MPN (Most Probable Number) assay for infectivity of algal viruses Version 3

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Abstract

Purpose: To quantify infectious viruses and evaluate the infectivity of a viral sample.

Summary: 50 µL of serially-diluted (10^{-3} to 10^{-10}) virus sample is added to 150 µL of exponentially growing host cells in triplicate 96-well microplates and incubated at normal growth conditions for ~2 weeks. Cell lysis is assessed every few days qualitatively by visual inspection and quantitatively by measuring optical density on a microplate reader. The MPN of infective viruses in each concentrate is estimated from the proportion of virus-positive (i.e., lysed) wells using the MPN_ver4.xls Excel spreadsheet from Jarvis, B., Wilrich, C., and P.-T. Wilrich (2010), *Journal of Applied Microbiology* **109** (2010), 1660 – 1667. Percent infectivity is then calculated by comparing the MPN-estimated abundance of infective viruses to the abundance of virus-like particles (VLPs) determined by flow cytometry analysis.

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Guidelines

Principle

The abundance of infective viruses in a sample can be estimated using a serial dilution approach. In theory, a single lytic virus can lyse an entire population of sensitive host cells, given enough time. The MPN approach uses a series of log-based dilutions of a virus sample added to aliquots of an exponentially-growing host culture. The abundance of infectious viruses can be estimated if the range of dilutions used includes when the mean viruses per aliquot ≈ 1. An estimate of infective units is calculated with software using the number of lysed host aliquots at each dilution level. Replication (and increased sensitivity) is achieved by using 96-well microplates (8 replicate wells per plate x triplicate plates = 24 replicates per dilution). The estimated MPN is then compared to the total virus-like particle (VLP) count determined by flow cytometry to calculate infectivity (i.e., the proportion of infective viruses in the sample).

Host Preparation

Initiate a host culture several days prior to assay setup to ensure exponential growth. Avoid transferring cells on the day-of or the day-before assay setup to minimize disturbance. Transfer/dilute host culture to pre-determined mid-exponential density daily (semi-continuous culture), including the day of assay setup.

Example *Ostreococcus lucimarinus* (CCMP2972A) host preparation:

Growth conditions: 18°C, 14:10 hour light:dark cycle, light irradiance ~100 µE m^{-2} s^{-1}

Growth media: L1 with natural seawater base (67-135)

Exponential range: $7x10^5$ – $2x10^7$ cells mL^{-1}

Day	**1**	**2**	**3**	**4**	**5**
Initial density (mL^{-1})	$5x10^7$		$1.5x10^7$	$9x10^6$	$1.2x10^7$
Growth rate (d^{-1})	NA		0.55	0.50	0.84
Dilution/Transfer	Transfer	*Skipped*	Transfer	Dilution	Dilution
Final density (mL^{-1})	$5x10^6$		$5x10^6$	$5x10^6$	$5x10^6$ **(use for assay)**
Volume (mL)	15		25	44*	104*

**Volume varies depending on growth*

Before start

Equipment and Materials.

Per virus sample tested:

5 mL round bottom polypropylene tubes	10
Culture medium	25 mL
96-well microplates	3
Exponentially-growing host culture	40 mL
Virus sample	0.2 mL (dilution) + 0.5 mL (FCM)
Sterile 50 mL sample reservoirs	2-3
Cryovials	2-3

Additional materials:

1000 uL pipette + filter tips
200 uL multichannel pipette + tips
Tube rack
Vortexer
Optical microplate reader
25% glutaraldehyde (EM grade)

Protocol

Virus Dilution Series

Step 1.

Virus Dilution Series

1. Label a series of 5 mL round bottom tubes from 10^{-1} to 10^{-10}.
2. Aliquot 1.8 mL culture media to each tube.
3. Dilute 200 µL virus sample into the "10^{-1}" tube and vortex to mix.
4. Use a clean pipette tip to transfer 200 µL from the "10^{-1}" tube to the "10^{-2}" tube and vortex to mix.
5. Repeat serial dilution to 10^{-10}.
6. Transfer 500 µL virus sample to a sterile 1.2 mL cryovial (to preserve for FCM counts).
7. In the chemical hood, add 5 µL 25% glutaraldehyde (0.25% final concentration) and gently vortex to mix.
8. Aliquot 250 µL to a duplicate cryovial and snap cryovials into cryocanes.
9. Incubate at 4°C for 30 minutes in the dark.
10. Flash freeze in liquid nitrogen and store at -80°C until analysis.

Plate Setup

Step 2.

Plate Setup

1. Label triplicate 96-well microplates as follows:

Column	1	2	3	4	5	6	7	8	9	10	11	12
Label	-3	-4	-5	-6	-7	-8	-9	-10	Control	(empty)		

2. Pour remaining culture medium into a sterile sample reservoir.
3. Use a multichannel pipette to add 50 µL medium to all wells in Column 9 ("Control") on all plates.
4. Discard unused medium and fill sample reservoir with host culture.

5. Add 150 µL host cells to all wells in Columns 1-9 on all plates. Discard remaining culture.
6. Pour "10^{-10}" viral dilution into new sterile sample reservoir.
7. Use a multichannel pipette to add 50 µL 10^{-10}-diluted virus sample to all wells in Column 8 on all replicate plates.
8. Discard remaining 10^{-10}-diluted virus sample and pour "10^{-9}" viral dilution into the same sample reservoir.
9. Use the same pipette tips to add 50 µL 10^{-9}-diluted virus sample to all wells in Column 7 on all plates.
10. Repeat additions of diluted virus samples from most dilute to most concentrated (moving from right to left across the microplates).
11. After final 10^{-3}-diluted virus sample is added to plates, measure optical density for T0 in plate reader (as described below) and incubate (unstacked to prevent shading) at standard growth conditions for 2 weeks, measuring growth every few days.

Data Collection

Step 3.

Data Collection

1. Turn on the Molecular Devices SpectraMax 340PC plate reader.
2. Log into the attached computer and open the SoftMax Pro 6 software.
3. Open or create a new "Basic Endpoint" protocol file (.spr).
4. Rename the experiment appropriately and configure a plate with the following settings:

- Read Type: Endpoint
- Wavelength: 750 nm
- Plate Type: 96-well standard clear bottom
- Read Area: All
- Pathcheck: Calibration on
- Shake: Once for 3 sec.
- More settings: Column priority

5. Add "New plates" as needed so there is one for each replicate plate in your assay (settings will copy to these new plates within the same experiment).
6. If the SpectraMax doesn't automatically connect, click on the instrument icon in the top left corner and manually select it from the menu.
7. Select the plate to be read in the software, and place the corresponding plate on the plate reader drawer (with Column 1 closest to the instrument).
8. Remove the plate lid and select "Read" to read the plate.
9. Read each plate individually, and copy the data (must right-click to do this) into an Excel spreadsheet.
10. Create a new data file (.sda) from the same master protocol for each time point.
11. Visually inspect plates and record observations.

Calculations

Step 4.

Calculations

1. Open the MPN_ver4.xls Excel spreadsheet from

http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html.

2. Enter the name and date of the experiment.
3. For “Total no. of test series,” enter 1 for each virus sample tested (i.e., two viruses tested = 2 total test series).
4. Enter “5” for “Max no. of dilutions” and hit Enter to generate a data table for each virus sample tested.
5. Enter the appropriate data in the yellow cells of each table generated. The “Dilution Factor” is the dilution ratio used for inoculating the wells of that column. The “Volume” is the volume of the dilution added to each well in that column.
    - Dilution Factor: Use results from a subset of the serial dilutions such that all host wells were lysed in the most concentrated of the dilutions (i.e., 24 of 24 wells were positive for viral activity) and all host wells were healthy in the most dilute of the dilutions (i.e., 0 of 24 wells were positive for viral activity). Common dilution factor range includes: $1x10^{-6}$ to $1x10^{-10}$.
    - Volume = 0.05 mL
    - tubes (wells) = 24
    - positive tubes = no. of cleared wells (out of 24; should be 24 for the most concentrated and 0 for the most dilute)
6. Once tables are completed, hit “Ctrl”+”m” to calculate MPN estimates. Program returns estimates of MPN, $\log_{10}$ MPN, Std Dev $\log_{10}$ MPN, Upper and Lower 95% Confidence Limits, and a Rarity Index (i.e., indication of the probability of the results).

LINK:
http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html