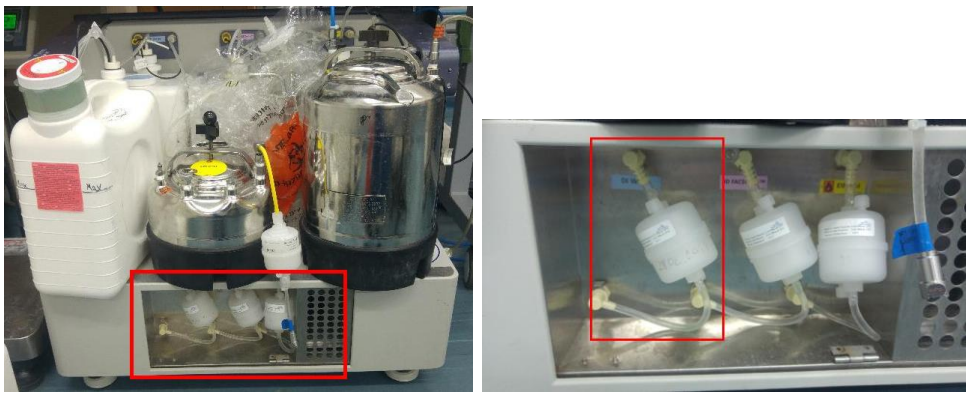
Preparing FACSAria for STERILE sorting

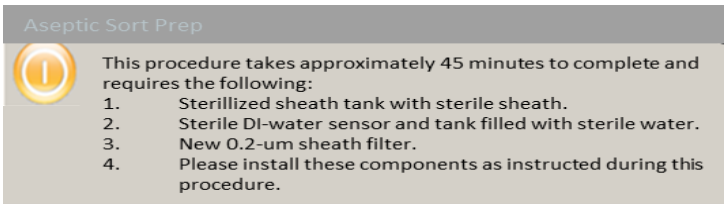
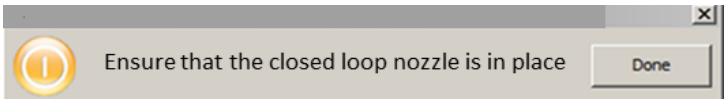
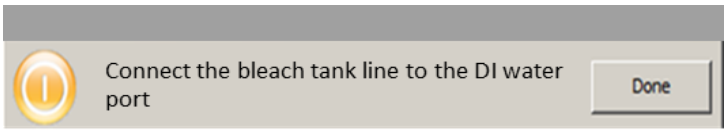
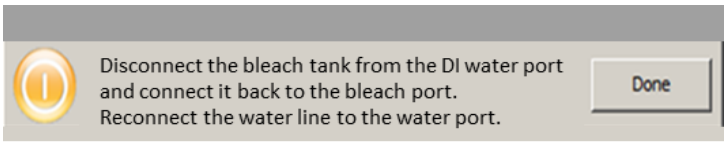
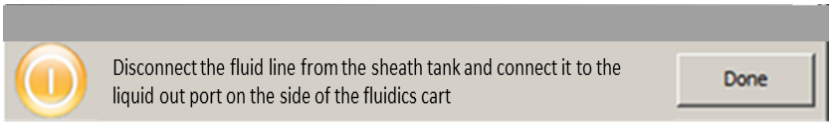
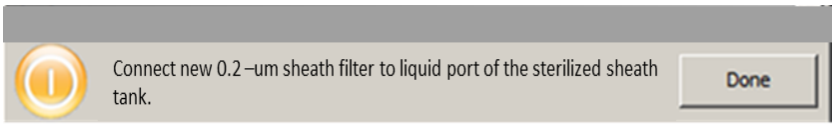
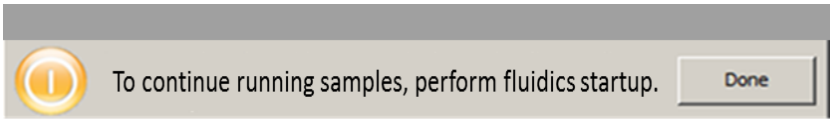
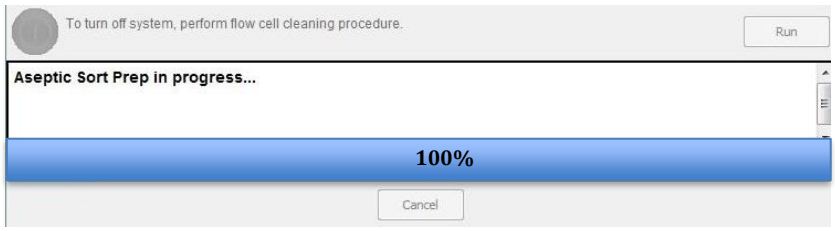
Fluids	Remarks
➢ Prepare 10L of **PBS (Mg- Ca-)** in a stainless- steel-sheath-tank & Place it in a biohazard bag. ➢ Fill the designated glass container with 500ml DW & Place it in a biohazard bag. ➢ 1 FACS tube with at list 3ml sterile DW ➢ 1 FACS tube with at list 3ml sterile PBS	➢ Each tank has a number. Document tank number and your details in the tanks logbook. Document also the date of return. ➢ Following autoclave, Close the lids of the DW and PBS containers. ➢ Fill the required information in the designated stickers. ➢ **Do not stick anything directly on containers.** Instead, attach stickers to the plastic bags.

	STEPS	NOTES
1.	If the computer is off, turn it on, login with: Username - **Operator** Password – **public**	
2.	**Turn on the FACSAria**	green button on the left side of FACSAria
3.	**Turn on the lasers**	White button / mechanical knobs / Software
4.	**Upload BD FACSDiva software**	Diva is a heavy & slow program. Anything within 3 minutes response is normal. Be patient!
5.	**Log in** with your labs' username and password **In the popup window, choose "use CST setting"**	For public use, login with Username - **public** Password - **public**
6.	**Turn on the cooling system** ("LAUDA") (beep sound for a couple of seconds is normal)	It takes **~15 minutes** to reach default settings of 4°C -8°C. **Confirm** temperature before you begin to sort.

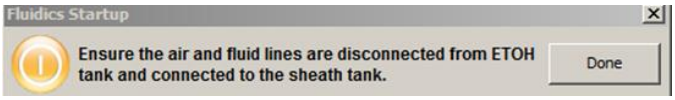
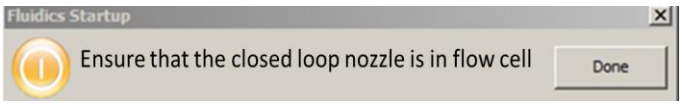
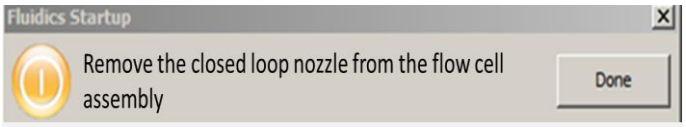
Checking the fluids of the cart

7.	**Check the waste container status.** To pass start-up with no problems, the waste tank should be no more than ¾ full.	
8.	**Check Ethanol tank status.** Ready-made 70% Ethanol is near the sink, in 5 L plastic containers.	
9.	**Place sterile steel Sheath tank on the scales.**	The scales allow tracking the PBS level as you run.
10.	**Check DW filter status.** Confirm it if full. When needed fill as you were instructed. 	

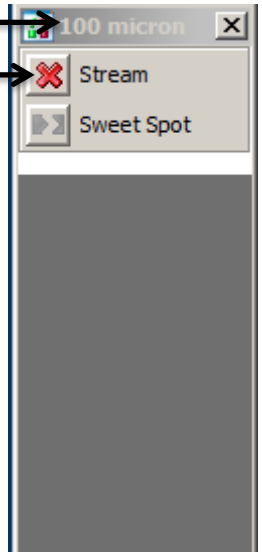
11.	**Make sure the container lid is firmly closed.** Make sure the filter on top of the DW container is standing straight.	
12.	**Confirm no air in the DW container and Replace the previous sterile DW container with the fresh one you prepared.**	For that use the designated syringe, or execute "prime after tank refill" [instructions in page 10]
13.	Go to "cytometer" ➡ "cleaning mode" ➡ **"prepare for aseptic sort"** **Pay attention to the notes**.	Follow the steps in the pop-up window.
14.	**Open the sorter cover**	**Total** sterilization procedure takes 45 minutes

15.		**Ignore this step and press 'Done'**
16.		
17.		**No remarks**
18.		**No remarks**
19.	 **Prepare sterile work environment**: change diaper on bench. Bring a sterile petri-dish (used as sterile space for tube caps)	**No remarks**
20.	 **Instead,** disconnect the blue line from the side of the cart and connect it **directly** to the sterile sheath tank. Then, connect the transparent (air) line to the sterile tank. Press Done for the coming 2 steps And then press following 'Run'. **This step takes 25 minutes.**	**Ignore this step.**
21.		**Ignore this step and press 'Done'**
22.	 **The software will direct to a new instructions-window of "Fluidic start-up"**	**Ignore this step and press 'Run'**

Fluidics start-up:

23.	**Go to "cytometer" ➡ "fluidic startup"**	Instructions will pop up.
24.	Follow the steps directed by the software with this protocol notes.	After each step you performed press 'done'
25.		Press **"DONE"** you have already performed it.
26.		Press **"DONE"** you have already performed it.
27.	 **Prepare the nozzle you will use** For instructions go to page 12	~5 minutes. At 30%, Airflow enter the sheath tank – at this point **Fix air leaks**, if any.
28.	After removing the closed nozzle loop and BEFORE you insert the required nozzle: **validate that all the slots, sockets, camera area and deflection plates are dry**	
29.		
30.	**Validate ND filter no 1.**	You can find ND filter types in SORTER tool drawer. No 1 is essential for accuracy of RB and AC calibrations

Stream operation

31.	**Validate configuration**. In the 'stream window' you can see configuration set up (picture 1a). If there is a mismatch between the nozzle you inserted and the configuration set-up, go to: **1) 'Cytometer' → 'view configuration'** (if a configuration window doesn't appear, minimize the DIVA, you'll find the configuration window behind) **2) Choose the correct nozzle configuration** **3) Press 'set configuration'** **4)** a message window pops up: **Press 'Ok'.** **5) Press 'Ok'** at the right side of the window. **6) Close this window at the windows' X** **7) Choose "use CST settings"** in the pop up window	**Picture 1 – Stream window** a) Speed configuration b) Turn on stream by clicking red x 
32.	**Turn on the stream and center it** *****Attention*** beware!!! Do not touch the deflection plates when high voltage warning light is ON.**	Click the red **X** in the "stream window" (picture 1b). It will change to green √. For instructions on how to center a stream, go to page 10.
33.	Close the deflection plates door	
34.	Close the sorter cover	

Setting the sweet spot

35.

A Table of settings is attached physically on the sorter

Validate that the frequency of the nozzle is according to Table setting values.

Validate that the left value of the gap is according to Table setting values.

Setting drop 1. Drop-1 is the breakoff point of the stream to separated drops (the location of the first separated drop). You can control the breakoff point by changing the amplitude, scroll up or down the amplitude to set the breakoff point. There are two fields for 'drop-1' values: the left shows the last saved value. The right shows current value. Current value will change when the amplitude is changed.

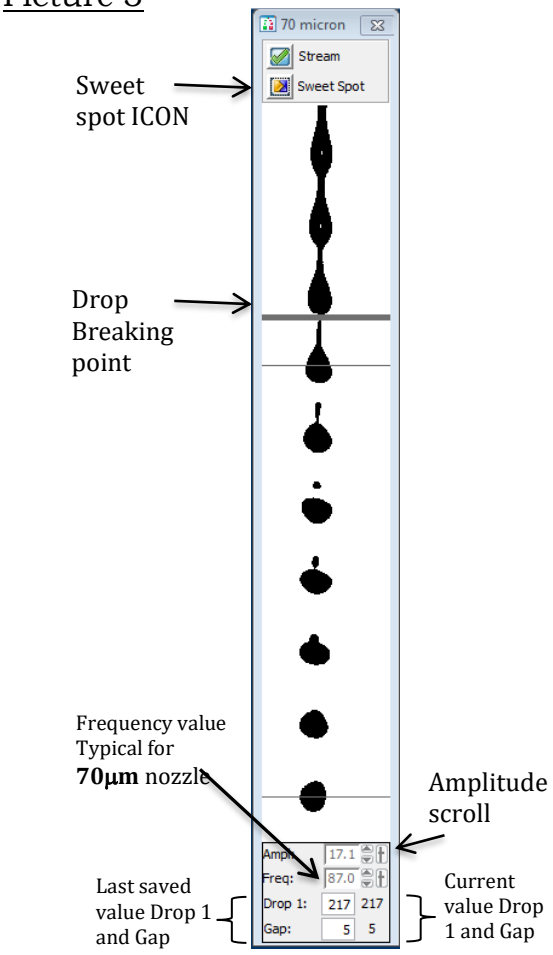
Recommendations for choosing the breaking point:

- Prefer to locate drop-1 breakoff point at the distance range (*i.e.* Drop-1 value) between 200-300 pixels.
- If you have a couple of possible break of points within that range, prefer the value, closest to the last saved value. It is not required to reach the exact value, but to find the closest one.
- **Confirm satellite merge.**

Setting the gap - fine tune the amplitude so that the Gap right value match the left value. Once the gap values match (+-2), go to the left field of Drop -1 and type the value you have on the right field of Drop-1.

Activate the 'sweet spot' to keep the stream conditions stable.

Picture 3



Calibrations:

Calibrations can take a few minutes, if the system is clean, and up to an hour if it needs cleaning. Consider it in your time planning.

Rainbow Calibration

Rainbow beads (RB) are used to check the system performance and that it is sufficiently clean- You should find a 'ready to use' tube, marked RB, in "FACS ONLY solution rack" near the sorter. And…If you must, Prepare new RB tube:
1 drop of RB from the stock vial into ~0.5ml either DW or PBS

36) In the Browser window open 'Shared view' (+), **DoubleClick** on the **notebook** named Rainbow or **RB** - If a window pops up, choose "continue".
37) Click (+) in the specimen of the current month.
38) Insert a new tube and name it with the current day. Activate the new tube (activated tube turns from gray to green).
39) Go to "cytometer" → "cleaning modes" → **"sample line backflush"** → **"start";** let it **wash** the sample line for **30 seconds**. Press **"Stop"** and then **"cancel".**
40) **Load** a tube containing FRESH **DW** and let it run for **30 seconds**.
(This step is essential to wash residues from the sample line that destroy the APC signal of the RB)
41) **Validate that all the lasers are turned ON**
42) **Vortex & Load a tube with RB**
43) **Acquire** at a **speed of no more than 200 events/sec and no more than flow rate 2.**
44) **Press Record**
45) **Unload** the RB tube and **analyze the result as followed**

- Confirm the gate P1 is set on the correct single bead population
- Confirm that P1 is more than 65%
- Confirm that each one of the histograms has 8 peaks
- Confirm that the CV of the last right peak is no more than 5%

46) If RB results are satisfactory, proceed to AccuDrop calibration. Do not proceed to AccuDrop calibration if RB calibration is faulty.

Run RB whenever:

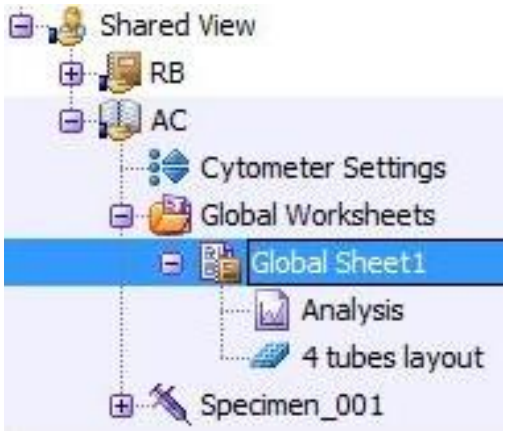
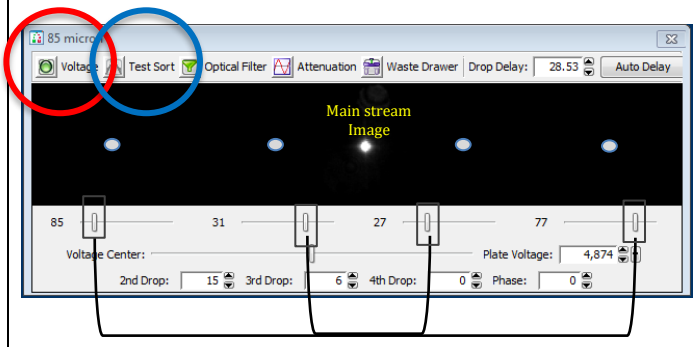
1) Following "start-up" procedure.
2) If you changed a nozzle.
3) As part of troubleshooting step.

AccuDrop Calibration

AccuDrop beads (AC) are used to set the optimal drop delay for sorting.

You should find a 'ready to use' tube, marked AC, in "FACS ONLY solution rack" near the sorter.

If you must….Prepare new AC tube: transfer 1 drop of AC from the stock vial into ~0.5ml either DW or PBS

Steps	Notes
47) Go to 'Shared view' **Open 'AccuDrop /AC'** experiment; by DoubleClicking on the notebook. If a window pop-up, choose "continue".	
48) **Press '+' of folder 'Global worksheet'**	
49) **Press '+' of page 'Global sheet1' and DoubleClick 'Sort layout'**	
50) **Confirm the sort layout conditions:** **Tube device**: 4 tubes. **Precision**: FINE TUNE. **Target events**: Continuous. The gate "NOT P1" should be positioned in the left stream	
51) **Press '+' of the specimen** and **activate the tube.**	
52) **Click on voltage** (red circle-picture 4) ➡ **press 'test sort'** (blue circle – picture 4). Four side streams should appear. The location of the side streams can be adjusted if needed, with their sliders.	 Side streams sliders – use them to reposition side streams
53) Close side streams by pressing again 'test sort' & close voltage, by pressing again voltage icon.	
54) **Vortex & Load AC tube**	
Assure to have a fit range of events/second: For 70 micron = 1,700 to 3,000 evt/s For 85 micron = 1000 to 2,000 evt/s For 100 micron = 800 to 1,500 evt/s For 130 micron = 600 to 1,200 evt/s	

If evt/sec is lower than desired rate, increase the 'flow rate', you can go up as much as flow rate-5 on the other end, if beads are too concentrated dilute them with DW/PBS.

Press 'Sort' ➡ **Choose 'cancel'.** When you choose 'cancel' beads are sorted and delivered to the waste.

Turn on the voltage; a side stream appears on the left ➡ Confirm that the **Image of the main stream and left-side streams in the 'sort window' are focused**. Do so by turning the AccuDrop laser adjust (Picture 2, silver knob) either clockwise or counterclockwise until the two streams are brightest/strongest.

When streams are focused, press on 'optical filter' icon.

Two squares appear. Each stream should be inside its square.

Confirm that the right & left sum ~100% altogether.

Press **'AutoDelay'** in the sorting window.

You can also do **manual setting of the drop delay;** in the 'sorting window', gradually increase or decrease the drop delay by clicking its up/down arrows. Choose the drop delay that yields 95-100% of the events in the left square. For each change you make in the drop delay wait ~2 seconds to let the sorting values stabilize.

55) **Unload AC tube**

56) properly Close the AC protocol by double clicking its notebook

DIVA 8 randomly deletes gates from experiments that are not closed properly. Opening the next protocol or pressing, "Log out" or "quit" is not a proper way to close the experiment.

Run AC whenever: **1)** Following "start-up" and only after RB test passed. **2)** When changing a nozzle. **3)** When 'Drop 1' has changed in more than 20 units. **4)** If you performed "clean the flow-cell" protocol.

57) Run bleach [FACS clean] for 2 min.

58) Backflush 30 sec

59) Run sterile PBS for 1 min.

60) Run sterile D.W for 1 min.

Biohazard safety: Safety level 2 and Human origin cells should Turn on The AMO system (safety regulations to protect people in the sorter area from breathing aerosols)!!! Remember to turn it off when you are done

The system is now ready for sterile use

Changing waste container:

A full waste container should be replaced with a reserve empty one, placed by the side of the cart. Confirm the empty container has 500ml sodium hypochlorite (EKONOMICA). Leave the full tank near the cart, but if you must. Empty the full waste tank to the sink and add 500 ml of sodium hypochlorite (that can be found under each sink). Place the empty tank by the cart. A full waste container will stop the stream without warning, giving a "clog" message. Keep track of the waste status.

Cleaning a nozzle: put the nozzle in a tube containing 70% ethanol or DW, sonicate for 1 minute and air dry. Sometimes this step has to be repeated several times until the nozzle is clean **Always validate that the nozzle is clean under the microscope**

Centering the stream

*****Attention*** beware!!! Do not touch the deflection plates when high voltage is ON.**

Open the deflection plates' door. The stream between the two deflection plates should hit the center of a narrow tab (waste drawer). If not, center it by first loosening two adjustment screws and then manually moving the sort box to center position. Re-tighten the screws to lock the position of the box. To handle the screws use an "Allen" type screwdriver (yellow handle), found in the tool drawer of the Aria.

When the stream is centered