimated burst size (we used a range from 20 to 50) to determine the number of bacterial cells killed $L^{-1} day^{-1}$. The researcher can divide the number of bacterial cells killed $L^{-1} day^{-1}$ by the rate of bacterial production in cells $L^{-1} day^{-1}$, to determine the portion of the bacterial community killed due to viral lysis. All of the provided calculations assume steady state.

Protocol

FLV tracer experiment

Step 1.

Prepare FLVs for tracer assays.

PROTOCOL

. [Concentration of viruses and preparation of FLVs for tracer assays](#)

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Step 1.1.

Collect up to 20 L using either four 5-L Niskin bottles (or other permutation) or by a triple acid- and sample-rinsed bucket into an acid-rinsed 20-L low-density polyethylene carboy.

Step 1.2.

The sample should be filtered at 5 kPa through a 142-mm-diameter, 0.22- μ m-pore-size Durapore filter to remove bacteria and protists. The virus-sized fraction (material between 0.22 μ m and 30 kDa) is concentrated to 150 mL using a spiral cartridge concentration system (Suttle et al. 1991). Further concentration should be conducted using Centriprep-30 centrifugal concentration units (Millipore) to a final volume of 5 mL.

Step 1.3.

The virus-sized fraction (material between 0.22 μ m and 30 kDa) is concentrated to 150 mL using a spiral cartridge concentration system (Suttle et al. 1991).

Step 1.4.

Further concentration should be conducted using Centriprep-30 centrifugal concentration units (Millipore) to a final volume of 5 mL.

📌 NOTES

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Alternative: The sample can be directly concentrated using a tangential flow filtration spiral cartridge concentration system with either a 30- or 100-kDa cutoff (both have shown excellent recovery rates for marine viruses in past experimental procedures; e.g., Suttle et al. [1991], Breitbart et al. [2002]; GE Healthcare, Inc.) and then filtered using a 0.2- μ m Sterivex-type filter (Millipore) to remove unwanted protists and prokaryotes. If desired, further concentration should be conducted using Centriprep-30 or similar centrifugal concentration units (Millipore) to a final volume of ~5 mL.

Step 1.5.

To each of the virus concentrates, SYBR Green I should be added at a final concentration of 2.5% vol/vol and incubated in the dark for at least 8 h at 4°C.

🕒 DURATION

08:00:00

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Alternative: To each of the virus concentrates, SYBR Gold (Molecular Probes, Inc.) should be added at a final concentration of 2.5% vol/vol and incubated in the dark for at least 4 h at 4°C.

Step 1.6.

After the staining period, the unbound stain can be rinsed away by adding an equal volume of 0.02- μ m filtered seawater (prepared by filtering fresh seawater from the same location through an acid-rinsed, autoclaved Nalgene filtration unit housing a 47-mm, 0.02- μ m Anodisc filter) to the concentrate and centrifuging it in Centriprep-30 ultraconcentration units at 3,000g for 15 min.

🕒 DURATION

00:15:00

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After labeling, the FLVs can be diluted into 1 L of 100-kDa filtrate from the sample site and re-concentrated using tangential flow filtration (TFF). This process is repeated three times to ensure removal of all stain.

Step 1.7.

This rinse is done three times. Each time, the labeled virus particles are resuspended in a total of 5 mL of 0.02- μ m filtered seawater while reusing the same Centriprep-30 unit.

Step 1.8.

The final concentrates should be resuspended in a total of 5 mL of 0.02- μ m filtered seawater.

Step 1.9.

To determine the concentration of viruses in the concentrate, 10 μ L concentrate is diluted to a final volume of 2 mL with 0.02- μ m filtered seawater, filtered through a 0.02- μ m Anodisc, and counted by epifluorescence microscopy under blue excitation (Noble and Fuhrman 1998, Patel et al. 2007).

FLV tracer experiment

Step 2.

Collect seawater samples from the desired location.

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The FLV concentrates should be freshly prepared at each new site and for each new experiment.

FLV tracer experiment

Step 3.

After determining the concentration of the FLVs in the concentrate, and the ambient concentration of viruses in the seawater, the proper amount of FLV concentrate should be added at tracer levels (<10% of original ambient virus concentration) into sample volumes of no less than 400 mL.

📌 NOTES

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The recommended sample volume is 1 L.

FLV tracer experiment

Step 4.

Designate a control treatment.

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Several approaches can be used for controls. Formalin-treated (FT) killed controls consist of 0.02- μ m filtered formalin added at a final concentration of 2%. Heat-treated (HT) controls are seawater boiled for 10 min and then cooled to ambient seawater temperature. The heat treatment denatures active proteins and enzymes and kills most vegetative bacteria (Karner and Rassoulzadegan 1995). If using TFF, the filtrate can also be used as a control treatment. Any measurable rate of disappearance of FLVs in FT, TFF-filtered, or HT treatments is subtracted from that seen in the untreated bottles. Because SYBR Green I stain fades quickly in sunlight, the samples should be incubated at ambient seawater temperatures in the dark. Experiments can be started at dusk so that the beginning of the experiment is done under simulated in situ conditions.

FLV tracer experiment

Step 5.

At each time point, total viral abundance and FLV numbers should be determined in duplicate from small volume subsamples (5–30 mL) taken into sterile, 15- or 50-mL polyethylene tubes.

📌 NOTES

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A suggested framework for the experimental approach might be to sample at time 0 h and at 4, 8, 12, and 16 h after initiation of the experiment.

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The volumes of the subsamples depend on the final concentration of the FLVs, the expected concentration of the native viruses in the sample, and the type of microscope to be used for enumeration.

FLV tracer experiment

Step 6.

Subsamples are immediately fixed with 1% to 2% (final concentration) 0.02- μ m filtered formalin and stored at 4°C.

FLV tracer experiment

Step 7.

Slides should be prepared according to Noble and Fuhrman (1998) or Patel et al. (2007).

📌 NOTES

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Attention should be paid to the mounting solution chosen, as it has been observed that different mounting solutions (p-phenylenediamine, ascorbic acid, ProLong) perform differently in different water sample types and different environments (R. T. Noble, data not published). Breitbart et al. (2004) suggested that samples can be held without adverse fading for up to 2 weeks; however, we do not advocate sample storage for longer than a few days unless absolutely necessary.

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Slides should be prepared immediately after sample collection for best results, particularly to avoid fading of the FLV signal.

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Patel, A., R. T. Noble, J. Steele, I. Hewson, M. Schwallbach, and J. A. Fuhrman. 2007. Virus and prokaryote enumeration from planktonic marine environments by epifluorescence microscopy with SYBR® Green I. *Nat. Protoc.* 2:269-276.

Calculation of virus production and removal rates

Step 8.

Production and removal rates are calculated from the equations of Glibert et al. (1982) and Fuhrman (1987) (see guidelines for equations).

📌 NOTES

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