

Oct 09, 2022

Virion quantification by flow cytometry, without fixation or freezing

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ABSTRACT

This protocol uses flow cytometry to measure the concentration of virions in a sample, and was based on [Brussaard \(2004\)](#) and [Wen et al. \(2004\)](#). This protocol works without fixing or snap-freezing the samples. The protocol is designed for chloroviruses (virion diameter ~190nm), and should apply to other large viruses. The protocol can handle up to 96 samples at a time. Repeatability is 97-99% (EJPL, unpublished data).

Using high-quality materials is relevant for this protocol; see *Materials* for more information. The protocol uses a DNA stain, see *Warnings*.

DOI

dx.doi.org/10.17504/protocols.io.6qpvr6q93vmk/v1

PROTOCOL CITATION

Eva JP Lievens 2022. Virion quantification by flow cytometry, without fixation or freezing. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.6qpvr6q93vmk/v1>

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CREATED

Jun 07, 2022

LAST MODIFIED

Oct 09, 2022

MATERIALS TEXT

- 1X Tris-EDTA buffer (TE), autoclaved and stored at room temperature. We use a 100X stock solution (VWR International, USA), diluted to 1X in ultrapure water (Milli-Q, Merck, DE).
- SYBR Green I nucleic acid gel stain (ref. S9430, Merck). We use aliquots diluted to 100X in autoclaved TE and stored at -20°C.
- Dry bath with heat block for 96 wells and heated lid, or thermal cycler with heated lid. The heated lid reduces condensation, and this helps prevent contamination and sample loss. We use a ThermoFisher Compact Digital Dry Bath S (ref. 88871003, ThermoFisher, USA) with 96-well block (ref. 88871107). Instead of a heated lid, we use four '6x1.5ml' blocks (ref. 88871103) which we heat and place on top of the plate (see below).
- An appropriate 96-well PCR plate and sealing film for use in the dry bath or thermal cycler. The plate should fit the 96 well block perfectly: there should be no air between the wells and the heating element, and the wells should not extend above the heating element. We have also found that the brand of the plate affects the precision of the results. We use low profile PCR plates from Starlab (ref. E1403-0200, Starlab, DE) and adhesive PCR film (ref. 732-3212, VWR).
- An appropriate 96-well plate for use in the flow cytometer, and a seal for this plate. We use Rotilabo microtitration plates (ref. 9291.1, Carl Roth, DE) and aluminum foil seals (ref. 391-1282, VWR).
- A flow cytometer that can record the analyzed volume, and ideally with an automated loader. We use a BD FACSVerser (BD Biosciences, USA).
- Vortexer and plate centrifuge.
- Multichannel pipettes are highly recommended, particularly when measuring samples stored in a 96-well plate.

SAFETY WARNINGS

SYBR Green I is a DNA stain. It is not known to be dangerous, but caution is advised. We do all steps involving SYBR Green in a separate lab area, with separate materials. We also wear gloves and a lab coat.

BEFORE STARTING

This protocol stains virus samples that are obtained directly from algae/virus cultures. Fixation and snap freezing are unnecessary. However, storage conditions do need to be taken into account.

The maximum storage duration depends on how the samples were obtained (EJPL, unpublished data).

- Samples that were filtered out of a mixed algae/virus solution (through 0.2 or 0.45µm) can be stored at 4°C for at least 3 months, and likely much longer, without changes in the virion concentration.
- Samples that were collected from the supernatants of a centrifuged algae/virus solution (e.g. in an mOSG assay) contain more bacteria and other contaminants. They can only be stored at 4°C for 1 week before the samples become noisy.
- Samples where viruses were not separated from algae (e.g. a direct aliquot of an

algae/virus solution) should be measured immediately. The algal concentration should also be fairly low, otherwise the samples will be too noisy.

It is typical to see a slight drop in virion concentration in the first 24h after fresh samples are transferred to 4°C (EJPL, unpublished data). After this slight drop, the virion concentration remains stable. The effect may be due to temperature shock in a subset of the virions. Practically speaking, it means that all stored samples are comparable, but 'fresh' samples should not be compared with stored samples. We therefore recommend storing all samples at 4°C for at least 24h before measurement.

We find that in many storage containers, the virion concentration decreases over time (beyond the slight drop mentioned above; EJPL, unpublished data). This includes many types of PCR plates, eppis, and centrifuge tubes. The effect may be because [virions adsorb to certain plastics](#). Any new storage container should be tested before use! To store virion samples at 4°C, we use:

- 15ml PP centrifuge tubes from Sarstedt (ref. 62.554.502, Sarstedt, DE)
- 50ml PP centrifuge tubes from neoLab (ref. C-8216, neoLab, DE) or from Greiner Bio-One (ref. 227261, Greiner Bio-One, DE)
- 24-/48-/96-well polystyrene tissue culture plates from Techno Plastic Products (ref. TPP92696 and others in this family, Techno Plastic Products, CH). The tissue culture plates must be sealed with an adhesive film to prevent evaporation and spills.

Preparation

- 1 Calculate volumes of TE buffer and 100X SYBR Green to use. Use an n_{samples} that is slightly higher than your actual number of samples, to account for pipetting error.

- 1.1 If using high-concentration virion samples, use 7.5µl of sample and 142.5µl of 0.526X staining solution:

$$V_{\text{staining solution}} = n_{\text{samples}} * 142.5 \mu\text{l}$$

$$V_{100\text{X SYBR Green}} = (V_{\text{staining solution}} * 0.526\text{X}) / 100\text{X}$$

$$V_{\text{TE buffer}} = V_{\text{staining solution}} - V_{100\text{X}}$$

- 1.2 If using low-concentration virion samples, use 30µl of sample and 120µl of 0.625X staining solution:

$$V_{\text{staining solution}} = n_{\text{samples}} * 120 \mu\text{l}$$

$$V_{100\text{X SYBR Green}} = (V_{\text{staining solution}} * 0.625\text{X}) / 100\text{X}$$

$$V_{\text{TE buffer}} = V_{\text{staining solution}} - V_{100\text{X}}$$

- 2 Defrost 100X SYBR Green aliquot (keep in the dark).

- 3 Turn on dry bath, set to 80°C. Put the '6x1.5ml' heat blocks in the dry bath as well (on top of the 96-well heat block), so that they heat to 80°C too.

Staining

- 4 Prepare the staining solution.

- 4.1 Filter the TE buffer to reduce noise in the samples.

1. Syringe-filter a little more TE than necessary through 0.2µm into a centrifuge tube.

2. Pipette the precise $V_{TE \text{ buffer}}$ into a new centrifuge tube. This is the **staining solution tube**.

We do this step at a clean bench so that the stock TE stays sterile.

- 4.2 Add $V_{100X \text{ SYBR Green}}$ to the staining solution tube and shake.

We do this step in a SYBR Green staining area, with specific pipettes.

- 4.3 Keep the staining solution tube in the dark. The staining solution can be prepared a few hours beforehand if necessary; in that case keep it in the dark at 4°C.

- 5 Prepare the staining plate.

- 5.1 Carefully cut the edges off a Starlab PCR plate, so that it can fit into the dry bath cover. This is the **staining plate**.

- 5.2 Aliquot 142.5µl (high-concentration samples) or 120µl (low-concentration samples) of staining solution into the staining plate.

We do this step in a SYBR Green staining area, with specific pipettes.

- 6 Add the samples.

- 6.1 Vortex and spin down the samples.

- 6.2 Add 7.5µl (high-concentration samples) or 30µl (low-concentration samples) of sample to the staining plate. The final SYBR Green concentration is now 0.5X.

We do this step in a SYBR Green staining area, with specific pipettes.

- 6.3 Make sure there are no drops on top of the plate, then seal the plate tightly with PCR film. Run a pipette tip between all the rows and columns to be sure it seals well. Fold the edges of the transparent seal over so it doesn't stick to things.

We do this step in a SYBR Green staining area.

- 7 Vortex the staining plate and spin it down.

- 8 Stain the samples.

- 8.1 Put the staining plate in the dry bath, and place the '6x1.5ml' heat blocks on top (flat side down). Careful: blocks are hot! Put dry bath cover on top, and cover with aluminum foil to keep the samples in the dark.

We do this step in a SYBR Green staining area, with a specific dry bath.

- 8.2 Incubate the plate at 80°C for 10min in the dark.

We do this step in a SYBR Green staining area, with a specific dry bath.

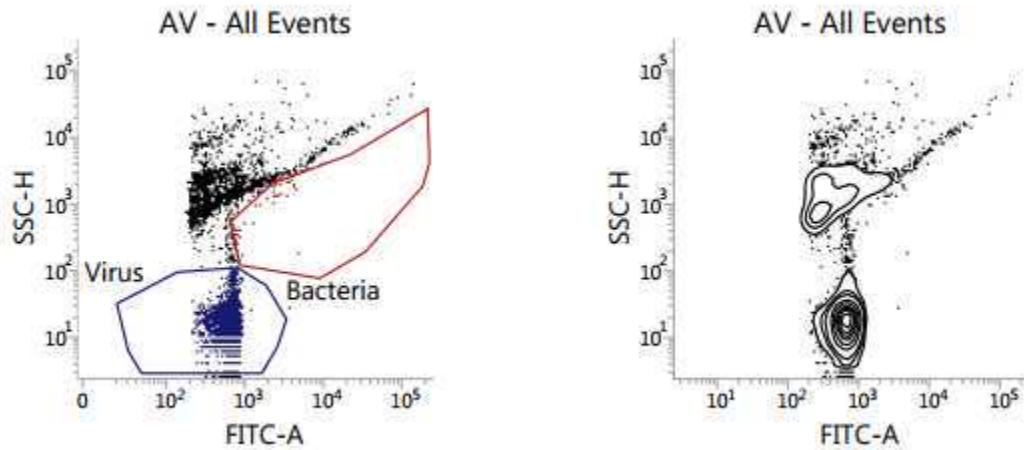
- 8.3 Carefully remove the staining plate from the dry bath, and spin it down very well.

- 9 Transfer the contents of the staining plate into a Rotilabo culture plate. This is the **flow cytometer plate**. Seal the plate tightly with an aluminum seal.

We do this step in a SYBR Green staining area, with specific pipettes.

- 10 Allow the samples to cool to room temperature for at least 15min (e.g. the time to go to the flow cytometer). Keep the plate in the dark.

- 11 Before starting the measurements, vortex and spin down the flow cytometer plate. The last samples should be measured at most 2h45min after staining (including the flow cytometer's run time!); after this time the results are less reliable.
- 12 The flow cytometry assay should measure side scatter vs. green fluorescence.



Example of flow cytometry results. The ungated points are caused by SYBR Green noise, and don't interfere with the measurements.