

Wet-Mount Method for Enumeration of Aquatic Viruses

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Cunningham, B.R., J.R. Brum, S.M. Schwenck, M.B. Sullivan, & S.G. John (*in review*).

An inexpensive, accurate and precise wet-mount method for enumerating aquatic viruses.

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 wet-mounted directly onto slides for epifluorescence microscopy after an optional chemical flocculation concentration step used for samples with anticipated virus concentrations of $<5 \times 10^7$ viruses mL^{-1} (samples with $>5 \times 10^7$ 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 $\leq 1 \times 10^6$ viruses mL^{-1} cannot be enumerated, as the abundance of viruses is too low for efficient enumeration.

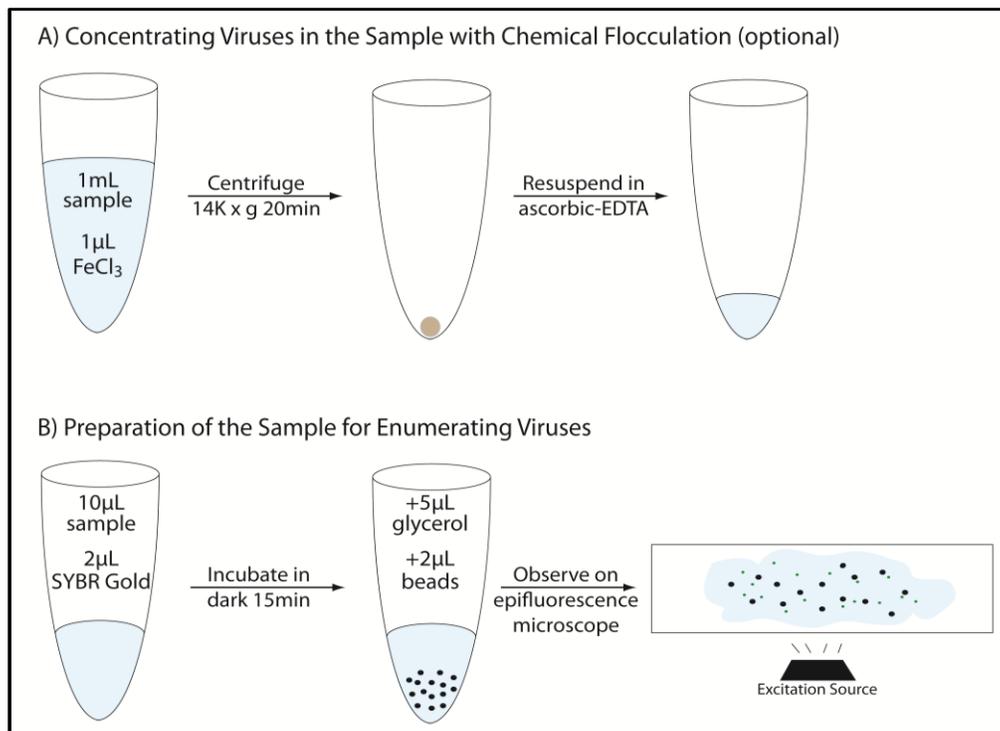


Figure 1. Overview of the wet-mount method for enumeration of aquatic viruses.

Optional Viral Concentration Method

Samples with $<5 \times 10^7$ 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 ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$)
- ultrapure water
- magnesium EDTA (Mg_2EDTA)
- ascorbic acid
- sodium hydroxide (NaOH)

Reagent Preparation:

Iron Chloride Solution:

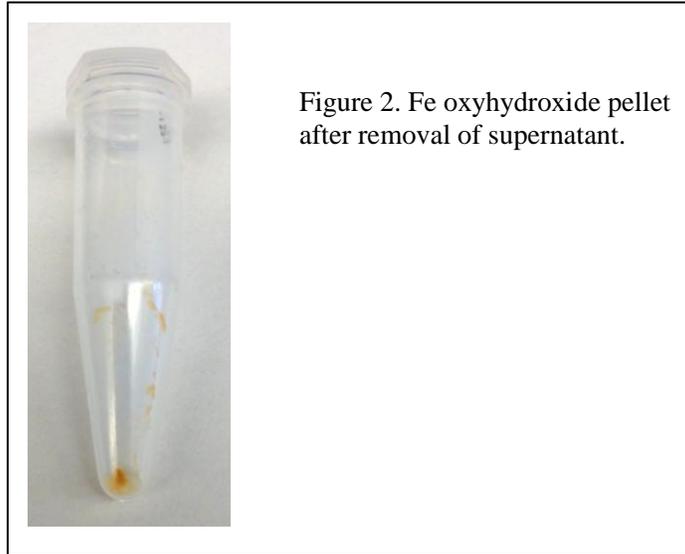
- Dissolve $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ into ultrapure water to form a solution of 10 g Fe L^{-1} (i.e., $0.484 \text{ g FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolved in 10 mL of ultrapure water).
- Solution has expired if a cloudy precipitate forms.

Ascorbic-EDTA Buffer:

- Combine equal parts of $0.4 \text{ M Mg}_2\text{EDTA}$ and 0.8 M ascorbic acid, adjust with 10 N NaOH to reach a pH of 6-7.
 - $0.4 \text{ M Mg}_2\text{EDTA}$ prepared by dissolving $1.434 \text{ g Mg}_2\text{EDTA}$ in 10 mL ultrapure water
 - 0.8 M ascorbic acid prepared by dissolving 1.409 g ascorbic acid in 10 mL ultrapure water
- Prepare fresh within 48 hours of use.
- Note: An alternative Ascorbate-EDTA Buffer can be made with MgCl_2 and Na_2EDTA if Mg_2EDTA is unavailable:
 - Combine equal parts of 0.125 M Tris-base, $0.1 \text{ M Na}_2\text{-EDTA}$ dihydrate, 0.2 M MgCl_2 hexahydrate, and 0.2 M ascorbic acid, adjust with 10 N NaOH to reach a pH of 6-7.
 - 0.125 M Tris-base prepared by dissolving 0.151 g Tris-base in 10 mL ultrapure water
 - $0.1 \text{ M Na}_2\text{-EDTA}$ dehydrate prepared by dissolving 0.372 g $\text{Na}_2\text{-EDTA}$ dehydrate in 10 mL ultrapure water
 - 0.2 M MgCl_2 hexahydrate prepared by dissolving 0.407 g MgCl_2 hexahydrate in 10 mL ultrapure water
 - 0.2 M ascorbic acid prepared by dissolving 0.352 g ascorbic acid in 10 mL ultrapure water

Steps:

1. Place 1 mL of sample into a 1.5 mL microcentrifuge tube.
2. Add 1 μL Iron Chloride Solution and vortex to mix.
3. Centrifuge the sample at $\sim 14\text{K}$ RCF for 20 minutes.
4. Remove supernatant using a pipette, leaving a small, undisturbed pellet of Fe oxyhydroxides behind (Figure 2).
 - Note: Removing all of the supernatant is not critical, as a tiny bit remaining will not affect results.



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.
 - 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.

Virus Counting Procedure

This part of the protocol starts with either 10 μL of unconcentrated sample (if the anticipated virus concentration is $>5 \times 10^7$ 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. 7.845×10^9 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:

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

Working Bead Solution:

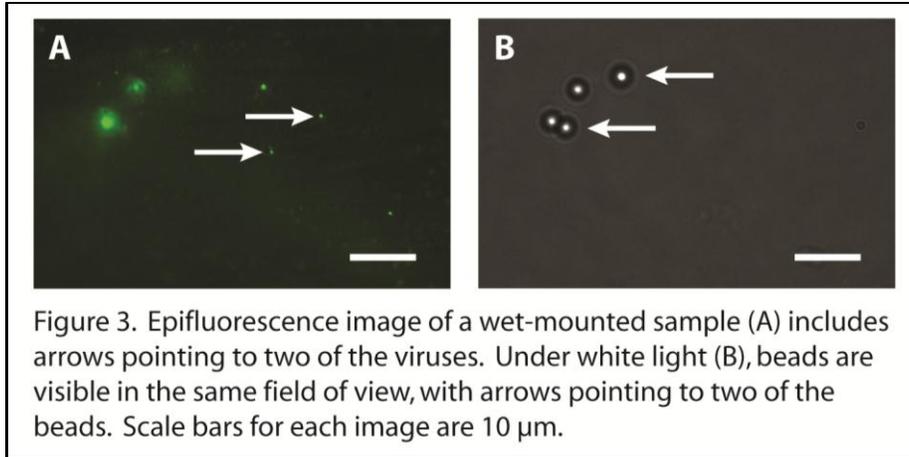
- Dilute stock bead solution 10-fold into PBS to obtain a concentration of $\sim 10^8$ beads mL^{-1} ; store at 4°C .

Ascorbic Acid Antifade Solution:

- Dissolve 0.1 g ascorbic acid into 1 mL PBS creating a 10% (wt/vol) solution.
- Prepare fresh daily.
- This solution is needed only if analyzing unconcentrated samples.

Steps:

1. Combine 10 μL sample (concentrated or unconcentrated) and 2 μL SYBR Gold Working Stock, vortex to mix, and place in dark for 15 minutes.
2. If sample is unconcentrated, add 1 μL of Ascorbic Acid Antifade Solution.
3. Add 5 μL of glycerol to stained sample and vortex to mix.
4. Add 2 μL Working Bead Solution to sample.
 - 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.
5. Clean glass slides and cover slips with isopropanol and Kimwipes.
6. 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.
7. View viruses under ~ 495 nm excitation at 1000X magnification using an epifluorescence microscope. Count the number of viruses in one defined field of view. 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).



8. Repeat Step 7 by counting viruses and beads in multiple fields until at least 100 of each have been counted.

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

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

where:

C_{virus} = concentration of viruses (viruses mL^{-1})

n_{virus} = total number of viruses counted in all fields

n_{beads} = total number of beads counted in all fields

v_{beads} = volume of Working Bead Solution added

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

C_{beads} = concentration of beads in Working Bead Solution (beads mL^{-1})

10. Prepared samples can be stored at -20°C either in the microcentrifuge tube (i.e., after completing Step 4) or after mounted on slides (i.e., after completing Step 6) with no significant change in the calculated virus concentration. Note: these storage conditions have currently been validated for a time period of 7 days.

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.