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BD Influx Cell Sorter Start Up and Shut Down for Viral Tagging and Grow

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ABSTRACT

The microbiome is now seen as the engine behind Earth's nutrient and energy cycles, bioreactor and crop yields, and many diseases in humans, plants and animals. However, evidence is accumulating that viruses of these microbes modulate microbiome impacts. While virus discovery is occurring at a blistering pace, scalable experimental methods to link viruses to hosts, culture them, and phenotype them are needed. Here we improve understanding of a sequencing-enabled 'Viral Tagging' (VT) approach, and establish a variant 'Viral Tag and Grow' (VT+Grow) to rapidly capture and characterize viruses that infect a cultivated target bacterium, Pseudoalteromonas. First, benchmarking using model systems provided baseline cytometric and microscopy data for understanding how infection conditions and host physiology impact populations in VT flow cytograms. These advances are critical to interpreting, and increasing robustness across this challenging and finicky method. Next, we extensively evaluated and added an 'and grow' capability to evaluate where VT signals reflect adsorption alone or wholly successful infections that lead to lysis. Third, we applied VT+Grow to a clonal virus stock, which, coupled to traditional plaque assays, revealed significant variability in burst size – findings that hint at a viral 'individuality' parallel to the microbial phenotypic heterogeneity literature. Finally, we established a live protocol for public comment and improvement via protocols.io to maximally empower the research community. Together these efforts provide a robust foundation for VT researchers, and establish VT+Grow as a promising technology to scalably capture and characterize viruses from mixed community source samples that infect cultivable bacteria.

PROTOCOL CITATION

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null, BD, Influx, Cell, Sorter, Start, Up, Shut, Down, Viral, Tagging, Grow, FCM

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MATERIALS TEXT

BD influx cell sorter (Becton Dickinson, San Jose, CA) with lasers at 488 nm (blue) and 642 nm (red) and nine optical detectors to analyze the size, granularity, and seven fluorescences per cell
Falcon Round-Bottom Polypropylene Tubes with Caps, 5 mL (catalog #352063)
Sphero Ultra Rainbow Fluorescent Particles, 3.0-3.4um (catalog #URFP-30-2)
Spherotech 8 Peak Validation Beads (FL1, FL3) (catalog #653144)
BD Diagnostics Accudrop Beads (catalog #345249)

SAFETY WARNINGS

The lasers are high powered and should be directly looked at when turned on.

BEFORE STARTING

Are the tanks and sonicator clean? If not then wipe down with ethanol.

Start up from Dry Shutdown

1 After connecting to cytometer, go to file->workspace->QC Daily-> Courtney URFP file + click “restore laser delays”

2 Place empty sheath tank on scale (with lid) and zero the scale and put the waste tank next to it

3 Add 3L of MSM to sheath tank for a 1 day run

3.1 If the sheath tank runs dry during your run you have to recalibrate the machine with more sheath

4 Before connecting the lines, clean the connections with a wet kimwipe and MQ then connect all the lines

5 Reconnect the fluid lines around the filter

6 Rinse O-ring of waste tank before screwing the lid on (to help form a seal)

7 Turn on vacuum then Jun Air then the air switch on the right side of FCM

7.1 Wait for PSI of 10 in the waste tank

7.2 Close the pressure valve on the sheath tank and turn the air switch on

7.3 Wait for 20 PSI in sheath tank

7.4 Add water to O ring of waste tank to help seal form (to be ~10 psi)

7.5 If the psi of waste tank does not reach ~10 (e.g., ~5 psi), disassemble and reassemble the lines and lid

8 Add MQ to sonicator and add MQ to syringe + 0.2uM double filter

9 Sonicate nozzle tip upright for 30 seconds then upside for ~15 seconds then right side up again for ~15 seconds

10 Check stream by using the syringe on the nozzle tip and spraying against the edge of the sink

11 Hold syringe upright and suck out excess water then VERY gently shake the rest of the excess out of the nozzle tip

12 Without the nozzle tip, add the flush bucket to nozzle area then press rinse and let rinse for two seconds then run for two seconds

13 Add nozzle tip

14 Click rinse then take out filter from holder and tap it against the metal frame three times and replace the filter then run and backflush for 30 sec to see if sample line is clogged

14.1 If clogged, add falcon tube filled with MQ and start sample, if sample light won't turn on then leave the tube for 1-2 minutes then remove the tube and back flush for 1-2 minutes; repeat process until sample light + backflush work as expected

14.2 Leave backflush on during stream calibration

15 Bubble prime the nozzle:

15.1 If the machine has been shut down for over a month: add 3.5mL of 0.2uM filtered 70% ethanol to the debubble bucket then purge + pulse to clean the line, rinse the bucket 3x with MQ to clean it

15.2 After the ethanol wash add debubble bucket with MQ then purge/pulse three times then remove the debubble bucket and allow the machine to run for 10-15 minutes before proceeding to actual bubble prime

15.3 Put the debubble bucket under the nozzle and submerge the nozzle tip in MQ then purge till the line is full of MQ then pulse, repeat a few times

15.4 If there are still bubbles appearing between the nozzle tip and the line then remove the bucket while purging then replace the bucket with more MQ

15.5 Repeat this process till you get three purge/pulse cycles with no (or a very tiny) bubbles in the nozzle.

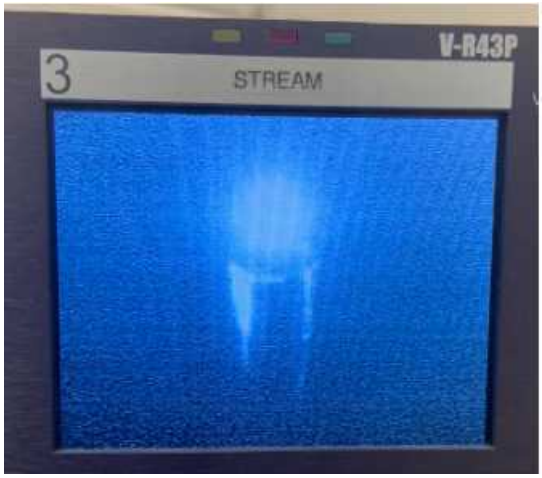
16 Turn on the lasers and make sure both lasers are at 100% power before proceeding

Calibrating the Stream

17 Remove flush bucket

18 Turn on illum button

19 While watching the stream pane, first adjust the black knobs above the nozzle to get the light centered above the waste drain then adjust the light intensity so it is slightly less than the most intense light possible



19.1 knobs can move the stream forward/backward (to adjust the brightness and focus) and left/right.

19.2 Remove any water from drain, make sure to not get kimwipe in the drain

20 Watch pin hole and drop pane, use silver knobs (behind the black knobs just used) to finely adjust the stream

20.1 In the pinhole pane, the stream should be sharply in focus and in line with the pin holes

20.2 Also you want the top of the nozzle to be slightly visible at the top of the pinhole pane, adjust this with the silver knob pointing straight up

20.3 In the drop pane, the stream should be close to the red tape marker

21 If the stream and pin hole can't be aligned try sonicating the nozzle again and restarting at the bubble prime

22 Keep the back flush on and close shutters around nozzle, and press the open circle above where the shutter handle is to turn the laser on

Laser calibration with Ultra Rainbow Bead

23 Before applying rainbow bead sample, adjust sample pressure to +1 what the sheat pressure is at

23.1 Sheath pressure should be 22, and the sample pressure around 22.9

23.2 Use the knob facing the front to adjust sample pressure and knob on the right side of FCM for sheath pressure if it is not 22 (but this will not happen often).

24 Adjust the default display count to 200 and turn the red laser off with the blue laser on (the protruding silver poles on the left side of FCM)

25 Vortex ultra rainbow bead for 10 seconds

26 Backflush for thirty seconds then add the tube with ultra rainbow beads

27 Click acquire on computer then sample on FCM

28 Adjust the blue laser with the silver knobs with blue labels to the left of FCM. You want the laser to appear in the middle of the top pin hole

29 You want the value of the median around 45k and the RCV around 2

29.1 Prioritize making the readings of the beads cluster together in the plots by adjusting two knobs for the blue laser

29.2 Adjust the parameters if necessary (the parameters move the plots)

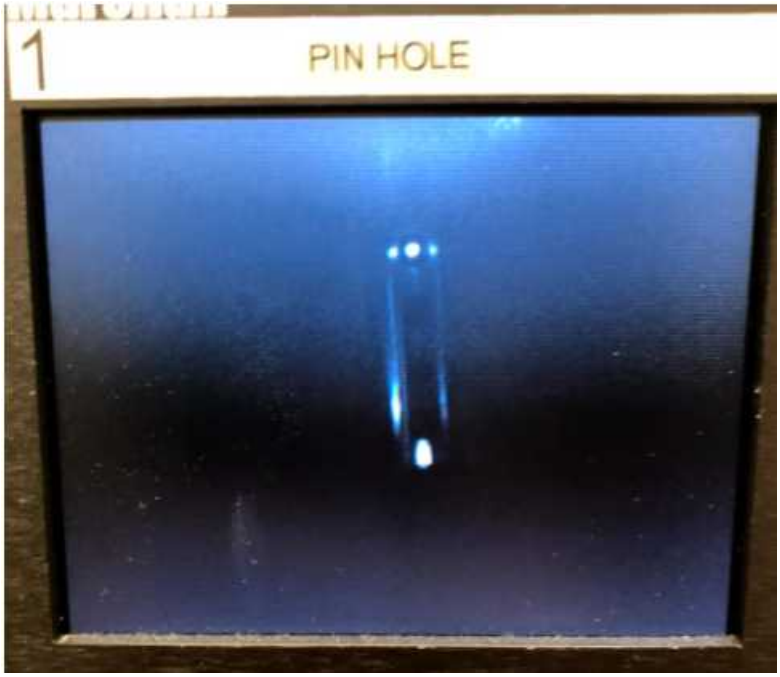
29.3 Also finely adjust once the beads are clustering and the numbers are close to what you want

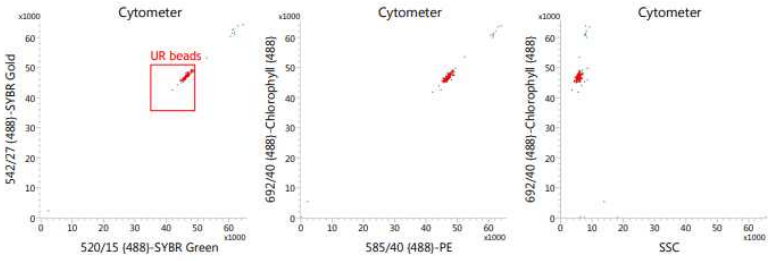
30 Turn the video knob next to the pinhole pane to video 2, adjust with right hand knobs to get an oval shape from the laser not a circle then place the laser over the top pinhole, this is the FSC adjustments and you'll want a median around 25k for FSC with RCV around 2

30.1 Prioritize making the readings of the beads cluster together in the plots

30.2 Adjust the parameters if necessary

31 Turn on the red laser, turn back to video 1, and adjust the red laser over the last pin hole with the red, silver knobs to the left of the machine, the value of the median around 45k and the RCV around 2.



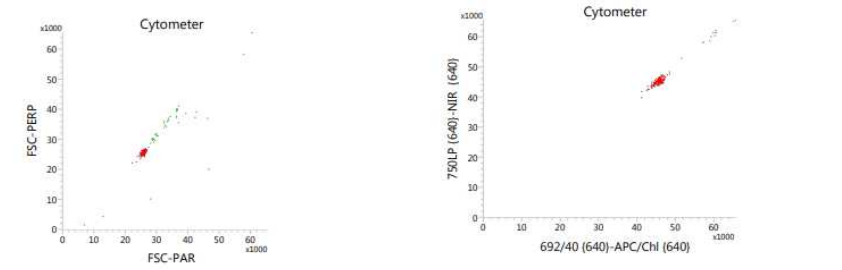


Statistics: Cytometer

Populations	Events	SSC Median	520/15 (488)-S... Median	542/27 (488)-S... Median	585/40 (488)-PE Median	692/40 (488)-C... Median
All Events	200	5,785	46,452	47,003	46,898	46,496
UR beads	156	5,574	46,226	46,755	46,691	46,247

Statistics: Cytometer

Populations	Events	SSC RCV	520/15 (488)-S... RCV	542/27 (488)-S... RCV	585/40 (488)-PE RCV	692/40 (488)-C... RCV
All Events	200	14.51	2.96	3.07	2.67	2.89
UR beads	156	10.11	1.93	2.03	1.74	2.01



Statistics: Cytometer

Populations	Events	FSC-PAR Median	FSC-PAR RCV	FSC-PERP Median	FSC-PERP RCV
All Events	200	25,771	2.47	25,923	3.19
UR beads	156	25,602	1.84	25,712	2.18

Statistics: Cytometer

Populations	Events	692/40... Median	692/40... RCV	750LP (... Median	750LP (... RCV
All Events	200	45,730	3.02	45,442	3.23
UR beads	156	45,378	2.10	45,136	2.07

Drop Delay Calibration with Accudrop Bead:

32 Switch the file in QC daily to “Courtney sorting” with laser delays checked

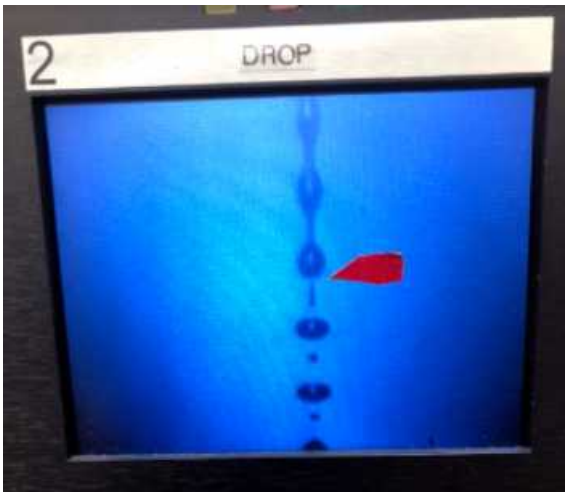
33 Close deflection plates and turn on plate button to electrify plates

34 **Set to 1.0 Drop Pure**

35 Set drop frequency to 49.4

35.1 When you change the frequency to 49.4, you can see that the shape of the mainstream is immediately changed (in the drop pane)

35.2 By adjusting drop frequency (to roughly tune drop; ~49.4) and piezo (finely; 2.0-3.5), make the shape, position, & number (5.5) of drops as the figure below.



36 Make sure the breakoff camera position is between 151-158

36.1 Caution: If you could not get the shape, position, and number of drop like the figure (above) with the piezo (~2.0-3.5), drop frequency (~49.4), and camera position (151-158), you need to clean the FCM with 5% contrad followed by MQ. Please see the clean the FCM with 5% contrad below.

37 Adjust the deflection angle to 67 degrees for both side streams

38 Turn “test stream” on (not flash charge and short flash).Then, you can see two side streams (left/right) and mainstream (middle), respectively. If two side streams from the mainstream are not bright enough, adjust first the black front facing knob to get all streams bright (if this is done, turn the lasers off and fine adjust the drop/pin hole panes again by adjusting silver knobs (behind the black knobs). Then, turn the test streams on again to check all the streams.

38.1 If you cannot still see the bright side stream even though adjusting the black/silver knobs, turn off the plate on, open the deflection plate, clean the deflection plate with a wet kimwipe, remove MQ from plate with a dry kimwipe, and let it dry for 10-20min (do not close the plate). If this does not still work well, clean the plates again just with a dry kimwipe. Please put the picture you took here to see what is the best condition.

39 Turn i) test streams ii) flash charge and iii) short flash on and at each step, adjust the piezo amplitude (not frequency) to maximize side stream’s distance from the main stream.

40 After adjusting streams, open the more tab and input the camera position number then +/- 20 to the other two variables. Then click set, this will adjust the drop delay number.

41 Clean drain

42 Set the default display count to 2000.

43 Take out the accudrop bead from the fridge (you can see the precipitate on the bottom of the falcon tube) and vortex vigorously for 10 sec until the precipitate is completely gone. Run the drop delay bead. Click the acquire.

44 Once you see the plot, adjust the daughter gates to what you expect will be 50/50 of the accudrop bead.

45 Make a contour plot with SSC y-axis and FSC x-axis

46 Click sort ready -> start then flip the knob with the orange tape above it from normal to the unlabeled orange tape (at this stage, you can see two side streams for P1 and P3, respectively).

46.1 Now decrease the drop delay to maximize the side streams and minimize the main streams

46.2 Then stop -> safe-> stop in the software window->stop run the bead

46.3 Remove paper towel under the two-tube sorting device with a new one and clean any water drops on the two tube sorting set up, which can cause the corrosion

47 Remove bead and backflush for 30 seconds

8 Peak Bead and Sorting Calibration

48 After backflush, label a P1 and P3 tube and check the streams make any adjustments if the stream is being unstable

49 set default display count to 1000

50 Set the deflection angle to 45 degrees

51 Adjust P1 and P3 gates to where you expect the populations to be

52 Take out the 8 peak bead and vortex it for 5-10 sec.

53 Run the bead and adjust the gates, first adjust the parent gate in the contour plot (tightly gate the parent population with a 300% zoom) then adjust daughter gates: P1 and P3

54 Add tubes, sort ready-> start, then use a paper towel to check that the deflection angle is good and the sorted beads are going into the tubes

54.1 Run for three minutes

54.2 Stop -> safe -> stop in the software window -> stop run the bead -> remove sample -> backflush -> add 150uL of MQ to each sorted population

55 Run P1 and P3 and make sure the its % of parent is high, >95% from the parent is acceptable, want 97-100%.

56 Backflush for 30 seconds between samples

Check 96 Well Tray alignment:

57 Adjust streams if necessary (by adjusting the piezo value at each step of test stream, flash charge, and short flash),

58 Change to 96 well tray sort and remove the 2 tube sorting apparatus then put the 96 well tray in

59 Set to deflection angle of 70 degrees then test streams, flash charge, short flash to make sure the piezo amplitude is good

60 Change deflection angle to 37 degrees (this will be flexible depending on how well the test stream is placed into the center of A1 well)

61 In the tray control make sure you see 12 dots representing the 12 columns

62 Sort ready

63 Open the tray control pane. Click the A1 in the sorting pane (not tray control) to use A1 for the sorting test. Use the tray control to move the tray around to get the dot in the center on the directional pad press the center dot to have a test drop come out

64 Click the blue circle in the tray control followed by home below the try control (make sure that the sort stream is

deflected well from drain). Check the drop of A1 well. Adjust the deflection angle as well as left/right button in the tray control pane to get the drop to be centered on A1.

65 Once the optimization of 96-well sorting is done, set up the FSC, SSC, SybrGold conditions for actual VTG.

66 After you're done with the sorting return to the 2 tube sorting option (click safe) then start shutdown

Wet Shutdown - If you plan on using the machine the next day

67 Stop running the sample then backflush for 30s

68 Turn off laser

69 Run a tube of MQ for 4 minutes

70 Turn up the sample pressure to around 23.4 so it rinses the line well

71 Leave the tube of MQ there but turn run off the sample button

72 Place the flush bucket under the nozzle and then the debubble reservoir

73 Fill the reservoir with MQ and make sure nozzle submerged

74 Stop the stream then purge for 1 minute to fill line with water

75 Stop the purge

76 Disconnect form cytometer

77 Shutdown cytometer

78 Close application

79 Shut down computer

80 Open plates and turn plate button off. Make sure stream is turned off.

81 Turn off auxiliary power

82 Remove fluid line on sheath tank

83 Set it on the FCM bench

84 Open pressure release on sheath tank

85 Wait till the pressure in sheath is 0

86 Turn air switch off

87 Turn main power off

88 Turn vacuum and jun air off

89 Remove lines from waste tank and empty waste tank, replace the waste tank but leave the lines disconnected

Dry Shutown with 5% contrad wash - if you are shutting the machine down for 3+ days

90 Wash the sample line with 5% contrad for 1 minute. Sample PSI can be increased to around 23.5.

91 Run/backflush for 30 seconds.

92 Open the shutters in front of the nozzle

93 Turn off laser

94 Wash sample line with MQW for 4 minutes

95 Occasionally (about once a month) place the flush bucket under the nozzle and then the debubble reservoir

95.1 Use 0.02m-filtered 70% EtOH followed by washing with MQW three times. Specifically, put 0.02um-filtered 70% EtOH to reservoir and wash the nozzle line by purge and pulse and stop. Take out the reservoir and wash with MQW. Then, place the reservoir under the nozzle and put MQW followed by purge and pulse. Finally, if there is no small bubble in the nozzle, stop the purge and pulse.

96 Stop backflush

97 Stop the run

98 Open the plates and turn the plate button off

99 Put the flush bucket under the nozzle

100 Depressurize the sheath tank and remove the sheath lines

101 If you think the waste tank is also already filled, you can empty the waste tank in this step and then reconnect the lines

102 Dump the rest of the sheath tank then zero the tank again

103 Add 1L MQ to the sheath tank and wash down sides w/ MQ, reattach the lines to the sheath tank, close the pressure

valve and allow the repressurize

104 Rinse and backflush for 1 minutes

105 Run and backflush for 2 minutes

106 Rinse and backflush until the scale reads 0.16kg then stop the backflush and rinse

107 Depressurize sheath tank

108 **DO NOT LET 5% CONTRAD INTO FILTER**, disconnect the fluid lines around the filter and reconnect them to each other

108.1 disconnect the blue-colored connectors (plastic) that are connected to the filters (one is from the sheath line of the sheath tank to filter and the other is from the filter to FCM). Then connect the blues connectors to each other so that 5% contrad from sheath tank can directly go to FCM but not through the filter

109 Add 1L of 5% contrad to the sheath tank

110 Rinse and backflush for a few minutes

111 Run and backflush for about 4 minutes to clean the sample line with a higher pressure

112 Rinse and backflush until 5% contrad runs out

113 Depressurize sheath tank

114 Disconnect lines on waste tank then dump the waste tank

114.1 Use the waste in the tank (since it contains contrad) to rinse the sides of the tank at least three times

115 Dump what is in the sheath tank as well

116 Reconnect the waste tank and the sheath tank

117 Zero the scale again (with lid) then add 4L of MQ

118 Rinse of the connectors then reattach the fluid and air lines to the sheath tank

119 If there is a bubble in the nozzle, do a bubble prime to clean the nozzle

120 Rinse and backflush till the tank runs out of MQ

121 Switch to run and backflush as well

122 Disconnect sheath and air lines from the sheath tanks and reconnect them to each other

123 Take off the nozzle and put inside the machine

124 Rinse and backflush for 5 minutes to dry the machine

125 After the dry time, disconnect the sheath tank to clean it by rinsing three times with MQ

126 Clean the sonicator by rinsing with ethanol

127 Stop the rinse and backflush

127.1 Double check that the two tube sorting set-up is in place

128

129 Disconnect cytometer -> turn off cytometer -> shut down computer

130 Turn off auxiliary power

131 Remove waste tank and dumping the contents then rinse with MQ that is inside the tank

132 Place both tank upside down next to the sink

133 Turn air switch off

134 Turn the vacuum off

135 Turn the main power off

Dry Shutown - if you are shutting the machine down for 3+ days and didn't work with sticky cells

136 Wash sample line with MQW for 5 minutes

137 Stop backflush

138 Stop the run

139 Open the shutters in front of the nozzle

140 Turn lasers off

141 Open the plates and turn the plate button off

142 Put the flush bucket under the nozzle

143 Depressurize the sheath tank and remove the sheath lines

143.1 If you think the waste tank is also already filled, you can empty the waste tank in this step and then reconnect the lines

144 Add 1L MQ water to the sheath tank and wash down sides with the MQ water, reattach the lines to the sheath tank, close the prssure valve and allow to repressurize

144.1 Rinse and backflush for 1 minute and immediately stop the rinse and backflush

144.2 Disconnect the blue connectors (above) from the filters and reconnect them to each other (caution: before disconnecting the blue ones, make sure that MQW completely stops. If not, when you disconnect the blue ones, MQWs spurts out of the lines)

144.3 Do bubble prime for nozzle line with MQW by purge and pulse just for several times since if you repeat this many times then mqw in the sheath tank run out (this bubble prime also uses the mqw in the sheath tank)

144.4 Rinse and backflush until the sheath tank run out of MQW

144.5 Stop rinse and backflush

145 Disconnect the sheath and air lines (silver ones) from the sheath tanks and reconnect them to each other.

146 Rinse/backflush to remove any remaining waters from the FCM lines (just for 5 sec?)

147 Remove the nozzle tips and put inside the machine

148 Rinse and backflush for 5 minutes to dry the machine

149 During the dry time, disconnect the sheath tank (open the depressurized valve on the sheath tank) to clean it by rinsing three times with MQ

150 Stop the rinse and backflush

151 Disconnect cytometer -> turn off cytometer -> shut down computer

152 Turn off auxiliary power

153 Remove waste tank rinse with MQ that is inside the tank then dump the rest

154 Place both tank upside down next to the sink

155 Turn air switch off

156 Turn the vacuum off

157 Turn the main power off