Epifluorescent Microscopy of Virus Particles Using SYBR Green Version 2

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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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Citation: Dr. Steven Wilhelm, Samantha Coy Epifluorescent Microscopy of Virus Particles Using SYBR Green. **protocols.io**
dx.doi.org/10.17504/protocols.io.hg4b3yw
Published: 29 Mar 2017

Protocol

Solutions

Step 1.

Make a 1% p-phenylenediamine (antifade) solution -- 0.01g + 1mL Milli-Q water. Vortex, keep in the dark.

Solutions

Step 2.

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

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

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

Solutions

Step 3.

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

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

Sample Preparation

Step 4.

Fix sample with 0.5x glutaraldehyde. 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 4ºC for 15-30 min, and flash

freeze in liquid nitrogen before storing at -80ºC.

Vacuum Setup
Step 5.

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

Vacuum Setup
Step 6.

Rinse wells with Milli-Q water.

Vacuum Setup
Step 7.

While the knobs are turned to open (vacuum on), use the tweezers to add the 0.45 um nitrocellulose backing filter. This filter is used to protect the more fragile 0.02 um Anodisc filter that the viruses are collected on. 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 8.

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

Vacuum Setup
Step 9.

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

SYBR Gold
Step 10.

Pipette 30 uL of the SYBR Gold as dots onto a glass petri dish. Make enough dots for each filter.

SYBR Gold
Step 11.

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

SYBR Gold
Step 12.

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

DURATION
00:20:00

Slide Preparation

Step 13.

During the wait, label slides with sample ID, dilution, stain, sample volume, and date. You may turn the white light on during this period switching it off anytime the stain is exposed to light.

Slide Preparation

Step 14.

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

Slide Preparation

Step 15.

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. Place the filter onto a Kim Wipe to dry.

Slide Preparation

Step 16.

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 17.

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

Storage

Step 18.

Store at -20ºC in the dark for up to four months (3-4 weeks recommended). 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 19.

Turn on all three computers and the shutter.

Microscope

Step 20.

Open the LAS AF program.

Microscope

Step 21.

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

Microscope

Step 22.

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 23.

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 24.

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

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

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

Computer Imaging Software

Step 25.

Under the acquire --> acquisition tab:

*To look at a preview of your image, click "live" (delay may occure 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 26.

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 clock 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 requried for your analysis software).

Enumeration of Virus-Like Particles

Step 27.

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 28.

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 29.

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 imprtant 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 30.

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:

(Avg VLP/field)*(28352.9)*(Dil. factor)*(1000/volume filtered (uL))*((1000+glu. volume (uL))/1000)