Enumerating algal viruses by flow cytometry

Amy Zimmerman

Abstract

Purpose: To quantify virus-like particles in culture or environmental samples.

Summary: Host cells and virus-like particles are enumerated from the same glutaraldehyde-fixed sample. For virus counts, an aliquot is diluted in 0.02-µm-filtered TE buffer prior to staining with SYBR Green I nucleic acid dye to improve staining efficiency and ensure an appropriately low event rate in the instrument. An unstained aliquot of sample is run for host cell counts.

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Guidelines

Principle

Combining high-fluorescence-yield nucleic-acid-specific stains with flow cytometry has significantly improved the speed of analysis and accuracy of counting virus-like particles. Robust quantification using this method relies on reproducible staining efficiency as well as appropriate instrument parameters to ensure all events of interest are on scale. This protocol builds on the work of Brussaard (2004) *Appl Environ Microbiol*. (doi: 10.1128/AEM.70.3.1506-1513.2004), optimizing conditions specifically for enumeration of Prasinoviruses.

Before start

Equipment and Materials.

Equipment:

BD Influx Flow Cytometer
Tube racks
10, 20, 200, and 1000 µL pipettes
Ice bucket

Light-safe box for incubation

Timer

Vortexer

Materials

10, 20, 200, and 1000 µL filter tips

High Intensity Green 0.5 µm fluorescent microspheres/beads (Polysciences #23516)

Plain YG 0.75 µm fluorescent microspheres/beads (Polysciences #17153)

1.7 and 2.0 mL microcentrifuge tubes

0.02-µm-pore-size sterile Anotop 25 Syringe Filter Plus (GE Healthcare #6809-4102)

0.2-µm-pore-size sterile Puradisc 25 PES Syringe Filter (GE Healthcare #6780-2502)

1X TE buffer pH 8.0 (Fisher BioReagents #BP1338-1), 0.02-µm-filtered

1X PBS (BioPioneer #MB1001), 0.2-µm-filtered

5 mL round bottom polypropylene tubes

Reagents and Solutions.

25% EM-grade glutaraldehyde:	Prepare small aliquots (1 mL) to avoid cross-contamination and store at 4°C protected from light.
0.02-µm-filtered TE buffer:	Dilute 0.5 mL 100X molecular grade Tris-EDTA (pH 8.0) in 49.5 mL MilliQ water. In a hood, filter through a 0.02-µm-pore-size sterile Anotop 25 syringe filter into sterile 15 mL tubes. Use within a few days of opening.
SYBR Green I working stock:	Dilute concentrated 10,000X SYBR stock (Invitrogen # S7563 or S7567) to 100X in molecular grade DMSO (Sigma D8418) (i.e., 10 µl concentrated SYBR in 990 µL DMSO). Prepare small aliquots (30-50 µL) and discard after thawing. Staining efficiency decreases from freeze/thaw cycles (stable for 2-3 thaws). Briefly spin stock solution (20,000 x *g*) to reduce noise levels.
Green 0.5 µm beads:	Prepare 2° stock by diluting 1 drop in 10 mL MilliQ water. Then prepare a 3° (working) stock by diluting 1 mL of 2° stock in 4 mL MilliQ.
YG 0.75 µm beads:	Prepare 2° stock by diluting 1 drop in 10 mL MilliQ water. Then prepare a 3° stock by diluting 200 µL of 2° stock in 10 mL MilliQ. Finally, prepare a 4° (working) stock by diluting 1 mL of 3° stock in 9 mL MilliQ

Dilution Table.

Staining efficiency depends on having an appropriately diluted virus sample.

Dilution	Sub-dilutions	Sample Vol	TE Vol	*Calc Check*
1:40,000	1:10	10	90	*10*
	1:10	10	90	*10*
	1:4	25	75	*4*
	1:100	5	495	*100*
1:25,000	1:10	10	90	*10*
	1:10	10	90	*10*
	1:2.5	40	60	*2.5*
	1:100	5	495	*100*
1:20,000	1:10	10	90	*10*
	1:10	10	90	*10*
	1:2	50	50	*2*
	1:100	5	495	*100*
1:15,000	1:15	6	84	*15*
	1:10	10	90	*10*
	1:100	5	495	*100*
1:10,000	1:10	10	90	*10*
	1:10	10	90	*10*
	1:100	5	495	*100*
1:8,000	1:8	12.5	87.5	*8*
	1:10	10	90	*10*
	1:100	5	495	*100*
1:5,000	1:5	20	80	*5*
	1:10	10	90	*10*
	1:100	5	495	*100*
1:4,000	1:4	25	75	*4*
	1:10	10	90	*10*
	1:100	5	495	*100*
1:2,500	1:2.5	40	60	*2.5*
	1:10	10	90	*10*
	1:100	5	495	*100*

	1:2	50	50	*2*
1:2,000	1:10	10	90	*10*
	1:100	5	495	*100*
1:1,500	1:15	6	84	*15*
	1:100	5	495	*100*
1:1,000	1:10	10	90	*10*
	1:100	5	495	*100*
1:500	1:5	20	80	*5*
	1:100	5	495	*100*
1:250	1:2.5	40	60	*2.5*
	1:100	5	495	*100*
1:100	1:10	10	90	*10*
	1:10	50	450	*10*

Protocol

FCM Sample Collection

Step 1.

FCM Sample Collection Fix samples of virus and TE buffer (i.e., for background noise) as described below.

1. Transfer 600 µL of culture sample to a sterile 1.2 mL cryovial tube.
2. Add 6 µL 25% glutaraldehyde (0.25% final concentration) and gently vortex to mix.
3. Aliquot 200 µL to two additional cryovials (i.e., triplicate samples).
4. Transfer 3 x 1 mL of 0.02-µm-filtered TE buffer to triplicate sterile 1.2 mL cryovials.
5. Add 10 µL 25% glut to each tube and gently vortex to mix
6. Snap cryovials into cryocanes and incubate at 4°C for 30 minutes in the dark.
7. Flash freeze in liquid nitrogen.
8. Store at -80°C until analysis.

Virus Dilution and Counting

Step 2.

Virus Dilution and Counting Dilute and stain a sample aliquot for viruses immediately after thawing. Store thawed sample on ice. An aliquot can be run for host cell counts while the diluted virus sample is incubating.

1. Dilute sample in 0.02-µm-filtered 1X TE buffer according to the Dilution Table.

   NOTE: For a new sample, prepare multiple dilutions to test and determine the most appropriate dilution factor. Stagger these dilutions/staining by 5 minutes to allow for sample loading and back-flushing between dilution tests.

2. Prepare a 1.7 mL microcentrifuge tube for staining by adding 2.5 µL of 100X SYBR Green I nucleic acid stain (this can be pre-aliquoted and kept in the dark at room temperature).
3. Add 497.5 µL of the diluted sample to the staining tube (0.5X SYBR Green I final conc) and mix

by pipetting and gentle vortexing.
4. Incubate the diluted/stained sample for 15 minutes in the dark at room temperature.
5. After incubation, transfer 480 µL of the stained sample to a 5 mL round bottom tube.
6. Add 10 µL 3° Grn and 10 µL 4° YG beads to the 5 mL tube.
7. Run immediately on HIGH voltage settings: Trigger = **520**, TFSC = **40**, PFSC = **27**, SSC = 40, 520 = 40, 572 = 40, 692 = 40. Load sample for 2 min, run for 2-4 min (depending on concentration), and back-flush for 2 min.

Host Cell Counts

Step 3.

Host Cell Counts Run an unstained aliquot of sample for host cell counts while the diluted virus sample is incubating.

1. Prepare a 5 mL round bottom tube with 10 µL 3° Grn and 10 µL 4° YG beads.
2. Depending on the host cell concentration, add 50 or 100 µL sample to the 5 mL tube.
3. Bring volume up to 500 µL by adding 430 or 380 µL 1X PBS to the 5 mL tube.
4. Run immediately on LOW voltage settings: Trigger = **TFSC**, TFSC = **27**, PFSC = **19**, SSC = 40, 520 = 40, 572 = 40, 692 = 40. Load sample for 2 min, run for 2-4 min (depending on concentration), and back-flush for 2 min.

Data Processing

Step 4.

Data Processing Analyze listmodes for host cell and virus counts using WinList 3D (Ver 7) software.

1. Open or create a Protocol file (*.wlx) to identify host cells by chlorophyll fluorescence (692 ± 35 nm filter, trigger) and Forward Angle Light Scatter (FALS).
2. Gate out all beads before drawing a region around the healthy cell population.
3. Under FILE, select “Next FCS File” to load a listmode (*.fcs) using these parameters.
4. Add results to the appropriate database (*.dbs) before opening the next listmode.
5. Save the database as a plain text file (*.txt) before closing the Protocol.
6. Open or create a separate Protocol file to identify virus-like-particles (VLPs) by green fluorescence (520 ± 35 nm filter, trigger) and FALS.
7. Gate out all beads before drawing 3 regions: (1) main virus population, (2) high-fluorescence virus particles, and (3) low-fluorescence virus particles.
8. Under FILE, select “Next FCS File” to load a listmode using these parameters.
9. Add results to the appropriate database before opening the next listmode.
10. Save the database as a plain text file before closing the Protocol.

Calculations

Step 5.

CalculationsCorrect virus counts for background noise from TE or uninfected controls.

1. Generate separate Excel spreadsheets for host cell and VLP counts so that each includes the following tabs: (1) all parameters from the Influx runs, (2) information on gates and regions used in WinList, (3) imported results from the appropriate Database, (4) calculations from events per region.
2. Calculate cells or VLPs per mL from events per region by accounting for the volume run and the

sample dilution. Calculations for host cell abundances are complete at this point.

3. Reevaluate the viral cytograms to determine in which samples the low-fluorescence VLPs appears.
4. Calculate “Total VLP mL^{-1}” for each sample by adding the counts from the main virus population with the high-fluor VLPs, and the low-fluor VLPs where appropriate.
5. For virus counts *NOT* associated with an infection time course (i.e., virus master stock), correct the “Total VLP mL^{-1}” count using the background noise values from TE blank samples (i.e., non-virus particles that show up in the main, low-fluor, or high-fluor WinList regions).
6. For all time course samples, determine whether there is a relationship between time and background noise in the uninfected control samples (i.e., non-virus particles that show up in the main, low-fluor, or high-fluor WinList regions) by regressing the counts for each population against time.
7. If the slope of the regression is significantly different from zero, use time-specific background noise values to correct the Total VLP counts from the corresponding infected treatment/virus population: for each individual time point, use the average of all uninfected control replicates for that treatment/virus population combo as the correction value.
8. If the slope of the regression is *NOT* significantly different from zero, correct the Total VLP counts from the infected treatment/virus population using the overall average (all replicates, all time points) of the regression-predicted values from the corresponding uninfected control treatment/virus population as the correction value.