

Epifluorescent Microscopy of Virus Particles Using SYBR Green Version 3

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Abstract

Adapted from: Ortmann and Suttle (2009) *Determination of Virus Abundance by Epifluorescence microscopy*. Ch. 10 Methods of Molecular Biology.

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Protocol

Solutions

Step 1.

Make a 1% p-phenylenediamine (antifade) solution -- 0.01g + 1mL Milli-Q water.

Solutions

Step 2.

Vortex, keep in the dark.

Solutions

Step 3.

Retrieve an 8 uL stock of 1,000x SYBR Green, DI water, and 225 uL 50:50 PBS/Glycerol solutions

Solutions

Step 4.

Add 6 uL SYBR Green to 235 uL filtered DI. Keep in the dark.

Solutions

Step 5.

Add 25 uL 1% p-phenylenediamine to the 225 uL aliquot of 50:50 PBS/Glycerol. Keep in the dark.

Solutions

Step 6.

Place the SYBR Green and antifade solutions into a drawer while working.

📌 NOTES

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Only the red light can be on when the solutions are out of the drawer.

Sample Preparation

Step 7.

Fix sample with 0.5x glutaraldehyde.

📌 NOTES

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If you are going to freeze your sample between slide preparation and visualization, then you need to cool the fixed sample in the dark at 4°C for 15-30 min, and flash freeze in liquid nitrogen before storing at -80°C.

Vacuum Setup

Step 8.

Turn on the vacuum (no more than 25 mm Hg).

Vacuum Setup

Step 9.

Rinse wells with Milli-Q water.

Vacuum Setup

Step 10.

While the knobs are turned to open (vacuum on), use the tweezers to add the 0.45 um nitrocellulose backing filter.

📌 NOTES

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This filter is used to protect the more fragile 0.02 um Anodisc filter that the viruses are collected on.

Vacuum Setup

Step 11.

Once the filter is on, you should add a few drops of Milli-Q and then turn the knobs to closed (vacuum off).

Vacuum Setup

Step 12.

Use tweezers to apply the 0.02 μ m Anodisc filter to the top of the backing filter.

Vacuum Setup

Step 13.

Turn the vacuum back on by turning the knobs to open and add the sample in two 500 μ L aliquots.

SYBR Green

Step 14.

Pipette SYBR Green as dots onto a glass petri dish. Make enough dots for each filter.



AMOUNT

30 μ L Additional info:

SYBR Green

Step 15.

With the vacuum on, use a needle and tweezers to remove the Anodisc filter and place onto a dot. Turn the vacuum off.

SYBR Green

Step 16.

Put the lid over the plate and back into the drawer to stain for 20 min.



DURATION

00:20:00

Slide Preparation

Step 17.

During the wait, label slides with sample ID, dilution, stain, sample volume, and date.



NOTES

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You may turn the white light on during this period switching it off anytime the stain is exposed to light.

Slide Preparation

Step 18.

Re-apply Milli-Q to the backing filter and place the stained Anodisc on top. Add another drop of Milli-Q.

Slide Preparation

Step 19.

Turn the vacuum on again. After the stain has been washed away, use the needles and tweezers to remove the Anodisc--again, with the vacuum still on.

Slide Preparation

Step 20.

Place the filter onto a Kim Wipe to dry.

Slide Preparation

Step 21.

On the labeled side, add 18 uL antifade solution, apply the dried Anodisc filter and apply another 18 uL of antifade to the top.

Slide Preparation

Step 22.

Apply a coverslip and lightly press down so there are no bubbles.

Storage

Step 23.

Store at -20°C in the dark for up to four months (3-4 weeks recommended). .

📌 NOTES

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Thawing the sample repeatedly will result in fading of the stain, but you can attempt to re-stain the filter if it fades

Microscope

Step 24.

Turn on all three computers and the shutter.

Microscope

Step 25.

Open the LAS AF program.

Microscope

Step 26.

On the second menu, use the FLUO--Filter cubes L5 and the FLUO incident settings.

Microscope

Step 27.

On the fourth menu, click set/clear focus position to desired bottom using the up and down arrows. Once positioned, click "set". You can then move the microscope slide to the desired position to visualize the slide and click "set". These settings will then be saved within the microscope.

*Z: up/down (turn the back knob)

*X/Y: Side to side (turn the top or bottom half of the front knob)

Microscope

Step 28.

Once the options are set and the microscope slide is set into place, you can then turn the shutter on/off on the first menu--'IL--Shutter ON/OFF'.

Microscope

Step 29.

Rod all of the way in on the microscope -- image only on the microscope itself

Rod half way in -- image on the microscope and the computer screen

Rod all of the way out -- image only on the computer screen

Computer Imaging Software

Step 30.

Under the acquire --> acquisition tab:

*To look at a preview of your image, click "live" (delay may occur due to exposure)

*Set desired exposure, gain and intensity.

*To save a desired image, click "capture image". The image will appear on the right screen, where you can adjust the background fluorescence by moving the bar on the black to green scale at the bottom lefthand corner.

Computer Imaging Software

Step 31.

Under the acquire --> experiment tab:

*The captured images will automatically save to the experiment .lif file. This can only be viewed using this software.

*Right click on an image to rename/delete it.

*Click "save all" to save images to the desired directory.

*To save images in a different format, right click, select export, and chose your desired format (e.g. JPEG, TIFF, or the format required for your analysis software).

Enumeration of Virus-Like Particles

Step 32.

With the rod all of the way in, use the fine adjustment knob to locate and resolve the viruses into focus.

Enumeration of Virus-Like Particles

Step 33.

Count all of the viruses in the grid. The ideal concentration is between 20-40 viruses per grid so that you have enough time to count the viruses before they fade.

Enumeration of Virus-Like Particles

Step 34.

After counting all of the viruses in the field, look up from the microscope, and move the stage to a different part of the slide to count viruses in another field. It is important to do this without looking so that you can randomly sample your filter and not incur a bias in your counts.

Enumeration of Virus-Like Particles

Step 35.

Repeat this process until you have counted enough fields to total between 200-300 viruses. Calculate the average, and determine the VLP/mL by the equation:

$$(\text{Avg VLP/field}) \times (28352.9) \times (\text{Dil. factor}) \times (1000/\text{volume filtered (uL)}) \times ((1000 + \text{glu. volume (uL)})/1000)$$