**General note:**
Registration with the Flow Cytometry Facility as well as attending the technical introduction by the FCF is a prerequisite to use the BD LSR Fortessa.

For questions and reporting technical problems, please contact the FCF staff by email cytometry@uochb.cas.cz or in an emergency call **+420 776 700 601 (Jana G.)/ +420 774 684 423 (Alžbeta M.)**

**Sample preparation:** Use enough sample volume/well and filter your cells prior to analysis to avoid sample line clogging. Recommended concentration of cells: $2x10^5$ cells/200µL.

## I. Switching the instrument on and general handling

1. Turn on the computer and log in using Admin account (password: BDIS#1).
2. Press the green button on the right side of the instrument.
3. Turn on the Fluidics cart and press prime.
4. Go to Coherent Connection and start laser you need for experiment.
5. Start FACSDiva Software, no password required.
6. Wait for the software to connect with the cytometer.
7. Accept "Use CS&T Settings" if prompted.
8. Perform a "Prime" on the instrument without a water tube attached.

## II. Installing the HTS on the Fortessa

- Verify that the instrument is in STANDBY mode.
- Check the sheath fluid level in HTS sheath container. Please refill sheath container with sheath fluid (DI $H_2O$) if necessary.
- Make sure that the lid of sheath tank is tightened.
- Remove the tube of DI $H_2O$ from the SIT arm.
- Turn the collection switch located above the BD LSR Fortessa power button from tube to plate.
- Attach the HTS sample coupler to the injection needle.
- Connect the HTS to the BD LSR Fortessa by screwing the sample line from the HTS to the sample port on the BD LSR Fortessa.
- Turn on the HTS.
- Switch the instrument to Run mode and Low flow rate. In the Diva Workspace menu select > HTS > Prime. After priming cytometer, the computer will show that the HTS is ready.

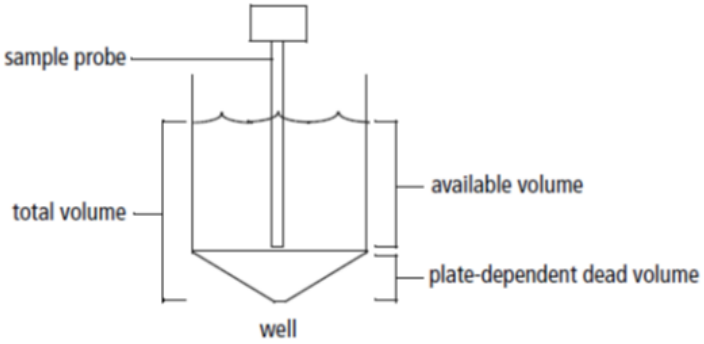
## III. Setting up the Plate in the Diva Software

- In the Experiment menu, click New Plate and choose the plate type that corresponds to the plate you will be using.
- In the Inspector frame, select Standard or High Throughput Mode.

- Double click on the Plate in the Browser to open the Plate window frame if not already open.
- Modify the well settings as needed. Add wells to the plate as needed: setup controls to optimize PMT voltage settings, compensation controls to calculate spillover, and samples.
- Click and drag to select the wells you want to acquire. The Wells should turn blue with the Specimen Number in the upper right hand corner. Wells will be acquired in the order they were created/selected.

## IV. Loader settings for the well

- The loader (HTS) settings can be modified for the selected well or plate. Default loader settings are provided for each throughput mode. You will need to optimize these settings for the plate type and assay you are running. **You cannot change HTS settings during acquisition or when a sequence is in process**.
- Understanding volumes:




Well volume: Volume that well can hold filled to the brim
Total volume: Volume pipetted into well – aspirated excess volume
Aspirated excess volume: Standard volume = 20 µL, High Throughput mode = 22 µL
Available volume: Volume pipetted into well – aspirated excess volume – dead volume
Minimum volume: 50 µL for both standard and high-throughput modes for 96-well plates
Mixing volume: is one-half the available volume
Dead volume: Volume in the bottom of the well that the probe cannot reach (20 µl)

- Select throughput mode by clicking the corresponding mode button in the Setup view.
- **Standard Throughput Mode:** HTS aspirates the selected sample volume (2–200 µL) plus an additional 2 µL from the well and can process a 96-well plate in approximately 44 minutes. We recommend preparing your plate with a minimum 250 µL/well for a 96 well plate and 50 µL/well for a 384 well plate.
- **High Throughput Mode**: HTS aspirates a fixed 22 µL per well even though you can select a sample volume between 2–10 µL and can process a 96-well plate in approximately 15 minutes. We recommend preparing your plate with a minimum 100 µL/well for a 96 well plate and 50 µL/well for a 384 well plate.

- **HTS settings for standard and high throughput mode:**

| Setting | Standard Mode | | High Throughput Mode | |
|---|---|---|---|---|
| | Default | Range | Default | Range |
| Sample Flow Rate (µL/sec) | 1 | 0.5–3.0 | 1 | 0.5–3.0 |
| Sample Volume (µL) | 10 | 2–200 | 3 | 2–10 |
| Mixing Volume (µL)[a] | 100 | 5–100 | 50 | 5–100 |
| Mixing Speed (µL/sec) | 180 | 25–250 | 200 | 25–250 |
| Number of Mixes (cycles) | 2 | 0–5 | 2 | 0–5 |
| Wash Volume (µL) | 400 | 200–800 | 200 | 200–800 |

- **To achieve optimal throughput, we recommends:**
  - Make sure each well on your plate contains sufficient sample for mixing. Insufficient volume can introduce air bubbles into the system.
  - Increase the Sample Rate to 1µL/sec or greater (Note: the higher the sample flow rate, the lower the resolution).
  - Set Mixing Volume to be one-half of the available volume (250 µL-20 uL-30 µL = 200 µL/2 = 100 µL).
  - Use BD's default Mixing Speed of 180 (Note: increased mixing speed could compromise the separator bubble between the sample and sheath, resulting in sporadic event rates and possibly higher carryover).
  - Set Number of Mixes to 2 or less (Note: a greater number of mixes could impact sample throughput).
  - Set Wash Volume to 400 µL or less (Note: the greater the wash volume, the slower the system throughput).

## V. Acquiring data

- Put your plate on the HTS plate holder with well A1 oriented to the back right corner of the stage.
- Put the LSR in Low and Run mode.
- Use the Acquisition Dashboard to acquire and record well data. **Run Plate** runs the wells from the current position to the end of the plate. **Run Well(s)** runs the selected wells only
- The sample probe goes through a homing sequence during initialization. The software automatically primes the HTS pumps during the homing operation. After the homing and priming operations, acquisition will begin.
- Wells will be acquired in the order they were created. At the end of the run, a dialog appears indicating the run is complete.
- When finished with your experiment, run Daily clean protocol as describe in [VI].

- **Pausing and stopping the HTS in the middle of a run:**
  - **To pause during a run**, click Pause in the Acquisition Dashboard. The HTS unit finishes processing the current well (or wells in high throughput mode), and then remains in a suspended state until you choose to continue. To continue the run, click Resume.
  - **To stop during a run**, click Stop Plate or Stop Well(s) in the Acquisition Dashboard. The HTS finishes the sequence in progress, stops, and then the following message appears: The Run Stopped. Click OK.

**Note:** *If you stop the HTS during a run in standard mode, the current well will be lost. If you stop the HTS in high throughput mode, the current and next well will be lost. Do not stop acquisition if your sample volume is limited.*

## VI. Daily clean

**Note: The HTS unit should be cleaned before each set of plates in an experiment is run, and after the final run on the unit**

- The Cleaning plate is located next to the HTS plate holder.
- In the Diva Workspace menu > Select HTS > Daily Clean - 96-well U bottom > OK.
- Install the 96-well U bottom cleaning plate on the HTS with wells A1-A4 filled with 200 µL BD FACS Clean, wells B1-B4 filled with 200 µL BD FASC Rinse, and wells C1-C4 filled with 200 µL BD DI water.
- Click OK in the Confirm dialog box.
- The cytometer goes through a homing sequence, and cleaning begins. Note: the cleaning procedure can take up to 15 minutes. Click OK when the completion message appears.
- Remove the plate and rinse it for use on another day.
- Turn off the HTS using the power switch that is located on the right side of the HTS unit and remove the HTS unit.
- Switch the acquisition control switch to Tube mode.
- Prime the cytometer a couple of times to remove any air bubbles that may have gotten into the lines while running the HTS unit.
- Put a tube of DI water on the SIP.
- Put the cytometer fluidics in Low and Standby mode

## VII. Data Export & Diva Database

- After measuring and cleaning, export your data as *.fcs files* to your fcf-files folder on our server (name: cytometry, data storage for 2 months).
- If you wish to preserve experimental settings for future experiments, additionally save them as experiment file (Export → Experiments; can be reimported into Diva; e.g. to public, Y-drive > sci_service > cytometry, 2GB permanent storage).

- **After exporting your data delete them from the Diva database.**
  A large Diva database will slow down the software significantly.
- **Please note: Only data stored on the server are secured by a backup**.
  The local computer and Diva database have no backup.
  **Locally saved data will be deleted without further notice**.
- Every user is responsible for securing their data directly after their measurement.
- The Diva database will be emptied by FCF Staff (D:\Exported Experiments) on a monthly basis without further notice to ensure the stability of the software.
- For your convenience, you may keep experiments without data marked as "template" in the Diva software. ("Duplicate without data" and rename to template_xxx)
- Do not use subfolder structures in Diva, this information will be lost upon export.
- Always rename your experiment files with e.g. date and your initials.

## VIII. Check the shared google calendar if there is a user booked after you!

## IX. Between different users of the day

- Close FACSDiva.
- **Please note:** The lasers should be switched off when the time between you and the next user is above 1 hour (Use Coherent Connection to turn off the lasers).
- Leave instrument and computer running for the next user.
- Clean the working area – do not leave used tubes, gloves, etc. behind!

## X. Last user of the day

- Even if a clean procedure has been performed on HTS, before shutting down the LSR, perform the 1 minute clean

  After cleaning the instrument and saving the water run pdf with your name and date **(Y-drive > sci_service > cytometry > Last User Clean),** close FACSDiva and turn off the computer.
- Turn off the instrument by pressing the green button on the right side of the Fortessa.

**Note:** Any violation of these rules will result in penalty points! **Users acquiring more than 3 penalty points will be banned from using the instrument for 1 month.**