**Analyzing/Sorting Sputum-Derived Bacteriophages via Flow Cytometry 11/12/19**

Materials:

* Sputum samples
* Flow cytometry analysis tubes
* Commercial stock solution of SYBR Green I
* FluoSpheres Carboxylate-modified Microspheres, 1.0µm, yellow-green fluorescent (505/515)
* 1.5mL microcentrifuge tubes
* 15mL conical tubes
* MilliQ/Nuclease-free water
* TE buffer 10:1 (10mM Tris, 1mM EDTA)
* 0.22µm pore size filters
* Heat block
* Sputasol
  + Add the Sputasol vial contents to a 92.5mL of sterile, distilled water (scale down if necessary).
  + Use right away or store at 4°C for 48H.

Steps:

*\*\*schedule time on the flow cytometer at the Flow Core with a prepared PO\*\**

**Prepare Working Stock Solutions**

**SYBR Green I**

1. Thaw the commercial stock of SYBR Green I in the dark @ RT. Vortex for 10s, then pulse centrifuge.
2. Prepare the working stock solution by aliquoting 5µl of the commercial stock into 10ml sterile MilliQ in a 15mL conical tube (1:2,000). Work with this solution in dimmed light, as SYBR Green is light-sensitive. Wrap tube in aluminum foil before labeling. You can prepare several working stocks at a time and store them at -20°C until use.

**Microsphere Standard**

1. Mix the beads by vortexing to ensure homogeneity.
2. Prepare a primary stock solution by adding 1-2 drops of the commercial stock into 10mL sterile MilliQ in a sterile 15mL conical. This can be stored at 4°C.
3. To prepare for use, add 10µL primary stock to 2.4 mL MilliQ or TE buffer (10:1, pH=8.0). This can be kept at RT for the entire day. Mix immediately prior to use.

**TE-Buffer 10:1**

1. Prepare 500mL TE buffer 10:1 by adding 5mL of 1M Tris (pH=8.0) and 1mL of 0.5M EDTA (pH=8.0) to 494mL of MilliQ. Mix well and check pH (should be 8.0).
2. Divide over two bottles of 500mL and autoclave for 20min with the lids closed. Store at RT.
3. Filter a small volume of the TE-buffer (50mL) through a 0.2µm filter into a sterile bottle or tube prior to use.
4. \*\*Check the quality of the solution by running through the FCM at the appropriate settings. \*\* This samples should give ≤ 30 events s-1 at a flow rate of approx. 35µl min-1.

**Checking to see if the FCM is clean enough to enumerate viruses**

1. Run MilliQ on the cytometer for 10 min.
2. Take away the MilliQ sample and replace with another tube of MilliQ. Check whether the FCM is clean enough to enumerate viruses by setting the trigger to SSC and the voltage below, but close to, the level where instrumental noise starts to become significant. Typically, the SSC voltage is set around 300; the event rate at a flow rate of 35µl min-1 should then be < 75 events s-1. If the event rate is higher, the machine is too dirty and should be cleaned before use and the above steps should be repeated until it is clean.

**Calibration of Flow Rate**

1. Sheath fluid recommended 🡪 MilliQ.
2. Select the appropriate flow rate. The typical flow rate is MED, between 25 and 35µl min-1.
3. Fill a FCM tube with 2-3mL of TE and determine its weight (Xi).
4. Run the sample for 15 min. Start the chronometer simultaneously when starting the run.
5. Remove the tube and stop the chronometer.
6. Weigh the tube and calculate the flow rate (µl min-1) using the formula below:

Where *Xi* = initial weight, *Xf* = final weight, *t* = time

**Enumeration of Viruses within Sputum**

1. Turn on the heat block and set one side to 80°C and the other side to 35°C.
2. Allow the SYBR Green I working solution to thaw a RT in the dark.
3. Choose sputum samples that are roughly 2mL in volume and aliquot each sample into its own 15mL conical. Add 2mL of Sputasol (dithiothreitol) to each sample for a 1:1 mixture.
4. Incubate the mixture at 37°C for 15 minutes. The resulting mixture should be visibly homogenous.
5. Dilute each sample 1:100 in TE: adding 50µL of sputum sample into 4,950µL TE.
6. Remove 1ml of the resulting mixture and filter through a 0.02µm pore-size filter into a sterile 1.5mL microcentrifuge tube.
7. Add 5µL working stock of beads (FluoSpheres carboxylate-modified microspheres, 1µm, yellow-green fluorescent) to the 1mL sample. (final concentration = 105 beads ml-1)
8. Mix the working stock of SYBR Green I well, spin briefly in a microcentrifuge, and add 111µL to the sample. (final dilution = 1:20,000)
9. Incubate the samples at 80°C in the dark for 10min, after which the samples should be allowed to cool in the dark for 5 min before analysis.
10. Acquisition: using MilliQ as sheath fluid, run the sample at an event rate below 1000 events s-1, at a flow rate between 20 and 50µL min-1 for 1-2 minutes. Before beginning data acquisition, make sure the discriminator is set on green fluorescence and the voltage level is such that no significant electronic noise is generated. Wait for the sample flow rate to stabilize before allowing acquisition of the data; typically after 15s.
11. Wipe the sample needle with moist tissue between each analysis to reduce contamination.
12. ANALYSIS: Viruses are discriminated on the basis of the scatter and fluorescence obtained after staining; green fluorescence vs SSC. Correct the raw data for the blank (background noise) before calculating the total virus abundance per ml talking into account the dilution factor.

Notes: