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Viral Tagging and Grow: a scalable approach to capture and characterize infectious virus-host pairs

Ho Bin Jang[1], Lauren Chittick[1], Fen Li .[1], Courtney M M Sanderson[1]

[1]Ohio State University, Columbus

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Sullivan Lab

Courtney M Sanderson
Ohio State University, Columbus

ABSTRACT

Viral tagging ('VT'), as shown in the **Fig. 1A and 1B**, entails fluorescently labeling the DNA of wild virus particles (upper, **A**), mixing these with a target, cultivable host bacterium (lower, **A**), and assessing the population of cells via flow cytometry for a fluorescent shift attributed to 'viral tagging' (VT) (**B**). VT was developed with culturable marine isolates (1, 2) and more recently applied to deduce virus-host pairing with unknown hosts in the human gut (3). However, all virus-host pairs detected (via phage adsorption) do not necessarily represent a productive lytic infection due to reversible and/or non-specific binding of lysogeny (phage genome replicates with the host genome). Additionally, post-adsorption cellular defenses could all prevent successful infection. To capture and characterize infectious, individual virus-host pairs, we developed 'Viral Tag and Grow (VT+Grow)' by incorporating a plate-based 'Grow' step (**C**) to VT (**A and B**).

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MATERIALS TEXT

Equipment
- BD influx cell sorter (Becton Dickson, San Jose, CA) with lasers at 488 nm (blue) and 642 nm (red) and nine optical detectors to analyze the size, granularity, and seven fluorescences per cell
- Falcon Round-Bottom Polypropylene Tubes with Caps, 5 ml (CatNo. 352063)
- Sonication bath (e.g., VWR Signature Ultrasonic cleaner B1500A-DTH)
- Refrigerated centrifuge (e.g., Eppendorf5417R)
- Amicon centrifugal ultrafiltration device (10-kDa molecular weight cut-off filter, Millipore, Cat No. UFC203024)
*We recommend the 2mL capacity units to reduce the number of separate MSM additions, but the 0.5mL units will work as well
- Digital vortex mixer (Fisher Scientific, Cat No. 02-215-418)
- white 96-well plate with clear bottom

Reagents
- SYBR Gold (Invitrogen, Cat No. S11494; initial dye stock of 10,000X)
- 0.2-µm-filtered 1% bovine serum albumin (BSA, Bioexpress, Cat. No. E531) in PBS
- SPHERO^{TM} Ultra Rainbow Fluorescence particles (Spherotech Inc., Cat No. URFP-30-2)
- BD FACS^{TM} Accudrop Beads (BD Biosciences, Cat No. 345249)
- Spherotech 8-Peak Validation Beads (BD Biosciences, Cat No. 653144)
- PZM media, 100% nutrient (1 liter) (*Pseudoalteromonas*-Zobell Media, 26g sea salts, 1g yeast extract, 5g proteose peptone no. 2, fill to 1 liter of qH_2O, Note: use 50% nutrient PZM for *Pseudolateromonas* culture)
- 0.1 µm-filtered MSM buffer (450 mM NaCl, 50 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, pH 7.5)
- Phosphate buffered saline (PBS)

SAFETY WARNINGS

The lasers of the flow cytometer are highly powered and should not be directly looked at when turned on.

BEFORE STARTING

-Additional details about the start-up, shut-down, and optimization of a flow cytometer are available in another protocol 'BD Influx Cell Sorter Start Up and Shut Down for Viral tagging and Grow' (https://www.protocols.io/view/bd-influx-cell-sorter-start-up-and-shut-down-for-v-bv8cn9sw).
-This protocol is based on *Pseudoalteromonas*-phage system. For other virus-host systems, the reagent (e.g., media) and others need to be optimized.

Amicon tube pre-treatment: 1h 15m

1 Prepare 0.02µm-filtered 1% bovine serum albumin.
* The BSA should be freshly prepared (1-2 days, stored at 4°C) before viral washing.
*The purpose of this is to better cushion the viruses during the subsequent wash steps and to aid in recovery

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2 Wet Amicon tubes by adding 500µl MSM buffer; let stand for 10 min, then spin until almost dry (5,000 x g, 10 min) and discard the flow-through.

3 Add 500µl of 0.02µm filtered 1% BSA, and let stand for 1 hr at room temperature. Proceed to viral staining during this period

Viral staining and washing 3h

4 Thaw prepared 10,000X SYBR Gold in the dark.
*We recommend making 1uL aliquots of stock SYBR Gold to avoid freeze-thawing of the reagent.

5 In a clean 1.5mL tube, stain the viruses by adding SYBR Gold to a final concentration of 10X. Perform in low light as SYBR Gold will degrade upon exposure to light. * Add 1µL stock SYBR Gold per 1mL of phage; if using a more concentrated phage stock, use 1uL of 1:10 diluted SYBR Gold to 100uL of concentrated phage
* We do not recommend adding more than 10^{10} viruses to the Amicon unit as this will greatly decrease the efficiency of excess SYBR Gold removal, which will result in more false-positive shifts during cell sorting
* If using a low titer of viruses, we recommend concentrating them to 10^{8}-10^{9} prior to staining to reduce the amount of SYBR Gold required. However, concentrating can be done concurrently with the initial wash step by repeating Step 11 until the entire volume of stained phages has flowed through the membrane.

6 Prepare a blank without virus to evaluate the efficiency of excessive SYBR Gold removal in later steps. This will be used as a negative control (SYBR-Blank, SYBR-Gold without viruses) for flow cytometric detection of bacterial cells. See step #6 in the Viral tagging and grow part below

7 Vortex the sample briefly to mix the dye and viruses.

8 Incubate for 20 min in the dark at room temperature.
* Practically speaking, viruses can be stained anywhere between 20-60 minutes; 20 minutes is a minimum

9 Spin down the BSA-treated Amicon tube (5,000 x g, 10-20 minutes) until all BSA has flowed through; discard the flow-through.

10 Rinse excess BSA from the Amicon filter by centrifuging 500uL filtered MSM buffer through the membrane (5,000 x g, 10 minutes)

11 Add the stained virus preparations and the SYBR-Blank in the pre-treated Amicon devices and centrifuge 3,000 x g for 30 min, 18°C.
* Following each centrifugation step, the volume remaining in the Amicon device should be reduced to 50-100uL for adequate washing. Depending on the phage concentration, you may need to adjust the length of centrifugation (may range between 20-60 minutes)

12 Wash the reduced volume of stained virus or SYBR-Blank by adding 1mL MSM buffer to the Amicon filter and gently pipetting up and down against the membrane. Add an additional 1mL of MSM and centrifuge as in step #11.
* If using a 0.5mL capacity Amicon filter, add 500µL MSM and wash the membrane by very gently pipetting up and down; proceed to centrifugation step

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13 Repeat #9 3 times so that a total of 8mL of MSM has flowed through the membrane (be sure volume falls <100 µl each time).

14 Following the final wash step, remove the Amicon filter from the collection tube, and vortex each side of the membrane for 5 seconds. Collect all liquid from the bottom of the membrane into a clean tube.

15 Add 100µL MSM to the filter and gently pipet up and down against the membrane to dislodge additional viruses. Without the collection tube, vortex the membrane as in Step 11, and collect all liquid.
* Depending on the starting concentration of viruses, the resuspension volume can be adjusted to maximize recovery. We found that recovering ~10^9 stained viruses with three final recovery washes of 300µL, for an estimated final volume of 1mL averaged the best recovery (70-80%) and simplified subsequent VBR calculations

16 Repeat Step 12 twice.

Viral tagging and grow 3h

17 Quantify your recovered SYBR-stained viruses to determine the amount to add. The multiplicity of infection (MOI, see below) will depend on the virus-host systems used (e.g., MOIs of 0.5 to 10). Cells are most susceptible during mid-log phase, so timing of your culture is crucial. Transfer 200uL of your overnight culture in 19.8mL of media prior to FCM setup with enough time to allow your culture to reach mid-log phase. We recommend completing an OD-CFU correlation of your target bacterium prior to this experiment.
* For environmental virus system where the phage's infection efficiency is unknown, measure the virus-to-bacterium ratio (VBR, see the reference #2)

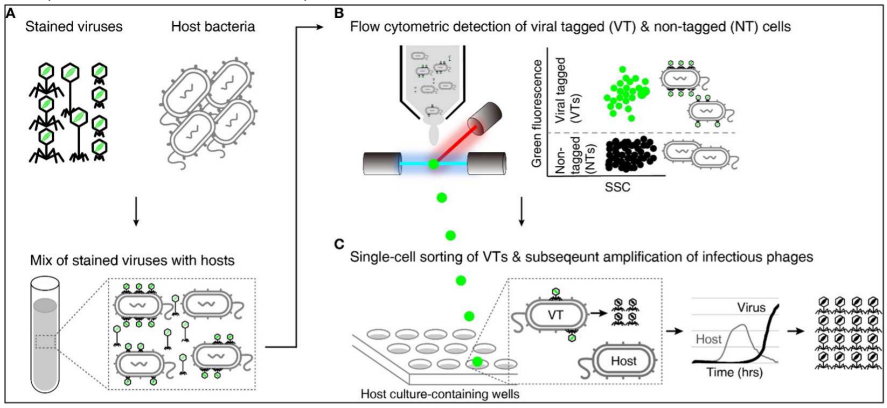

FIG 1 Overview of viral tagging, and the variant developed here – viral tag and grow. **(A)** Viruses are labeled with a green fluorescent dye and then mixed with potential host bacteria. **(B)**Fluorescence detection of individual cells with fluorescently labeled viruses (FLVs) by flow cytometer. The flow cytometry plot (side scatter or forward scatter versus green fluorescence) shows the expected locations of FLV-tagged (VTs) and nontagged cells (NTs), which are flow-cytometrically green positive and negative, respectively. **(C)** Single-cell sorting of VTs is followed by subsequent amplification of infectious viruses. Single VTs are sorted into a 96-well plate that contains host culture. Culture growth is monitored by measuring optical density (OD) over time. A decrease in the OD curve from VT-containing wells (relative to the phage-negative control) indicates cell lysis by progeny viruses produced from a single isolated VT cell.

18 Before viral tagging, flow cytometric (FCM) conditions and parameters should be adequately optimized as below. For more details, see the sections below: 'BD influx Cell sorter Start Up and Shut Down for Viral Tagging and Grow' and 'Flow cytometer optimization for cell detection and sorting'.
* Stream/laser alignment, drop-charge delay, and sorting calibration are optimized using Ultra Rainbow particles, Accudrop Beads, and 8-Peak Beads, respectively (see the reagents).
* Finely align the 96-well plate tray for single-cell sorting.
* Set up the flow cytometric gating and conditions (e.g., forward and side scatters and SYBR-Gold, respectively) to avoid the overlap of the cell signals with background noises.

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* Format your 96-well plate to allow the appropriate number of wells for VT and NT populations, as well as sufficient control wells (media blank, and non-infection (-) and positive-infection (+) controls) (Fig. 2)

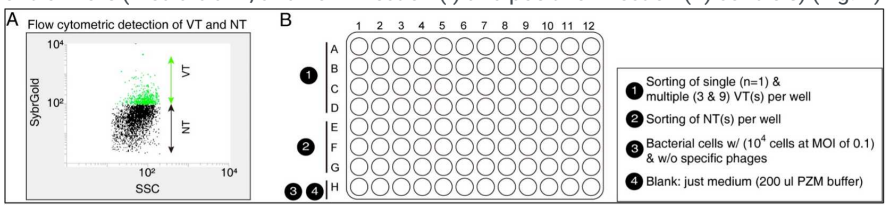

FIG 2 Flow cytometric sorting of viral tagged (VT) and nontagged (NT) cells into the 96-well plate. **(A)** Flow cytometric (FCM) detection of VTs and NTs. For the details about FCM optimization for detecting and sorting VTs and NTs, see the following section 'BD influx Cell Sorter Start Up and Shut Down for Viral Tagging and Grow'. (**B**) Schematic representation of plating for VT and NT sorting followed by host lysis observation. Each well contains 100 µL of Zobell media to prevent desiccation of sorted droplets during processing. For details about the titers of host cells and viruses and plating, see step #8.

19 Infect ~10^5-10^6 cells with stained viruses depending on your desired MOI and the estimated titer from Step 1 of this section
*The appropriate incubation time will vary between systems, and should be determined by latent periods of known infectious phages
* Ideally, the infection volume should be kept <<1mL to increase the probability of contact between viruses and hosts.
* Particularly for single-cell assay for burst size (4) using VT+G, the one-step growth curve for target bacteria using specific phages helps to decide the proper time for incubation, sorting, and plating (see Kirzner et al., (4)), respectively.

20 Immediately after incubation of viruses with hosts, add MSM buffer to bring the infection volume up to 1 ml. To remove free viruses, centrifuge the infection (12,000 ×g for 1 min at room temperature) and resuspend the pellet in 1mL MSM buffer.

21 Repeat Step 20 twice for a total of three centrifugations

22 Resuspend the infected cells to final concentrations ranging from ~5 x 10^5 ml^{-1} to 10^6 ml^{-1}. This process will decrease the coincident sorting of multiple cells per droplet and achieve optimal spacing between sorted cells.

23 Before sorting, add 100 µl of Zobell media to each well of a 96-well plate to prevent desiccation of sorted droplets during processing.
*We recommend sorting NT populations in parallel with VT populations to assess the false-positive cell lysis of VT-containing wells by the free virus(es).

24 Following FCM sorting of cells into the 96-well plate, monitor bacterial growth by measuring OD600 nm at 15-min intervals for 24 h. Remove any contaminated blanks, and determine the lysis curves of your wells by plotting OD against time.
*Details about FCM gating strategy and single-cell sorting are described in 'Flow cytometer optimization for cell detection and sorting' below.

25 Compare the lysis patterns of the wells containing sorted VT and NT cells to those of positive and negative controls (Fig. 3).

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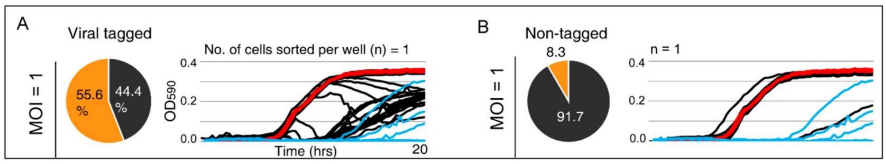

FIG 3 Viral growth assay for single VT- and NT-containing wells. Liquid culture of *Pseudoalteromonas* strain H71 (10^5/ml) in the late-logarithmic growth phase were infected by specific phage HM1 at an MOI of 1. From this infected culture, single (# of sorted cell per well) viral tagged (VT) and nontagged (NT) cells were sorted into individual wells of a 96-well plate containing growth medium followed by the addition of fresh host cells (10^4cells per well, see **Fig. 2**). Positive and negative controls (host culture with HM1 at an MOI of 0.1 and without HM1, respectively) were included in each plate (see **#7**). From left to right in panels (A) VT and (B) NT, respectively, pie charts depict the percentages of lysed (yellow) and nonlysed (gray) wells from the total wells containing the given number (n = 1) of isolated VTs and NTs. Culture lysis for VT- and NT-containing wells was determined by comparing their growth curves (next to each pie chart, black lines) to those of negative (red) and positive controls (blue) (see **#7**). The X-axis indicates the OD_{590nm} and the Y-axis, the time in hours.

Flow cytometer optimization for cell detection and sorting

26 The instrument we used is a BD influx cell sorter (Becton Dickson) equipped with two high-power lasers at 488 nm (blue) and 642 nm (red) and nine optical detectors, respectively. Each detector uses a high-performance photomultiplier (PMT) to amplify the low-intensity signals of the nano-sized particles (e.g., viruses, (**5**)). This influx sorter has the capability of 2- to 6-way population sorting into tubes and single-cell sorting directly into plates or slides. The fluidic system was only run using sterile solutions as a sheath (e.g., 0.02-µm-filtered and autoclaved MSM, see Reagents) and is always cleaned and air dried before shutdown. Additional details are described in the 'BD influx Cell sorter Start Up and Shut Down for Viral Tagging and Grow'

27 Details about system startup, alignment, optimization, shutdown, and maintenance are described in the 'BD influx Cell sorter Start Up and Shut Down for Viral Tagging and Grow'

28 Detect the cells using hierarchical gating. For example, as shown in Fig. 4A, a parent gate is drawn on Pseudoalteromonas sp. H71 cells in the forward (FSC) vs. side scatter (SSC), from which viral tagged (VTs, green positive) and nontagged (NTs, green-negative) cells are further gated as a sub-fraction in the green fluorescence (SYBR-Gold) vs. SSC. Events were detected using a forward scatter trigger, and data were obtained in logarithmic mode then analyzed with BD FACS software version 1.2.0.142 (Becton Dickinson, San Jose, CA).

29 Adjust trigger and the PMT voltages for relevant parameters (FSC and SSC) to avoid the overlap of the cell signals with background noises from the instrument (electronic noise), micro-particles in the buffer, and/or cellular debris (5) (Fig. 4B and 4C).

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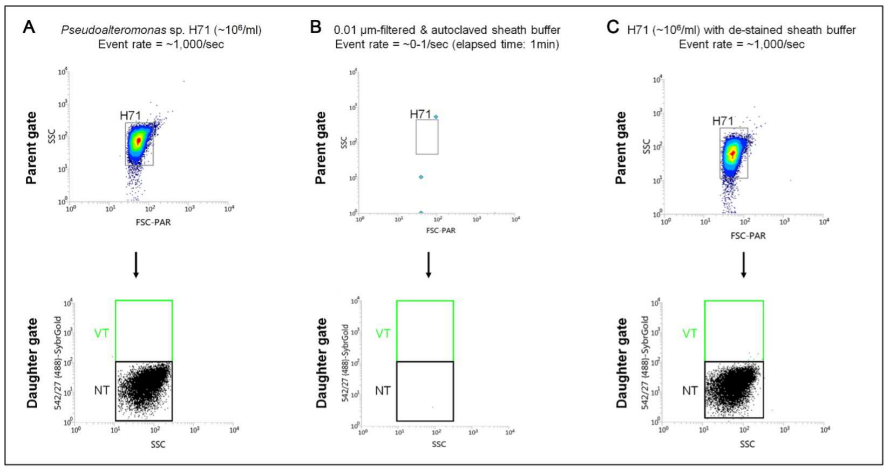

FIG 4 Flow cytometric optimization of viral tagging signal detection. (A) Upper, *Pseudoalteromonas* strain H71 cells (~10^6/ml) were detected with a gate using forward (FSC) versus side scatters (SSC) (Upper). From this parent gate, the virus-tagged (VTs) and nontagged populations (NTs) were sub-gated, using green fluorescence (542/27 nm, SybrGold) and SSC (Lower). (B) Autoclaved and filtered sheath buffer without H71 cells on the influx. Note that there is the non-overlap of cell populations (gated) with background noises (electronic noise and/or microparticles in the sheath buffer) in a parent and two daughter gates (VT and NT populations, respectively). Detected events were shown for 1 min of sample running (elapsed time) with an event rate of 0 to 1/sec. (C) H71 cells with stained and washed sheath buffer on the influx.

30 4. Before sorting, finely align the 96-well plate by visually inspecting the deposition of droplets into the center of each well.

31 Sort single VTs or NTs into each well of the 96-well plate using the single sort mode of 1.0 drop purity.
* To prevent the coincidence of free viruses sorted with VT or NT cells, which would be too close to be separated, adjust the event rate for sorted particles from the VT and NT cell populations to ~40 events per second. The estimated ratio of sorted particles to generated drops (piezoelectric at 43.2 kHz, above) of ~1/1,180, ensuing a separation enough between sorted particles (6).
* To maximize the sorting purity of the false-positive population, we use the biased gating strategy (7).

32 After sorting, monitor bacterial growth as previously described (see #8 and Fig. 3 in 'Viral tag and grow').

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