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Viral isolation for SAR11 and OM43 hosts

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1 Works for me dx.doi.org/10.17504/protocols.io.bb73irqn

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ABSTRACT

Working protocol

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Phosphate buffered saline	P4417-50TAB	Sigma Aldrich
SYBR® Green I nucleic acid gel stain	S9430	Sigma Aldrich

EQUIPMENT

NAME	CATALOG #	VENDOR
Sartorius 50R Vivaflow	VF05H4	Sartorius
323Du RS232/auto/manual enhanced-control high-flow pump	323Du	
BD Accuri C6 Plus	BD Accuri C6 Plus	BD Biosciences

Sample Preparation

1 Take 2 liter water samples of environmental water you wish to isolate viruses from. To remove larger plankton and particles, filter through a GF/D filter membrane (14.2 cm Whatman GF/D filter) using a peristaltic pump. Collect filtrate in a sterile, acid-washed 2 liter Polycarbonate bottle.

323Du RS232/auto/manual enhanced-control high-flow pump
Peristaltic pump
Watson-Marlow Bredel Pumps 323Du

2 Filter filtrate through a 0.2 µm PC membrane (142mm Merck Millipore 0.2 µm PC filter) to remove larger sized bacterioplankton. Collect filtrate in a clean, acid-washed 2 liter Polycarbonate bottle.

3 Concentrate the 2 liter filtrate down to 50 ml, using a tangential flow filtration unit following the manufacturors protocol.

Sartorius 50R Vivaflow
Tangential Flow Filtration cassette
Vivaflow VF05H4

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4 Filter the 50 ml concentrated sample through a 0.1 µm PVDF membrane syringe-filter to ensure a cellular free viral fraction. Water samples can be stored at 4°C in the dark for approximately 8 weeks.

Plate preparation

5 Using a Cat II flow hood, pipette 1 ml of sterile ASM1 medium (Carini et al. 2013 http://dx.doi.org/10.1038/ismej.2012.122) into each well of a sterile, acid-washed 96-well 2 ml Teflon plate.

6 Inoculate column 1 to 11, with exponentially growing bacterial cultures to a cell density of 1*10^6 cells/ml (T0). Leave column 12 blank as a medium control to check for contamination.

Sequential viral enrichment

7 Add 100 µl (or 10% v/v) of concentrated water sample as viral inoculum (step 4, section "Sample Preparation") to each well in columns 1 to 8 of a prepared 96-well plate (Section "Plate preparaton"). These are your **Viral Enrichment culture** sets. Each row is one set. Wells in column 9-11 are left without adding an inoculum for a no-virus control.

8 Incubate the plate at 18°C in an incubator without rotation for 2 weeks until no-virus controls reach stationary phase. 2w

336:00:00 This depends on the average growth rate of the bacterial host that is used.

9 Count all wells of the 96-well plate on a flow cytometer following steps (Section 4: "Observing viral infection"). Make a note of all wells that show signs of infection.

BD Accuri C6 Plus
Flow Cytometer
Accuri BD Accuri C6 Plus

10 For each of the viral enrichment culture sets: If none of the wells in column 1 to 8 show signs of infection, **proceed with step 11**.

If one or more wells in a Viral Enrichment culture set show signs of infection, **proceed with step 12** (Section "Viral Isolation)".

11 For each Viral Enrichment culture set: Pipette all liquid from wells in columns 1 to 8 into a 15 ml falcon tube.

11.1 In a sterile Cat II flow hood: Filter all liquid through a 0.1 µm PVDF membrane syringe-filter to ensure a cellular free viral fraction. Collect filtrate in another sterile 15ml falcon tube. This is the new viral inoculum.

11.2 Prepare a new 96 well plate as described in section 2 ("Plate preparation").

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11.3 Add 100 µl (or 10% v/v) of the new viral inoculum from step 11.1 to each well in columns 1 to 8 of the newly prepared 96-well plate (11.2). These are your new **Viral Enrichment culture** sets. Each row is one set. Wells in column 9-11 are left without adding an inoculum for a no-virus control.

11.4 Follow instructions from step 8 to 9.

11.5 For each of the viral enrichments culture sets:

If based on the flow cytometer results from the previous step (11.4) none of the wells in column 1 to 8 show signs of infection, this isolation attempt is deemed a failure.

If one or more wells in column 1 to 8 show signs of infection, proceed with step 12 for each well that shows signs of infection.

Viral Isolation

12 In a sterile Cat II flow hood: Pipette each **individual well** that shows sign of infection in step 11.5 into sterile 1.5 ml Eppendorf tubes. Each well/tube is treated as an individual **viral culture**.

13 Filter each viral culture through a 0.1 µm PVDF membrane syringe-filter to ensure a cellular free viral fraction. Collect filtrate in another sterile 1.5 ml Eppendorf tube. Make sure to change filter membranes and syringes every time.

14 Prepare a 96-well plate as described in section 2 ("Plate Preparation").

15 Each row of the 96-well plate will hold a serial dilution used to isolate only one viral culture.
Add 100 µl (or 10% v/v) of a viral culture to column 1. Mix well and gently by pipetting.

16 For each row: Pipette 100 µl of liquid in column 1 and add it to column 2. Mix again by pipetting. Then take 100 µl of liquid in column 2 and add it to column 3. Repeat this process to column 10. Do not add viruses to column 11 or 12. Column 1 has the highest concentration of viruses, column 10 has the lowest.

17 Incubate the plate at 18°C in an incubator without rotation for 2 weeks until no-virus controls reach stationary phase. 2w
336:00:00 This depends on the growth rates of bacteria used as host.

18 Count all wells of the 96-well plate on a flow cytometer following the steps described in Section 4 "Observing viral infection"). Make a note of all wells that show signs of infection.

19 In a sterile Cat II flow hood: Pipette viral lysate from the single **individual well** that shows sign of infection, but has the lowest concentration of viruses added to them (i.e. the well of each row from the highest column number) into sterile 1.5 ml Eppendorf tubes.

20 Filter the viral lysate through a 0.1 µm PVDF membrane syringe-filter to ensure a cellular free viral fraction. Collect filtrate in

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another sterile 1.5 ml Eppendorf tube. Make sure to change filter membranes and syringes every time.

21 Repeat steps 14 to 20 two more times using the viral lysate from step 20 as new viral culture each time.

22 The viral culture is now considered a pure viral isolate and is ready for downstream applications.

This was confirmed by sequencing 13 viral isolates, which all returned single viral genomes. We extracted DNA following the protocl for DNA isolation from phage lysate (Solonenko 2016)
dx.doi.org/10.17504/protocols.io.c36yrd

Observing viral infection 2h 45m

23 # Steps are for counting a full 96-well Teflon plate.

Working in a sterile Cat II flow hood: Use a flat bottom 96-well microplate and pipette 180 µl of autoclaved PBS solution into each well.

23.1 To make up the PBS solution: Dissolve one tablet of PBS in 200 ml deionized water to create a solution with 0.01 M phosphate, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25 C.

Phosphate buffered saline
by Sigma Aldrich
Catalog #: P4417-50TAB

24 Wipe down the outside of the 96-well teflon plate with 70% Ethanol and place in the Cat II flow hood.

25 Pipette 20 µl of each well on the Teflon plate into the corresponding well of the microplate.

26 Add 2 µl of SYBR green working stock solution to each well of the microplate.

26.1 To make a SYBR green working stock: Add 198 µl of sterile PBS solution (Step 23.1) and 2 µl of SYBR green in a 200 µl PCR tube to make up a working SYBR green stock. Make sure to wear gloves when handling SYBR green!

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SYBR® Green I nucleic acid gel stain
by Sigma Aldrich
Catalog #: S9430

SYBR green is carcinogenic, handle with care!

27 Incubate the microplate in the dark for 45 minutes. 45m

00:45:00

28 Using a flow cytometer: set up a working sheet with the following settings:

Primary threshold: FL1-H at 1000
Secondary threshold: none
Ungated sample

Medium flow rate
42 µl collection limit
OR
1 minute collection limit

29 Run the microplate on the C6 following the manufacturers instructions. This will take approximately 2 hours. Discard microplate as cytotoxic waste. 2h

02:00:00

30 To spot an infection compare the no-virus control to samples treated with viruses.

In liquid cultures viral infections can be difficult to detect and usually can only be detected by comparing controls to cultures treated with viruses. Look out for a combinations of these signs, descripted in the sub-sections below:

30.1: Increase in "noise"
30.2: Decrease in host cell abundance
30.3: Increase in green fluorescence signal of population peaks
30.4: "Smearing" of population peaks, or double peaks.

This is an example of bacterial host Pelagibacter ubique HTCC1062 and with and without infection over time.

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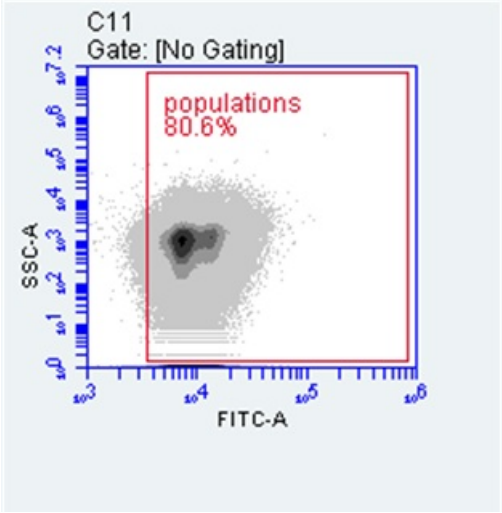

Figure 1. The cytogram of a no-virus control culture at maximum cell density ~10^8 cells/ml

30.1 Increase in "noise"

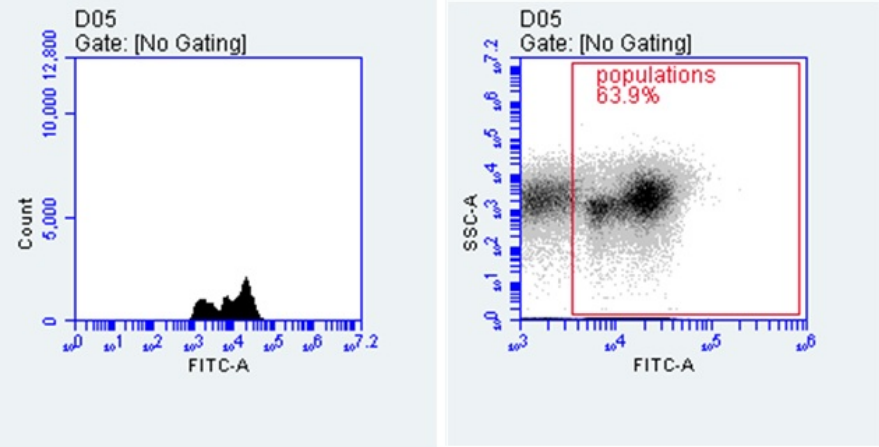

Figure 2. Cytogram of an infected HTCC1062 culture, note the reduced number of cells in the "populations" acquisition window and the "noise" outside the main population.

30.2 Decrease in host cell abundance.

By counting host cell abundance on the flow cytometer over time, growth curves can be used to spot differences between no virus controls and infected cultures. It is important to note, that growth curves alone are not sufficient evidence for an infection, as reduced growth in bacteria can have several reason unrelated to viruses.

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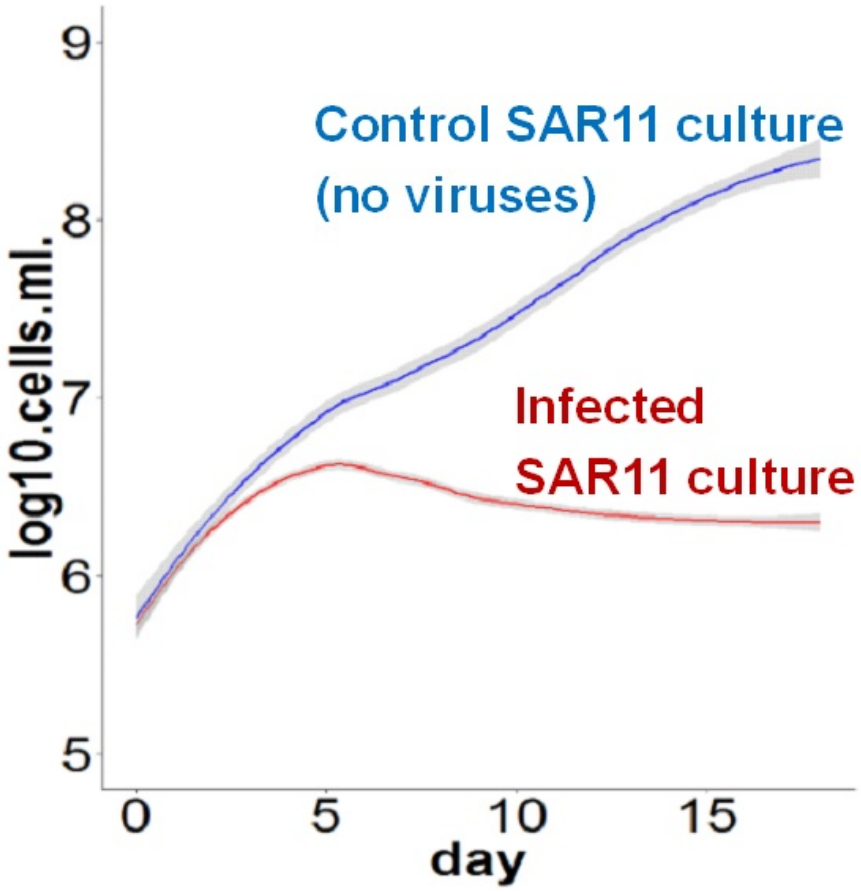

Figure 3. Growth curves of HTCC1062 with and without viral treatment

30.3 Increase in green fluorescence signal of population peaks

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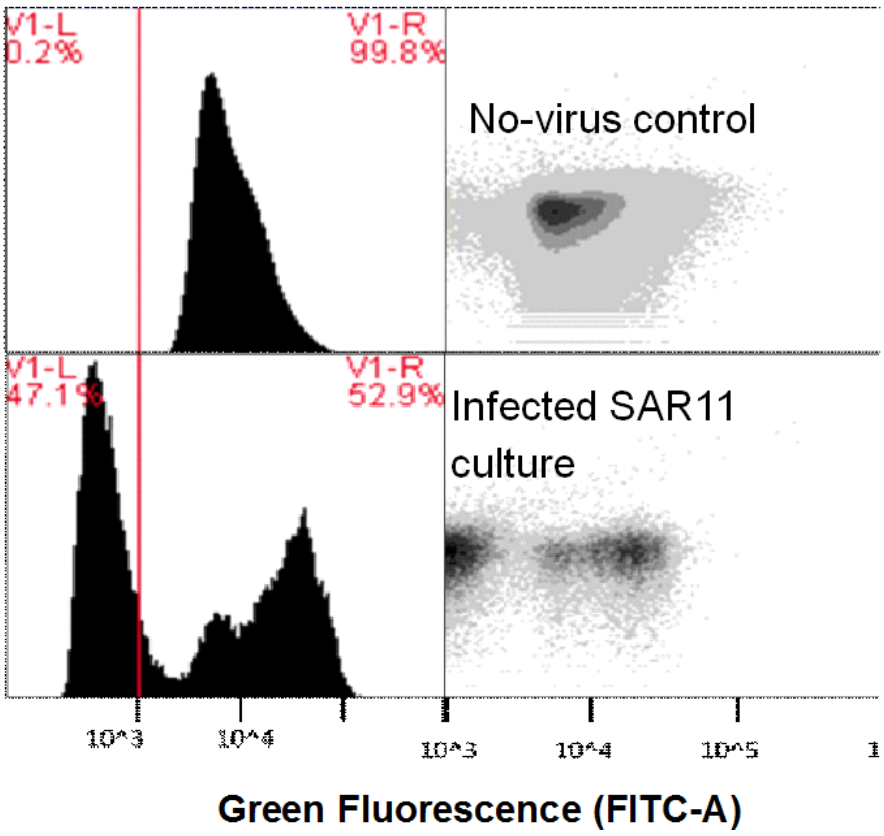

Figure 4. HTCC1062 cultures with and without viral treatment. Note that the infected culture has both a peak with a higher green fluorescence signal, a small secondary peak that presents as a "ridge" next to the main peak, and a large amount of background scatter in the low green fluorescence area (the left-hand side)

30.4 "Smearing" of population peaks, or double peaks.

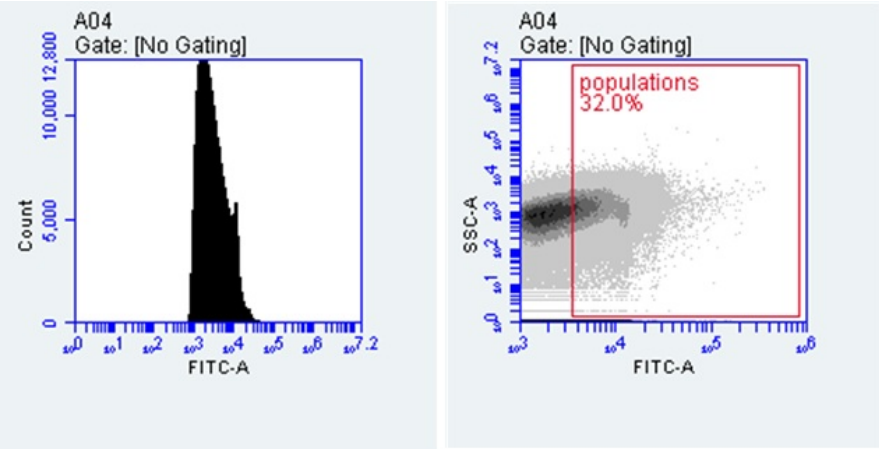

Figure 5. OM43 culture H5P1 infected with the virus Venkman. Note that the population peak is almost indistinguishable from the noise and just presents as one long smear.

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