Samples Preparation

- filter sample before sorting (70 or 100 nm filter)
- don’t dilute sample too much, as more dense as faster the sort.
  You can always add some PBS when it is too dense.
- you need unstained and single stained controls for compensation.
Sorter

- open the grey door which covers flow cell

**REMEMBER:** when the door is closed without stream the Laser will burn the flow cell.

It’s better to always open that door, when you are not running a sample.
- turn Sorter on, the green button is for both, Cytometer and Laser, **don’t** touch the small white button underneath the green one, labeled “Laser”.

**REMEMBER:** Laser need 30 minutes to warm up

- Computer should be on, open “FACSDiva” software (icon on desktop), log in with you name
- do “fluidic start up”:
  “Instrument” in Menu bar, “fluidic start-up” in drop down menu
- fluidic-start up takes ~5 min
- **Look for salt/liquid deposition/dripping around the nozzle holder:** IF SALTS OR LIQUID IS VISIBLE, THE NOZZLE NEEDS TO BE CLEANED. DO NOT START THE STREAM in case of visible salts/liquid

---

**Look for salt/liquid deposition/dripping around the nozzle holder.**

- **STOP the stream**
- **Nozzle Holder**
  - remove. NOZZLE HOLDER WET IS AN INDICATION THAT THE STREAM IS NOT CORRECT AND THE THE NOZZLE IS NOT PROPERLY CLEANED/INSTALLED
  - clean with DI
  - dry
- **Nozzle**
  - remove
  - locate the O-ring; on the nozzle or stuck somewhere in the sort block
  - remove the O-ring and put it in DI
  - clean the nozzle; DI, if not sufficient, sonicate (lab 13) + rinse in DI
  - dry the nozzle
  - put back the O-ring with a sharpened Qtip
  - gently press the O-ring with a finger
  - dry the O-ring
- **Put back in gently the nozzle, then nozzle holder**
- turn stream on

- **Turn on stream:**
  click “Stream” (red cross icon) in “Stream” window, stream appears, first as a line, then starts forming drops. Now click on “Sweat spot” icon, to reset the stream to the setting used the last time for sorting. The stream need some time stabilize, so do this immediately after the fluidic start-up, then prepare samples during the time the Laser need to warm up.

**IF THERE IS A PROBLEM WITH THE STREAM** (no stream; spraying; water dripping), **stop the stream immediately. Nozzle (see above) AND sorting chamber (see below) need to be cleaned**
- Clean the sort block:

1/ Clean the plates:
   - use a wet kimwipes to remove salts
   - dry the plates with a clean Qtip

2/ Clean the posts that hold the plates (BEHIND THE PLATES):
   - gently clean with a wet Qtip (to remove salts)
   - dry with a clean Qtip

- when the stream looks all good with "Sweet spot" On, if possible, let run for 30 minutes so that the stream stabilizes itself
Pre Sorting Set up

- Connect tube holder to the cooling system (green tubes)
- turn on cooling water bath (underneath FACSARia unit, next to rolling cart with buffer)

- create a sorting experiment:
  - click “Experiment” in menu bar, choose “New Experiment” from drop down menu,
    a window with several pre-set Experiments appears
  - choose “Doublet Discrimination Gating”, double click to open
  - rename
  - expand Specimen
  - expand tube, rename Presort (or whatever you like…)
  - point on tube (the arrow on the very left side next to the tube has to be green+ activated),
    go to “Experiment” in menu bar, choose “experimental Layout” in drop down menu
  - in the “experimental layout” window, click on ‘Acquire’ tap, and set the number “ events to
    record” next to the presort tube to 10’000.
  - In the “Instrument” window, go to ‘Parameter’ tap and delete all the parameter you don’t need

Do Compensation Control:
  - choose “Instrument” in menu bar, “Instrument setup” in drop down menu,
    then choose “Create Compensation Control” in sub-menu
  - do Compensation Control

after Compensation Control:
  - point on the Presort tube again
  - change to the “DD Gating” worksheet by clicking on the small worksheet icon in the very left
    upper corner on the worksheet window
  - the “DD Gating” worksheet shows 3 different dot plots (and a population hierarchy window):
    FSC-A vs SSC-A, with a rectangle gate
    SSC-H vs SSC-W with a SSC gate - shows only the FSC-A/SSC-A gated population
    FSC-H vs FSC-W with a FSC gate – shows only the SSC gated population
    Just the cells gated with the FSC gate will be sorted
    These gates should be set very narrow, use the magnification view to discriminate doublets
    (set the upper boarder of the gates as close to your population as possible)
  - set the ‘flow rate’ in the dashboard to 1
  - run your sample, as soon as you see some events, record the data. Don’t adjust the gates now,
    you don’t want to lose too much of your sample. All the adjustments and new gates can be
    done with the recorded data.
  - after recording unload sample and keep on ice
  - adjust the SSC and FSC gates, then use your own dot blots/ histogram to define the
    population/s you want to sort. You can use as many blots as you want, and as many gates you
    want. Always make sure that you display the right population in a new blot (check in hierarchy,
    all blots must show FCS gate, or must show a partial population of the FSC gated population
Setting up side streams

When you open the sort block, you see one stream in the middle, which should be nice and straight and hit the waste without any spraying. The side streams have to hit the middle of the tube you want to sort in, and you have to set them up every time. Even if they are still perfect from the last sort and you don’t have to adjust anything, the next time they can be really out of range, or something got stuck in the flow chamber and makes the side streams really ugly.

You adjust the side streams with the side stream window “high”

- put some collection tubes in the tube holder, and install the tube holder
- turn on deflection plates: click on the small icon next to ‘Voltage’ (green dot in the icon turns red)
- click on the icon next to ‘Test Sort’
- click on the icon next to ‘Waste Drawer’, you can hear that the waste drawer get moved back
- open the sort block door (always try to avoid touching the two deflection plates!)
- you can see the middle and the 4 side streams
- adjust the side streams with the slider controls in the “high” window
- when you close the sort block door you can see the image of all 5 streams in the “high” window
- when the side streams are adjusted, click on ‘Waste Drawer’ icon to close it
- click on ‘Test Sort’ and the ‘Voltage’ icons to turn them off
- close sort block door
- remove tubes from tube holder

Close the “Doublet Discrimination Gating” Experiment
Optimizing Drop Delay

To make sure that all the cells you want to sort are in the side stream, you use lead beads and optical filter to set the side stream to 100%. You need to turn on the deflection plates to “sort” this beads.

For this control use a new, pre-set experiment:

- click “Experiment” in menu bar, choose “Accudrop Drop Delay” Experiment in drop down menu
- expand specimen
- expand tube, open “Sort Layout” (new window “Sort Layout” appears)
- vortex tube with Accudrop beads very well, load
- adjust flow rate to an event rate of 2000-3000 events

“high” sort window:
- turn on deflection plates by clicking on ‘voltage’ icon
- click ‘Optical Filter’ icon, two squares appear in the ‘high’ sort window

“Sort Layout” window:
- set ‘precision mode’ to “Initial”
- click ‘Sort’ button in the “Sort Layout” window (click cancel at prompt to keep aspiration drawer closed)

The square on the left (left side stream) should show 100%. Wait a few seconds for a complete response to the delay change, at least 3 seconds.

To optimize the drop delay, move the values up/down in the drop delay field (upper right corner)

DON’T MOVE TOO FAST. THE CYTOMETER ALWAYS NEED AT LEAST 3 SECONDS TO RESPOND.

The system is very sensitive, when you move too much up or down, the value can drop down to 80% (or whatever, but it’s not 100 % anymore) you have to go back in that case.

- when the left window shows 100% (or close too, the value will always change a bit), change the ‘precision mode’ in the “Sort Layout” window from “Initial” to “Fine tune” (drop down menu), WAIT, the value should adjust pretty close to 100% again.

- when done, stop sort by clicking on ‘Sort’ in “Sort Layout” window
- close ‘Optical Filter’ (click on icon)
- turn off deflection plates by clicking on ‘Voltage’ icon
- unload Accudrops

- close “Accudrop Drop Delay” Experiment

Note on sorting in 15 ml conical tube and 2-way sorts
In case of 2-way sorts, only the left and right streams close to the center are used.

If you plan to do a 2-way sort in 15 ml conical, you have to re-adjust the side streams at this point. Most likely, you will have to deviate the streams a slightly further away from the center.
Set up Sort Layout

- open “Doublet Discrimination Gating” Experiment again
- point on “presort” tube
- expand “global worksheet” and select “DD Gating” worksheet
- open a new “Sort Layout” window  
  (click on the icon underneath the menu bar on top of the “browser” window)

In “Sort Layout” window:

Sorting 1 or 2 fractions:
- set ‘Device’ to “2 tube”
- set ‘Precision’ to “Purity”
- add populations to each sort location field,
  click on white field under “left” “right”, chose ‘add’, pick population to sort in this position
  from drop down menu

Sorting 3 or 4 fractions:
- set ‘Device’ to “4 tube”
- set ‘Precision’ to “Purity”
- add populations to each sort location field,
  click on white field under “far left” “left” “right” “far right”, chose ‘add’, pick population to
  sort in this position from drop down menu
Start and Monitoring the Sort

- Install collection tubes (with PBS, Media, Serum) in tube holder to collect your sorted fractions.
- set flow rate to 1
- vortex sample, load, and wait until you see that it gets acquired
- click ‘Sort’ button in “Sort Layout” window to start the sort, click ok at prompt.

Monitor sort progress in “Sort Layout” window.
The number of sorted events for each fraction is displayed in the corresponding white field
(where you chose the population).
Sort Rate and Sort Conflict Rate are displayed in the counter fields underneath.

Changing collection tubes:
- Click on ‘Pause’ in “Sort Layout” window. Sort counts are retained.

Changing sample: (vortex, changing sample tube)
- You have to stop and unload sample. Don’t forget to write down the number of sorted events
  after stopping the sort, the counts will be reset to zero when you stat the sort again.

Try to stay in the room when you sort. Nothing should happen, but you might want to control the
flow rate (SLOW), and you will have to mix the sample in between.

Cytometer shuts down the sort, the stream, and unloads the sample automatically when
- sample is empty (will never suck in air)
- sample line or flow cell is clogged

In case of clog, bad stream, spraying of the stream

- STOP THE STREAM IMMEDIATELY
- Clean the nozzle (see figure)
- Clean the sort block:

1/ Clean the plates:
- use a wet kimwipes to remove salts
- dry the plates with a clean Qtip

2/ Clean the posts that hold the plates (BEHIND THE PLATES):
- gently clean with a wet Qtip (to remove salts)
- dry with a clean Qtip

- Clean any remaining liquid with kimwipe
- Turn stream back on
- IF STREAM STILL NOT GOOD, STOP IT IMMEDIATELY

Note on purity modes and volumes

The "Yield" mode is not stringent for purity but it will recover all sorted cells. Very useful to enrich a population.

The "Purity" mode is very stringent for purity. However, in order to provide maximum yield, for each drop sorted it will sort the previous and the next drops, if they are empty, to be sure that the cell is caught. As a consequence, the volume of liquid accumulate rapidly. Typically, a 5 ml FACTS tube is filled with 1 million cells.
To decrease volume, at the cost of yield, you can use the "Purity ms" mode. This will sort only the drop supposedly containing the cell. Volumes are drastically reduced, typically a 5 ml FACS tube will hold 5 million cells. Yield is slightly decreased - typically you loose 1% events compared to "Purity".
After the Sort

After Sort:
- stop ‘Sort’
- unload sample tube
- go to “Sort” in menu bar, chose “Sort Report” from drop down Menu.
  Print the “DD Gating” worksheet with your set up and the statistics.
- export and save your Experiment
- delete your Experiment

Cleaning procedure
- go to “Instrument” in menu bar, choose ‘backflush sample line’, click on ‘start’, let run for ~1 min, then ‘stop’
- run a full FACS tube of fresh FACSRinse for ~10 min at sort rate 11, then run a full FACS tube of fresh water for ~10 min, at sort rate 11, to rinse the sample line.
- put flow rate back to 1

When you are the last person that day:
- go to “Instrument” in menu bar
- select “fluidic shutdown” from drop down menu
  (stay there, system will ask you to load a tube with FACS Rinse, and a tube with water)
- when fluidic shutdown is finished, remove tube, quit FACS Diva software, switch off sorter (green button)
- remove tube holder, take tubes with sorted cells out of the holder
- turn off cooling system (underneath FACSaria unit, next to rolling cart with buffer)
- Disconnect green cooling tubes from tube holder, plug them in underneath the sorting chamber
- Look for salt/liquid deposition/dripping around the nozzle holder: IF SALTS OR LIQUID IS VISIBLE, THE NOZZLE NEEDS TO BE CLEANED

- nozzle holder
  - remove. **NOZZLE HOLDER WET IS AN INDICATION THAT THE STREAM IS NOT CORRECT AND THE THE NOZZLE IS NOT PROPERLY CLEANED/INSTALLED**
  - clean with DI
  - dry
- nozzle:
  - remove
  - locate the O-ring: on the nozzle or stuck somewhere in the sort block
  - remove the O-ring and put it in DI
  - clean the nozzle: DI, if not sufficient, sonicate (lab 13) + rinoc in DI
  - dry the nozzle
  - put back the O-ring with a sharpened Qtip
  - gently press the O-ring with a finger
  - dry the O-ring
- leave the nozzle and the nozzle holder under the sorting chamber; the next person will install it the next day
- CLEAN THE SORT BLOCK:

1/ Clean the plates:  
- use a wet kimwipes to remove salts  
- dry the plates with a clean Qtip

2/ Clean the posts that hold the plates (BEHIND THE PLATES):  
- gently clean with a wet Qtip (to remove salts)  
- dry with a clean Qtip

- Dry tube holder with tissue, place it turned over on some tissue to dry out the inside  
- Clean and dry tube holder unit on lifting arm, and under the sort block with a tissue  
- Clean up desk, please don’t leave any unused tubes with media or cells there, no used gloves, tissues…