

**INSTITUTO DE MEDICINA MOLECULAR JOÃO LOBO ANTUNES
FLOW CYTOMETRY FACILITY
SORTING PROCEDURES**

(BD FACSARIA III & FACSARIA FUSION)

AUTHORS: Andreia Santos
Rute Gonçalves
Mariana Fernandes

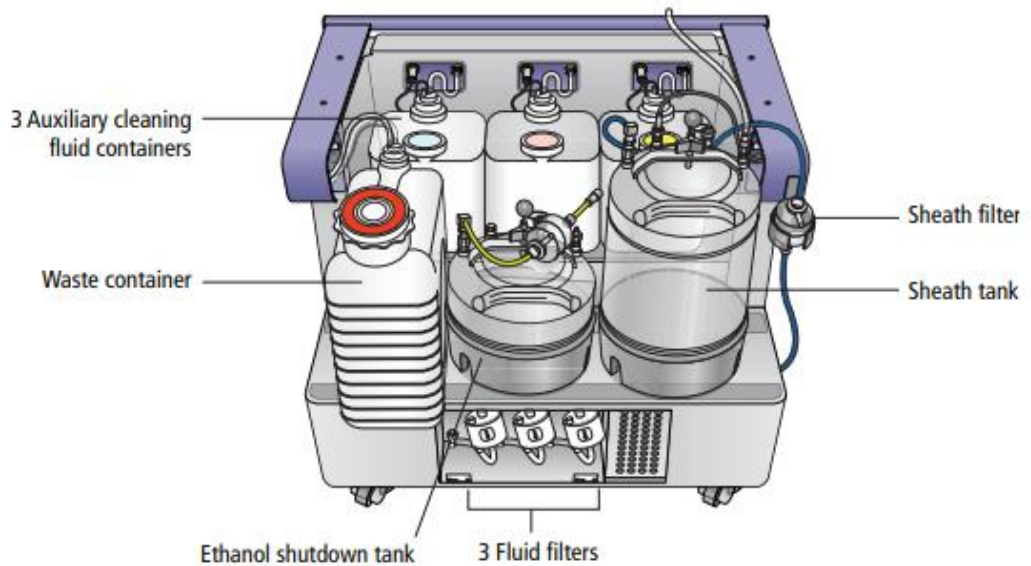
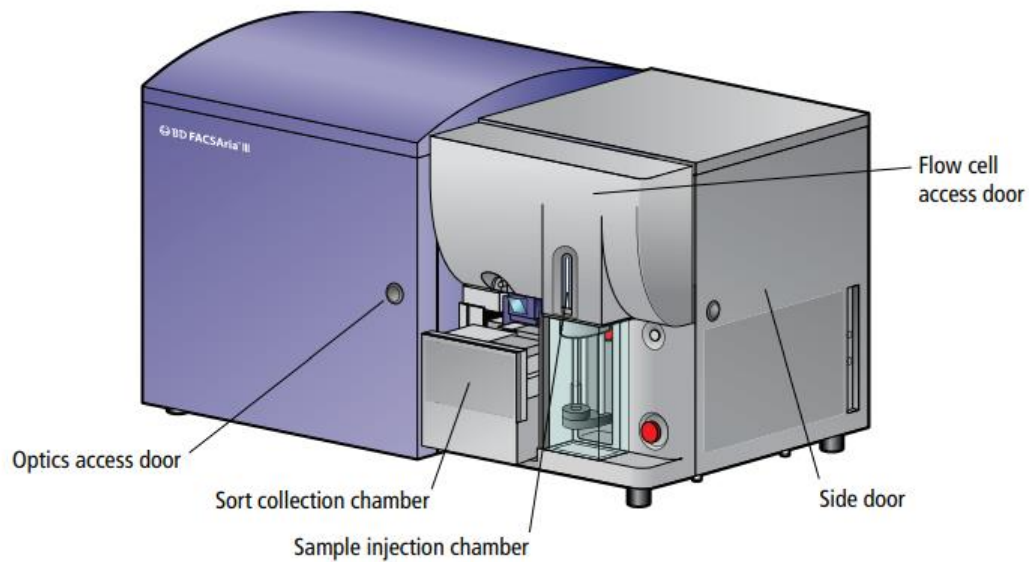
APPROVAL: Mariana Fernandes

CONTENTS

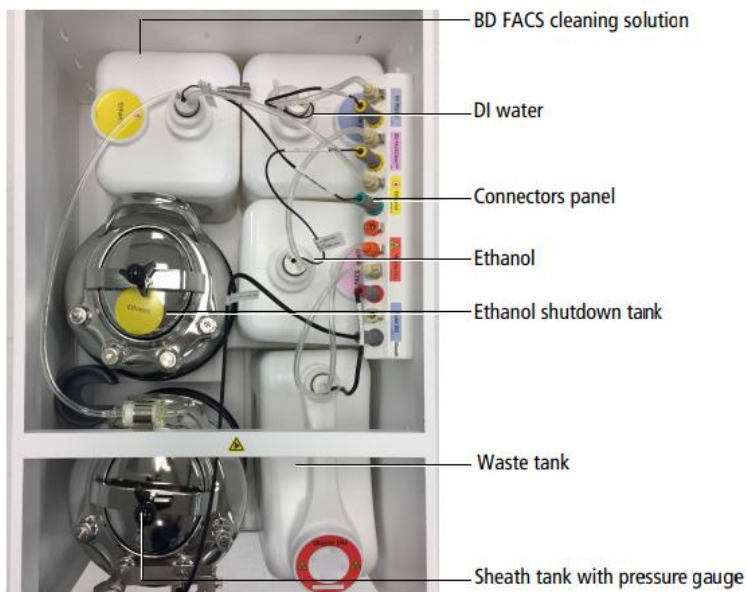
	Pag.
EQUIPMENTS.....	3
SORTING SETUP AND QUALITY CONTROL.....	6
CELL SORTING.....	11
SORTING SHUTDOWN AND MAINTENANCE/ DECONTAMINATION.....	14

Equipments

BD FACSAria III



BD FACSAria Fusion



Sterile hood (BD FACSAria Fusion)

The hood has three buttons (Off – Low – High) on the left to control the AMS (Aerosol management system) to exhaust the aerosols from the area around the collection tube holder. It is recommended to turn on the AMS if human samples are being processed.

On the right, there are three “Cabinet Control” buttons: the first to switch on the light, the 2nd to turn ON the airflow of the sterile hood and the last one to pause the alarm (if the window is open).

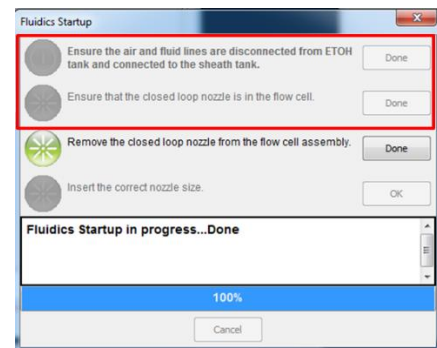
The light should be switched off during acquisition.

The airflow should be always switched on.



Sorting Setup and Quality Control

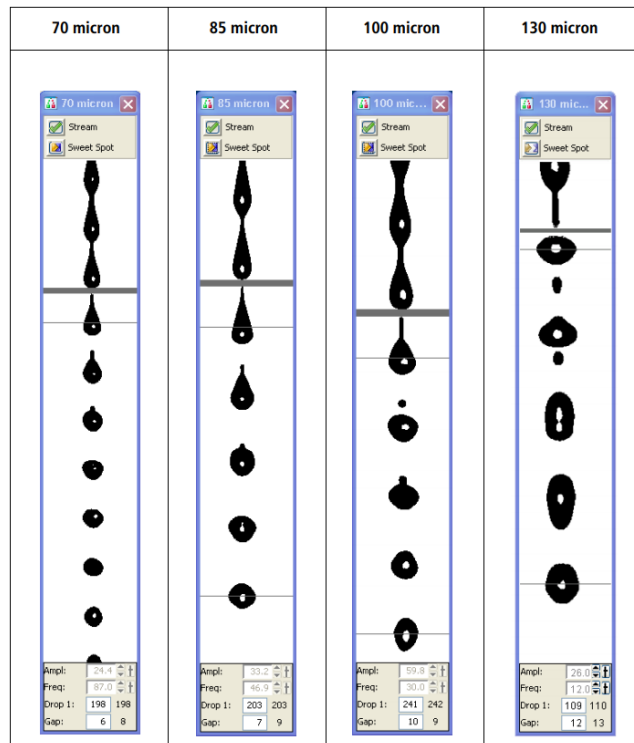
1. Switch on compressed air.
2. Switch on the computer.
3. Wait a couple minutes.
4. Switch on the instrument (BD FACSAria III – Green button on the left panel; BD FACS Aria Fusion - green button on the right side of the sterile hood). For FACSAria Fusion, turn ON the airflow of the Sterile hood (by clicking on the 2nd button on the right panel). If working with human samples, also turn ON the AMS system, selecting the Low option (use the High option only if there is a clog issue).
5. Login on windows username: Flow Cytometry User; Password: BDIS
6. Lift flow cell access door. (This blocks laser emission, avoiding fixation of PBS crystals to the flow cell walls or burning the flow cell gel, which can be damaging)
7. Launch FACSDiva software.
8. Log in in Administration account.
9. Wait a couple of minutes while the system connects to the cytometer.
(On Mondays, before the Fluidics Startup (step 10), we perform a Prepare for Aseptic sort and we install a new autoclaved PBS tank. Also, the PBS filter was connected to the Ethanol tank the whole weekend, so we empty it and we reconnect it to the new PBS tank. Make sure that in the end, the lines/filters are all in the right place)
10. Perform Fluidics Startup: **Cytometer Menu > Fluidics Startup**, following the wizard instructions.
 - a. Be sure that the sheath tank is filled and the waste tank is empty.
 - b. Disconnect the blue fluid line (without the filter) and the transparent air line from the Ethanol tank.
 - c. Connect both lines to the sheath tank (resp. Sheath filter).
 - d. After confirming each step in the software, startup procedure will start.
11. Remove the closed loop nozzle from the flow cell.
12. Insert the correct nozzle (red o-ring must point upwards), ensuring the cytometer pressure settings are appropriate for the nozzle size:
 - a. To change the configuration, go to the Instrument **Menu > Cytometer Configuration>Set configuration** to select the correct configuration.
 - b. Press “ok” and close the window.
13. Wait for cytometer’s connection and click “Use CST Settings”.
14. Turn ON the stream.
15. Open the sorting chamber door and ensure stream is falling in the center of the waste aspirator.
16. If not, use a hexagonal screwdriver to adjust the sorting chamber position so that the stream is aligned with the waste.
17. Close the sorting chamber door.
18. Perform a quick clean cycle of 10 minutes FACS Clean and 10 minutes dH2O.



19. Close the flow cell access door to open the laser shutter.
20. Before running the quality control beads, ensure the lasers had enough time to warm up (ideally 30 minutes).
21. Evaluate the instrument optical alignment and detector sensitivity by running Quality Control beads – CS&T and/or Rainbow Beads.
22. Close the CS&T menu, wait for the cytometer’s connection and click “Use CST Settings”.
23. Establishing a stable drop pattern in the breakoff window is one of the most important steps in getting optimal results from the system. Adjust the amplitude until the drop breakoff is approximately in the top third of the Breakoff window.
24. Do not exceed 70 volts. If you cannot achieve a drop breakoff at <70 volts, do the following:
 - Check the flow cell for air bubbles. If you see bubbles, turn the stream off and back on.
 - Make sure the sheath pressure and drop drive frequency are appropriate. See *Table* below for starting values.
 - If the amplitude is <10 volts, turn on attenuation in the Side Stream window.

Setting	70 micron	85 micron	100 micron	130 micron
Sheath Pressure	70	45	20	10
Amplitude	60	32	12	24
Frequency	87	47	30	12
Drop 1	150	150	150	150
Gap (upper limit)	6 (14)	7 (17)	10 (21)	12 (21)
Attenuation	Off	Off	Off	Off
Drop Delay	47.00	30.00	27.00	16.00
Far left voltage	100	100	80	60
Left voltage	40	35	30	20
Right voltage	40	35	30	20
Far right voltage	100	100	80	60
Plate voltage	4,500	4,000	2,500	2,000
2nd Drop	20	20	10	0
3rd Drop	10	10	5	0
4th Drop	0	0	0	0
Laser Delay (blue)	0.00	0.00	0.00	0.00

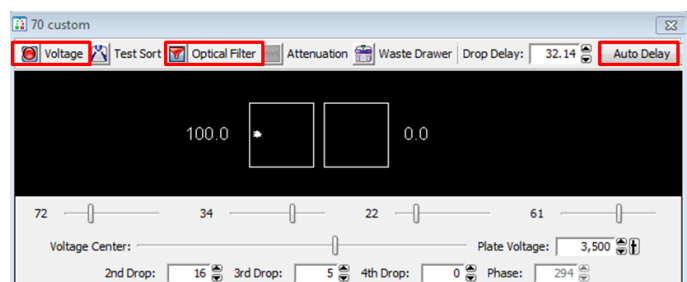
25. Verify that the small satellite droplets are merging with the large droplets. The satellite drops should merge into the drops in 5 satellites or fewer. If the satellites are not merging, you might need to clean the nozzle by sonicating.
26. Enter the actual Drop 1 value as the target in the Drop 1 field.
27. Confirm that the stream is stable and has a suitable profile for sorting, as in the *picture* on the right. Turn on the Sweet Spot when the drop pattern is stable.



28. Open Accudrop Charge Delay Experiment.
29. Turn ON the deflecting plates voltage and do a test sort.
30. Confirm that there is no fanning in the side streams.
31. Looking to the Side Stream window display, adjust the micrometer dial on the left side of the flow chamber to obtain the brightest bead spots.
32. If any fanning is observed, adjust the correction factors for the drop charge applied to the 2nd, 3rd and 4th drops at the bottom of the Side Stream window.
33. Calculate the drop delay; see section “*Calibrating Drop Delay*”.
34. Prepare the recipient holder for cell collection (2 falcons, 4 tubes, 96 wells...).
 - a. If sorting to a plate, do not forget to install the splash shield.
35. Turn the deflecting plate’s voltage ON and do a test sort.
36. Confirm that the stream is falling into the acquisition recipients.
 - a. If using Eppendorfs, tubes, falcons: while doing the test sort, open the waste drawer, do a quick test sort and check if the drop is falling into the center of the acquisition recipients. Change the aperture values using the voltage sliders of the far left, left, right and far right side streams to make them fall at the center of the recipient.
 - b. If using plates or slides, see section “*Align for plates or slides*”.
37. Log in to the user’s account.
38. Open the Sort Report file and fill the fields accordingly.

Calibrating Drop Delay

1. Open the “Drop Delay” experiment in FACS Diva, and select the tube called “Drop Delay”.
2. Prepare a tube of Accudrop beads (ideally 1 drop per 600 μL for the 70 μm nozzle; or 1 drop per 1 mL for the 100 μm nozzle) and install it on the loading port.
3. Open the Sort Layout named “Accudrop Drop Delay”.
4. Load the tube containing the Accudrop bead suspension.
5. Adjust the flow rate to achieve an event rate of 1000–3000 events per second for the 70 μm nozzle; 800–2000 events per second for the 85 μm nozzle; or 600–1500 events per second for the 100 μm nozzle.
6. Turn ON the voltage plates and the optical filter.
7. Click to Sort on the Sort Layout window and choose the option Cancel to avoid the waste drawer to open and sort the beads to the waste.
8. Adjust the micrometer dial to obtain the brightest bead spot on the center stream.
9. If the left side stream is not completely contained in the left region, adjust the voltage slider to place the stream in the center of the region.
10. Confirm that the sum of the intensity values of the left and central stream is 100%.
 - a. If not, confirm the event rate is within the recommended range (see point 5 above);
 - b. Adjust the micrometer dial on to obtain the brightest bead spots on the left and central streams.
11. Click the Auto Delay button on the Side Stream window.
12. Select Start Run in the Auto Drop Delay dialog.
 - a. To calculate the Drop Delay manually, use the precision mode “Initial” for an initial estimate, and at the end use the “Fine Tune” mode to account for very small changes.
13. After the Auto Delay calculation, confirm the Drop Delay value is optimal by increasing and/or decreasing its value in 0.03-drop increments (click arrow control). Ideally, the intensity of the left stream should be as close to 100% as possible, and the central stream close to 0 (the sum of the two values should be 100%).
14. Change the Drop Delay value to 1 drop above and below the optimized value. This should make approximately 100% of the bead brightness to fall into the right region, leaving the left region with approximately 0. Choosing one unit above or below should provide similar results. If not, optimize the Drop Delay value to ensure this happens.
15. IMPORTANT: return to the optimized Drop Delay value and confirm that approximately 100% of the bead brightness is detected on the left region, and 0 on the right region.
16. Stop the sorting; turn off the deflection plates; click the Optical Filter button to move the emission filter away from the camera; and unload the tube.



Align for plates or slides

1. Install the splash shield below the aspirator drawer.
2. In the Sort Layout window, click the Access Stage button to bring the ACDCU stage to the front.
3. Click the Voltage button to turn ON the deflection plates.
4. Open the sort block door and the aspirator drawer.
5. Do a test sort and adjust the far left stream to fall within the hole of the splash shield. Do not adjust the voltage sliders of the other side streams.
6. Go to **Sort > Home Devices** (if the collection device is standard) or **Sort > Custom Devices** (if the collection device is customized).
7. Select the collection device that will be used.
8. Click “Go to home”.
9. Place the test collection device on the tray support.
10. Turn ON the Voltage.
11. Double-click the Test Sort button to deposit a drop at the home location.
12. Inspect the collection device to see where the drop has fallen. The aim is to target the center of the slide – if sorting onto slides; or the center of the A1 well – if sorting to plates.
13. Wipe the collection device dry and place it back on the tray support.
14. Adjust the home location, if necessary: click the appropriate arrow buttons to move the tray support as needed. Large arrows move the tray by five-steps. Small arrows move the tray by one-step.
15. When the drop is falling in the correct position, click “Set home”.
16. If a custom device is being used, adjust the position of the last well by clicking “Go to end”, adjusting as described in step 12. At the end, click “Set End”.
17. Click the Voltage button to turn off the deflection plates.
18. In FACS Diva software, open the “Drop Delay” Experiment and run a tube of Rainbow beads suspension.
19. Sort 100 beads to:
 - a. The center of the slide, if sorting onto slides.
 - b. Each well of the first and last columns of the plate (A1, B1, ..., Z1 and An, Bn, ... Zn), if sorting into plates.
20. Check if the bead drop fell exactly in the center of the slide/well.
 - a. If not, repeat steps 8 to 15 until it does.

Cell Sorting

1. Under the User's account in FACS Diva, create a new experiment and name it "[YYYY_MM_DD] [User's name]".
2. In the "Cytometer" window, go to the "Parameters" tab and delete all parameters that will not be used in the experiment.
3. If the experiment needs compensation:
 - a. Go to **Experiment > Compensation Setup > Create Compensation Controls**.
 - b. Select/Unselect the box that asks whether you have an Unstained sample.
 - c. Without recording, acquire all single staining controls and check that there is good/maximal resolution between the negative and positive populations in the primary detectors.
 - d. Adjust the voltage for each detector whenever it would improve resolution of positive from negative populations, avoiding excessive spread of negative populations.
 - i. Note that the voltage of the fluorescence detectors cannot be changed after compensation setup.
 - e. Record all single staining samples (and unstained if present).
 - f. Make sure you have at least 300 events on the P2 (positive) gate of each single color.
 - g. Go to **Experiment > Compensation Setup > Calculate Compensation**
4. Create a new specimen and name it "Sort [YYYYMMDD]"
5. Create a new tube and name it "Presort [sample name]" (Name the sample tubes ensuring the type of tissue and the treatment or condition allowing the distinction between the total samples of the experiment)
6. Set up the experiment layout.
 - a. **IMPORTANT:** select FSC-H, FSC-W, SSC-H and/or SSC-W to enable doublet exclusion.
7. Label all the channels (e.g. CD8-FITC).
8. If necessary, set up voltages for FSC-A and SSC-A.
9. If necessary, adjust the FSC-A scaling factor and replace the FSC Neutral Density (ND) filter (for larger cells use ND filters with larger numbers and vice-versa).
10. Load the sample to be sorted and record 10,000 events using the Normal Worksheet for each tube, instead of a Global Worksheet. (Eventually record more events if working with rare populations and/or if the researcher asks for it)
11. Confirm the compensation performed automatically, checking all the colors in the sample. Adjust manually if needed.
12. Establish the gating strategy based on the FMO controls, if they are available.
13. Confirm the gating strategy applies well to the sample and define the populations to be sorted.
14. Perform the back gating of all populations of interest through the entire gating hierarchy.
15. Create a sort layout and set it to acquire the desired populations.
 - a. In the Sort Layout window, select the type of device from the Device menu.
 - b. Select the sort precision mode from the Precision menu.

- c. Regarding the options from the Save Sort Reports menu, select **Save All**.
- d. Enter the number of events to be sorted in the Target Events field or add limits to the number of cells to be sorted according to the type of collection tube:

Tubes	Precision mask	Collection volume	Max. number of cells		
			70 µm nozzle	85 µm nozzle	100 µm nozzle
1.5 mL tubes	4-Way Purity	250 µL	1 100 000	420 000	350 000
		500 µL	800 000	320 000	200 000
	0-16-0	250 µL	1 000 000		400 000
		500 µL	800 000		300 000
	Yield	500 µL	400 000		100 000
FACStubes	4-Way Purity	250 µL	3 700 000	1 700 000	1 200 000
		500 µL	3 500 000	1 400 000	1 000 000
		1000 µL	3 000 000	1 200 000	800 000
	0-16-0	250 µL	4 000 000	1 600 000	1 000 000
		500 µL	3 500 000		800 000
		1000 µL	3 000 000		600 000
	Yield	1000 µL	1 700 000		500 000
	15 mL tubes	4-Way Purity	500 µL	12 000 000	
1000 µL			11 000 000	5 200 000	3 750 000
2000 µL			10 000 000	4 800 000	3 500 000
3000 µL			9 000 000	4 400 000	3 200 000
0-16-0		500 µL	12 000 000	5 000 000	3 500 000
		2000 µL		4 200 000	3 200 000
		3000 µL			3 000 000
Yield		1000 µL	6 000 000		2 000 000

- e. Select the field(s) corresponding to the tube(s), well(s), or spot(s) where the population will be sorted and select a defined population from the Add menu.

16. Start sorting.

17. Throughout the sorting, ensure:

- a. There is a low number in the Events to Display tab, to quickly see any variation while acquiring the sample (e.g. variations in SSC-A and FSC-A pattern)
- b. The stream remains stable (look like at the beginning of the sort and the Drop 1 value is similar at the beginning). If not stop the sorting, adjust the stream and check the Drop Delay.
- c. That there is no fanning.
- d. The number of events is stable. If not unload the tube and agitate the sample.
- e. Sort efficiency (>90%). If not filter, dilute, vortex the sample or decrease flow rate.
- f. That the populations fall inside the gates that were set at the beginning.

18. Write down the number of collected cells for each sample in the Sort Report file.

19. Check purity of the sorted samples (see section “Purity Check”) and write it down in the Sort Report.
20. Export the Experiment form the Software to the folder *D:\Backup por Unidadelyear\semester\PI*
21. Complete all the information requested in the Sort Report file, save it and export as PDF to the folder *C:\Users\Admin\Desktop\AriaIII* or *C:\Users\Admin\Desktop\AriaFusion*.
22. Send an email with the Sort Report (and the Sort Layouts, if the researcher asks for it).

Purity Check

1. On the software, duplicate the sorting tube and name it “*Purity [tube name]_[population name]*”
2. Run the sorted sample at flow rate 1, and record 200 events inside the first gate (cells).
 - a. The percentage of events of the population of interest is expected to be higher than 95% when cell sorting was performed with a sort precision of Purity or higher.
3. In the sort report file, write all the purities in the purity check well for of all the samples. (If the sorted population has <3000 cells, we only check the purity if the researcher asks to)

After Sorting

1. Run FACS Clean for 10 minutes (flow rate 5.0).
2. Run H₂O for 5 minutes (flow rate 11.0).
3. Switch off the stream.
4. Remove the Nozzle from the flow cell assembly.
5. Insert the integrated closed-loop Nozzle into the flow cell assembly.
6. Clean flow cell with FACS Clean (3x) and H₂O (5x), especially if using viability dyes.
7. Shutdown the Cytometer.
(See next Section for the complete shutdown and other cleaning procedures)

Sorter shutdown and maintenance/decontamination

To ensure a proper performance of the instrument, and to establish a regular cleaning, the facility staff should do the recommended maintenance procedures below.

Cleaning the flow cell

Summary:

Cleans the sample path and the flow cell

Frequency:

As a daily basis, it is recommended to run a clean flow cell with H₂O in the beginning of the sorting/analysis and with FACS Clean and H₂O in the end, before the shutdown.

It can also be performed whenever the equipment shows poor optical performance – distorted scatter or high CVs

Sample line backflush

Summary:

Flushes the sample line with sheath fluid

Frequency:

After running samples with adherent cells or dyes.

Prime after tank refill

Summary:

Primes the fluid lines for the designated fluid(s)

Frequency:

When a fluidics line is unplugged to refill a tank (once a week during the weekly cleaning)

Fluidics Shutdown

Summary:

Removes sheath fluid from the lines and fills them with 70% Ethanol.

Frequency:

After finishing the sorting/analysis, in the end of the day.

1. Unload sample tube, if one is loaded.
2. Open the flow cell access door.
3. On the Diva breakoff window, turn the stream OFF.
4. Remove the Nozzle from the flow cell assembly.
5. Insert the integrated closed-loop Nozzle into the flow cell assembly
6. Clean flow cell with FACS Clean (3x) and H₂O (5x), especially if using viability dyes).
7. Go to the menu **Cytometer > Fluidics Shutdown** and follow the indications provided by the software.
8. Be sure that the ethanol tank is filled.
9. Disconnect the blue fluid line (without the filter) and the transparent air line from the sheath tank.
10. Connect both lines to the ethanol tank (respective ethanol filter).

11. After confirming each step in the software, shut down procedure will start.
12. At the end, you are prompted to “*Install a tube with cleaning solution on the loading port, and click OK*”. Prepare a new tube of H₂O, install it and select OK.
13. Switch off the instrument (green button).
14. Wipe down all cytometer surfaces that have been exposed to sheath fluid. Clean surfaces with a cloth dampened with 5% bleach solution or 70% ethanol, followed by H₂O. (The following surfaces should be inspected and cleaned when necessary: Sort chamber; Deflection plates; Sample loading port; Collection devices)
15. Switch off the hood controls.
16. Switch off compressed air.
17. Exit FACS Diva Software and switch off the computer.
18. Check if the waste tank is getting full. If so, swap it by an empty one.
19. Vent the air pressure from the sheath tank by pulling up on the ring on the pressure relief valve. Fill the sheath tank to the line with PBS.
20. The ethanol tank is refilled every second day. If it is a fill day, fill it.

Prepare for aseptic sort

Summary:

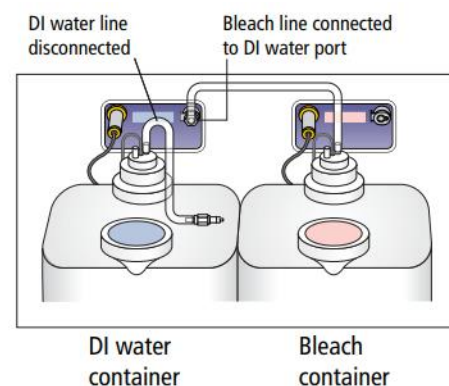
Decontaminates the entire sheath path and sample path. It removes sheath fluid from the lines and fills them first with FACS Clean, then with H₂O and finally with 70% Ethanol.

Frequency:

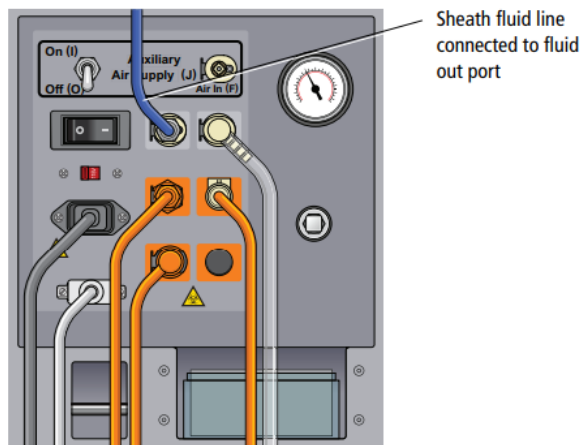
At least once a week, to avoid contaminations inside the equipment. We always perform it before installing a decontaminated and autoclaved sheath tank (Mondays) (*See next section on how to autoclave sheath tanks*).

Also, whenever needed to perform an extensive cleaning if the system is used to process different sample types, or if the system has contamination problems.

1. Select **Cytometer > Cleaning Modes > Prepare for Aseptic Sort**.
2. Follow the instructions on screen as you perform the procedure.
3. Make sure you have the integrated closed-loop nozzle into the flow cell assembly.
4. Remove the fluid line from the DI water port and connect the fluid line from the bleach container.
5. Disconnect the bleach fluid line from the DI water port, and connect it back to the bleach container port, then reconnect the fluid line to the DI water port, and click Done.



6. Disconnect the fluid line from the sheath/ethanol tank and connect it to the fluid out port on the side of the fluidics cart. Click Done.
7. The system cleaning takes approximately 20 minutes.
8. Disconnect the fluid line from the fluid out port on the side of the fluidics cart and connect it to a new 0.2- μm sheath filter and decontaminated and autoclaved sheath tank.
9. To complete the process, select one of the two options: to continue running samples, perform fluidics startup or to turn off the system, perform the flow cell cleaning procedure.



Autoclaving the Sheath Tank

1. Disconnect the sensor cable from the solutions cart.
2. Disconnect the ethanol filter line from its tank and put it aside.
3. Disconnect the filter from the PBS tank and connect it to the ethanol tank.
4. Disconnect the transparent air line from the sheath tank and connect it to the Ethanol one.
5. Start the Shutdown procedure (see previous section)
6. Prepare a lab cylinder and a beaker with 70% ethanol.
7. Remove the sheath probe:
 - a. Vent the air pressure from the sheath tank by pulling up on the pressure relief valve. Verify that all of the pressure is released by pulling up a second time.
 - b. Open the lid by loosening the black screws. Take the black o-ring from the lid and both screws into the 70% ethanol beaker prepared in step 3.
 - c. Loosen the nut at the top of the probe with an 11/16-inch wrench.
 - d. Loosen the thumbscrew on the containment device.
 - e. Pull the top section of the containment device straight up and out of the bottom section.
 - f. Finish loosening the 11/16-inch nut at the top of the probe and pull the probe straight up and out of the sheath tank.
 - g. Decontaminate the sheath probe using the 70% ethanol cylinder prepared in step 3.

8. Discard the PBS in the tank.
9. Add AquaClean Solution to the tank.
10. Take the tank to the Washing Room for decontamination and autoclaving.
11. Once the tank is autoclaved, we put it aside until next week.
12. Next week we will install it after the Prepare for Aseptic Sort procedure (Mondays).

