Wet-mount Method for Enumeration of Aquatic Viruses

B.R. Cunningham, J.R. Brum, S.M. Schwenck, M.B. Sullivan, S.G. John

Abstract

Purpose: This method for the enumeration of aquatic viruses is a low-cost alternative to the commonly used filter-mount method. Briefly, fluorescently-stained samples are wetmounted directly onto slides for epifluorescence microscopy after an optional chemical flocculation concentration step used for samples with anticipated virus concentrations of $<5\times10^7$ viruses mL^{-1} (samples with $>5\times10^7$ viruses mL^{-1} do not require this concentration step prior to analysis). Virus concentration in the wet-mounted sample is determined from the ratio of viruses to microsphere beads, which are added at a known concentration. This wet-mount method for enumerating viruses is significantly less expensive than the filter-mount method (i.e., the cost of microsphere beads per sample is ~500-fold lower than the cost of one filter per sample), and is appropriate for rapid, precise and accurate enumeration of aquatic viruses over a wide range of viral concentrations encountered in field and laboratory samples. The only limitation of this method is that samples with virus concentrations $\leq1\times10^6$ viruses mL^{-1} cannot be enumerated, as the abundance of viruses is too low for efficient enumeration.

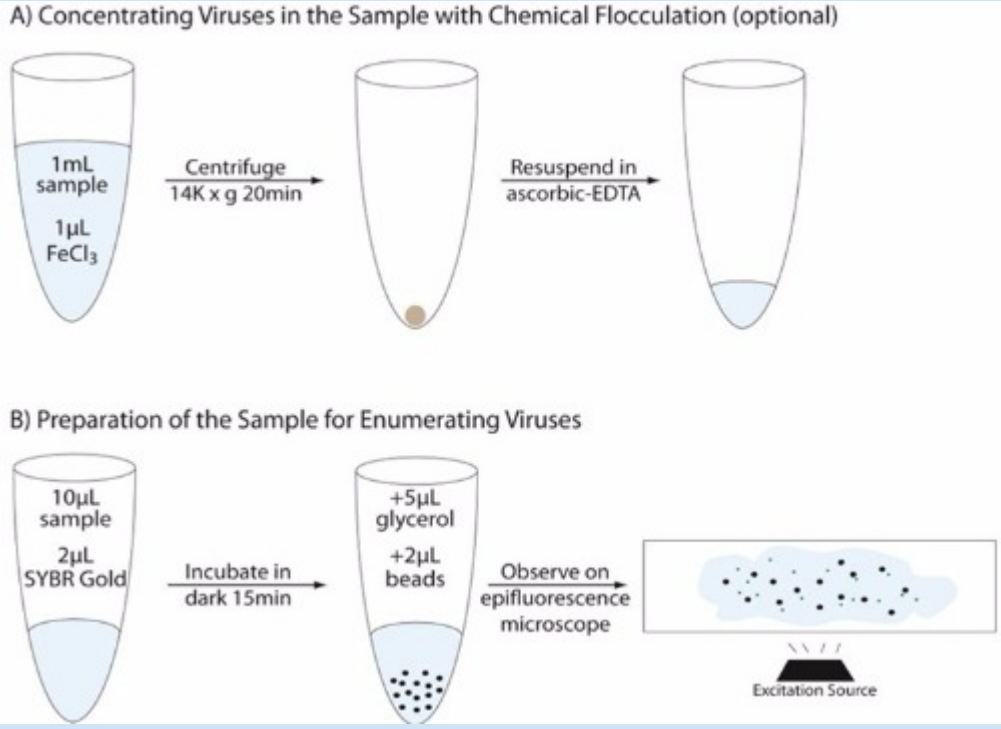

Figure 1. Overview of the wet-mount method for enumeration of aquatic viruses.

Citation: B.R. Cunningham, J.R. Brum, S.M. Schwenck, M.B. Sullivan, S.G. John Wet-mount Method for Enumeration of Aquatic Viruses. **protocols.io**

dx.doi.org/10.17504/protocols.io.c8pzvm

Published: 07 Jan 2016

Guidelines

Optional Viral Concentration Method:
Samples with <5×107 viruses mL-1 must be concentrated using this iron chloride flocculation method adapted from John et al. [1] prior to enumeration of viruses with the wet-mount method. The steps for this procedure are displayed in Figure 1A.

Materials Required:
- 1.5 mL microcentrifuge tubes
- microcentrifuge
- vortexer
- phosphate buffered saline (PBS)
- iron chloride ($FeCl_3 \cdot 6H_2O$)
- ultrapure water
- magnesium EDTA (Mg_2EDTA)
- ascorbic acid
- sodium hydroxide (NaOH)

Reagent Preparation:
Iron Chloride Solution
Ascorbic-EDTA Buffer

Virus Counting Procedure
This part of the protocol starts with either 10 µL of unconcentrated sample (if the anticipated virus concentration is >5×107 viruses mL-1) or 10 µL of concentrated, resuspended sample (see above). The steps for this procedure are displayed in Figure 1B.

Materials Required:
- epifluorescence microscope (1000X magnification and ~495 nm excitation)
- vortexer
- 1.5 mL microcentrifuge tubes
- isopropanol
- glass slides (25x75 mm)
- cover slips (24x60 mm)
- microsphere silica beads (Bangs Laboratories, 2.34 µm diameter, conc. 7.845x109 beads/mL, Cat. # SS04N/4186, Inv. # L060320A)
- glycerol
- phosphate buffered saline (PBS)
- SYBR Gold (Invitrogen)
- Kimwipes
- ascorbic acid (if analyzing unconcentrated samples)

Reagent Preparation:
SYBR Gold Working Stock
Working Bead Solution
Ascorbic Acid Antifade Solution

Related References

1. John SG, Mendez CB, Deng L, Poulos B, Kauffman AKM, Kern S, Brum J, Polz MF, Boyle EA, Sullivan MB (2011) A simple and efficient method for concentration of ocean viruses by chemical flocculation. Environmental Microbiology Reports, 3:195–202. doi:10.1111/j.1758-2229.2010.00208.x

Protocol

Optional Viral Concentration Method

Step 1.

Place 1 mL of sample into a 1.5 mL microcentrifuge tube.

Optional Viral Concentration Method

Step 2.

Add 1 µL Iron Chloride Solution and vortex to mix.

AMOUNT

1 µl Additional info:

PROTOCOL

. Iron Chloride Solution

CONTACT: VERVE Team

Step 2.1.

Dissolve $FeCl_3 \cdot 6H_2O$ into ultrapure water to form a solution of 10 g Fe L-1 (i.e., 0.484 g $FeCl_3 \cdot 6H_2O$ dissolved in 10 mL of ultrapure water).

Optional Viral Concentration Method

Step 3.

Centrifuge the sample at 14K RCF for 20 minutes.

DURATION

00:20:00

Optional Viral Concentration Method

Step 4.

Remove supernatant using a pipette, leaving a small, undisturbed pellet of Fe oxyhydroxides behind (Figure 2).

Figure 2. Fe oxyhydroxide pellet after removal of supernatant.

NOTES

James Thornton Jr 28 Jul 2015

Note: Removing all of the supernatant is not critical, as a tiny bit remaining will not affect results.

Optional Viral Concentration Method

Step 5.

Dissolve the pellet in 10 µL of Ascorbic-EDTA Buffer, creating a 100-fold concentration of the original sample. Vortex and then pipette up and down to ensure complete dissolution.

AMOUNT

10 µl Additional info:

PROTOCOL

. Ascorbic-EDTA Buffer

CONTACT: VERVE Team

NOTES

James Thornton Jr 28 Jul 2015

Note: A larger volume of buffer may be used to speed pellet dissolution or decrease concentration factor. If a larger volume is used in this step, solution volumes in subsequent steps must be increased accordingly.

Step 5.1.

Combine equal parts of 0.4 M Mg2EDTA and 0.8 M ascorbic acid, adjust with 10 N NaOH to reach a pH of 6-7.

Step 5.2.

Prepare fresh within 48 hours of use

Optional Viral Concentration Method

Step 6.

Vortex and then pipette up and down to ensure complete dissolution.

Virus Counting Procedure

Step 7.

Combine 10 µL sample (concentrated or unconcentrated) and 2 µL SYBR Gold Working Stock, vortex to mix, and place in dark for 15 minutes.

AMOUNT

2 µl Additional info:

DURATION

00:15:00

PROTOCOL

. SYBR Gold Working Stock

CONTACT: VERVE Team

Step 7.1.

Dilute SYBR Gold (Invitrogen; 10,000X stock) into PBS (phosphate buffered saline) to prepare 1000x solution. Prepare fresh daily. Store in dark between uses.

Virus Counting Procedure

Step 8.

If sample is unconcentrated, add 1 µL of Ascorbic Acid Antifade Solution.

AMOUNT

1 µl Additional info:

PROTOCOL

. Ascorbic Acid Antifade Solution

CONTACT: VERVE Team

Step 8.1.

Dissolve 0.1 g ascorbic acid into 1 mL PBS creating a 10% (wt/vol) solution. Prepare fresh daily.

Virus Counting Procedure

Step 9.

Add 5 µL of glycerol to stained sample and vortex to mix.

Virus Counting Procedure

Step 10.

Add 2 µL Working Bead Solution to sample.

AMOUNT

2 µl Additional info:

PROTOCOL

. Working Bead Solution

CONTACT: VERVE Team

NOTES

James Thornton Jr 28 Jul 2015

Note: The Working Bead Solution must be thoroughly mixed by vortexing prior to adding it to the sample so that the appropriate number of beads are added to the sample.

Step 10.1.

Dilute stock bead solution 10-fold into PBS to obtain a concentration of 108 beads mL-1 ; store at 4°C.

Virus Counting Procedure

Step 11.

Clean glass slides and cover slips with isopropanol and Kimwipes.

Virus Counting Procedure

Step 12.

Thoroughly mix the sample/bead mixture by pipetting up and down, then immediately pipette 10 µL of it onto a glass microscope slide. Place a coverslip over the mixture and avoid trapping air under the coverslip.

Virus Counting Procedure

Step 13.

Place a coverslip over the mixture and avoid trapping air under the coverslip.

Virus Counting Procedure

Step 14.

View viruses under 495 nm excitation at 1000X magnification using an epifluorescence microscope. Count the number of viruses in one defined field of view.

Virus Counting Procedure

Step 15.

Once complete, switch off the excitation and turn on the white light of the microscope to count the beads in the same field of view (Figure 3).

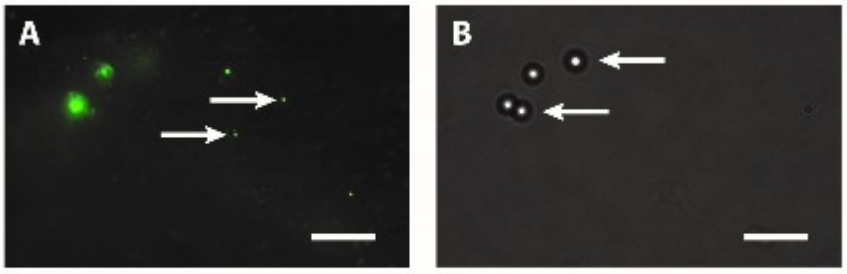

Figure 3. Epifluorescence image of a wet-mounted sample (A) includes arrows pointing to two of the viruses. Under white light (B), beads are visible in the same field of view, with arrows pointing to two of the beads. Scale bars for each image are 10 µm.

Virus Counting Procedure

Step 16.

Repeat Step 14 & 15 by counting viruses and beads in multiple fields until at least 100 of each have been counted.

Virus Counting Procedure

Step 17.

The concentration of viruses can then be determined with the following equation:

$$C_{virus} = \frac{n_{virus}}{n_{beads}} * \frac{v_{beads}}{v_{sample}} * C_{beads}$$

NOTES

James Thornton Jr 31 Jul 2015

cvirus= * * cbeads

where:

cvirus = concentration of viruses (viruses ml -1)

nvirus = total number of viruses counted in all fields

nbeads = total number of beads counted in all fields

vbeads = volume of Working Bead Solution added

vsample = volume of sample used (if the sample has been concentrated with iron chloride flocculation, use the pre-concentration sample volume here)

cbeads = concentration of beads in Working Bead Solution (beads mL -1)

James Thornton Jr 31 Jul 2015

cvirus= * * cbeads

where:

cvirus = concentration of viruses (viruses ml -1)

nvirus = total number of viruses counted in all fields

nbeads = total number of beads counted in all fields

vbeads = volume of Working Bead Solution added

vsample = volume of sample used (if the sample has been concentrated with iron chloride flocculation, use the pre-concentration sample volume here)

cbeads = concentration of beads in Working Bead Solution (beads mL -1)

Virus Counting Procedure

Step 18.

Prepared samples can be stored at -20ºC either in the microcentrifuge tube (i.e., after completing Step 4) or after mounted on slides (i.e., after completing Steps 12&13) with no significant change in the calculated virus concentration.

NOTES

James Thornton Jr 28 Jul 2015

Note: these storage conditions have currently been validated for a time period of 7 days.