



LSRFortessa User Guide

Tube Mode: Starting up (For HTS Plate Mode, See Page 4)

1. Turn on the computer. Wait for the computer to boot up.
2. Log in to the computer (facuser, password =BDIS) and open BD FACSDiva software. Log in to Diva at the prompt with your Diva username and password.
3. Turn on main power switch (Green button - right side).
4. Wait 30 minutes for lasers to warm up before running samples.
5. Watch for Instrument Connected status at bottom right of Cytometer window.
6. If using Tube mode, ensure **the acquisition control switch is on tube mode** (U),
7. Check that the sheath buffer tank has an adequate volume. If it is low, fill with sheath fluid. To fill the tank:
 - Verify that the cytometer is in standby mode.
 - Disconnect the green airline.
 - Depressurize the tank by lifting the pressure release ring.
 - Open the lid.
 - Fill with sheath fluid.
 - Close the lid.
 - Reconnect the green airline.
8. Check waste container, empty if necessary and add a small amount of bleach to inactivate pathogens.
 - Verify that the instrument is in standby mode.
 - Open the cap of the waste tank.
 - Empty the waste container into the sink.
 - Add approximately 1L of bleach to the waste container and close it.
9. If necessary, remove trapped air bubbles from the sheath filter and sheath line.
10. Press Prime on the machine, wait until it turns to Standby automatically. Repeat this step for 3 times, or as necessary to remove trapped air bubbles from the flow cell.

Checking Cytometer performance

1. Select Cytometer > CST. The cytometer disconnects from the BD FACSDiva interface and connects to the CS&T interface.
2. The setup tab indicates the time of the last Performance check for your configuration. If the check was done in the last 24 hours, you do not need to run the beads again. Proceed to step 12 below. If the Cytometer Performance indicates that it has expired, then you will need to run the check. Note that a performance check needs to be run every 24 hours.
3. Use CST beads. To prepare the beads, mix beads well by inverting the vial or gently vortex. In a 12x75mm tube, add 0.35ml sheath fluid and 1 drop CST beads. Label tube with “CST”, the lot number, the date and the time. Store the bead suspension at 2°C to 25°C in the dark until you are ready to use them. Notice: Beads are stable at 2°C to 25°C for no more than 20 minutes in direct light, and up to 8 hours if protected from light.
4. Select the correct bead Lot ID from the menu (bead lot is printed on each Bead vial).
5. If using tube-mode, be sure that “load tube manually” is selected. De-select for plate-mode (HTS).

6. Install tube onto the cytometer loading port.
7. Set Flow Cytometer to RUN on “LOW” and click “Run” under Setup Control to check the performance. The performance check takes approximately 5 minutes to complete.
8. Once the performance check is complete, click “View Report”.
9. Verify that the performance passed- In the Setup tab, the “Cytometer Performance Results” should have a green checkbox displayed and the word Passed next to it.
10. If any parameters did not pass, refer to the cytometry supervisor or the BD Cytometer Setup and Tracking Application Guide for help troubleshooting.
11. Select File > Exit to close the CST window and connect back to the BD FACSDiva Interface. Click the *Use CST Settings* in the settings mismatch dialog box that appears. By selecting “Use CST Settings”, the laser delay, area scaling, and other cytometer settings will be updated to the latest optimized settings from the performance check.

Setting Up Your Experiment

1. Create a new Blank Experiment by selecting Experiment > New Experiment > Blank experiment with sample tube.
2. Rename the Experiment (right-click > Rename) using a good descriptive term and the date.
3. **(First time only)** In Edit menu > user preferences, select the user preferences that you would like. Generally, this is to clear all preferences in the General tab except Load data after recording.

Creating New Application settings (First time you run an experiment)

Application settings are associated with a cytometer configuration and include the parameters needed for the application, area scaling values, PMT voltages, and threshold values, but not compensation. Each time a performance check is run for a configuration, the application settings associated with that configuration are updated to the latest run.

Using application settings provides an easy, consistent, and reproducible way to reuse cytometer settings for your commonly used applications.

1. Start with a new blank experiment (See above).
2. Select Cytometer Settings in the Browser.
3. Delete all parameters you will not be using:
 - a. Choose Parameters tab under the Inspector Window and click on small button to left of parameter name.
 - b. Click delete button (use control key and highlight for multiple deletions).
 - c. Repeat for each parameter you are not using.
4. Click the H and W checkbox to select Height and Width for FSC and SSC to enable doublet discrimination.
5. Right-click Cytometer Settings in the Browser, then select Application Settings > Create Worksheet. A second global sheet is added with the plots created according to your selections in the Parameters tab.
6. Install a tube of cells with all stains onto the cytometer.

Note: All samples should be filtered before installing onto the cytometer.
7. Click Acquire in the Acquisition Dashboard. During Acquiring, optimize the FSC and SSC voltages to place the population of interest on scale.
8. Verify that the positive populations are on scale. If a positive population is off scale, lower the PMT voltage for that parameter until the positive population is entirely on scale.

9. Stop acquiring. You do not need to record a data file. Unload the stained cells tube from the cytometer.
10. You now have your Application settings. To save, Right-click Cytometer Settings in the Browser, then select Application Settings > Save. Name the Application Settings appropriately and click OK. The application settings are saved to the catalog. Application settings do not include compensation settings.

Using previously created Application Settings

1. In a newly created experiment, ensure that the current CST settings are applied. Then, right-click the Cytometer Settings icon in the Browser and select Application Settings > Apply.
2. Select your correct previously created Application Settings from the catalog.
3. Click Overwrite in the dialog that appears.
4. If a message appears about area scaling, click Yes to accept all changes to cytometer settings.
5. The parameter list and PMT voltages are updated to match the application settings you previously created.

Compensation

1. Ensure that you have the correct Application Settings applied, or if you are not using them, ensure that you have the correct parameters and PMT voltages for your experiment.
2. Select Experiment > Compensation Setup > Create Compensation Controls.
3. Click OK to close the Create Compensation Controls dialog. A compensation controls specimen is added to the experiment, along with an unstained control tube, and a stained control tube for each parameter. Worksheets containing the appropriate plots are added for each compensation tube.
4. Install the unstained control tube onto the Cytometer.
5. Set the current tube pointer to the unstained control tube in the Browser.
6. Click “Acquire Data” in the Dashboard.
7. Move the P1 gate to fully incorporate the singlet population.
8. Right-click the P1 gate and select Apply to all Compensation Tubes.
9. All single-color controls are screened prior to recording data in the unstained tube area to verify all colors are on scale (below 10/5th) and the relationship between the fluorochromes is correct at selected voltages.
10. Click Record Data in the Dashboard to record the events from the unstained control tube.
11. Unload the unstained control tube.
Notice: Do not change the PMT voltages after the first compensation tube has been recorded. To calculate compensation, all tubes must be recorded with the same PMT voltage settings.
12. Click Next Tube in the Dashboard.
13. Acquire each compensation tube and record in this manner.
14. Verify that the snap-to interval gates encompass the positive populations
15. Select Experiment > Compensation Setup > Calculate Compensation. If the calculation is successful a dialog appears. Appropriately name the compensation setup.
16. Click Link & Save to close the dialog box and save the compensation setup and link it to the experiment’s cytometer settings.
17. Applying a previously recorded compensation matrix to your experiment is not recommended and does not follow best practice for flow cytometry standards.

Recording Sample Data

1. After compensation, Click the Worksheets View button on the Worksheet toolbar.

2. Create worksheet elements (dot plots, hierarchy, statistics, etc.) you want to view for your experiment.
3. If necessary, create a new specimen and tubes for your samples. Activate the first tube, install the tube, load it and record it.
4. Continue for all tubes you wish to run.

Data Export

1. Right-click the title of your experiment and go to “Export > FCS Files”.
2. Save to “C:\FACSDData”.
3. Go to the Desktop.
4. Open the “Data_Upload” program.
5. A command terminal will open.
6. Follow the on-screen instructions:
 - a. Type your SUnetID, hit Enter.
 - b. Type “yes” when asked, if your SUnetID was typed correctly, then hit Enter.
 - c. Confirm you are uploading the correct experiment, then hit Enter when prompted.
7. Leave the program to run until it closes on its own. **DO NOT CLOSE THE PROGRAM EARLY, OR TURN OFF THE COMPUTER.** Otherwise your upload will be cancelled.
8. (First Upload Only) You will receive an email from a FACS Core staff member containing a link and password to your data. This information will be used to access all subsequent uploads.


Shutting down/Preparing for Next User

1. Load and run a tube of 10% bleach (FACSClean) (no more than 2 mL) for at least 5 minutes at a High flow rate.
2. Repeat with a tube of FACSRinse (no more than 2 mL) for 5 min.
3. Repeat with a tube of DI water (no more than 2 mL) for 5 min. Leave the DI water tube on the SIT.
4. Place the instrument into standby.
5. Logout of the DIVA software.
6. If you are the last user of the day, or more than 4 hours until the next user, you will turn the computer and cytometer off.

Plate Mode: High-Throughput System (HTS) Start-Up and Experiment Set-Up


HTS Start-Up

Important! Ensure that the sheath tank is full and the waste tank empty prior to run HTS experiment. The HTS uses more sheath fluid than loading the tubes.

1. **Switch the acquisition control switch to plate mode  on side of the instrument.**
2. Remove the tube of DI water from the SIT.
3. Attach the HTS sample coupler to the cytometer SIT. Slide the sample coupler onto the SIT until you reach a hard stop. Make sure the sample coupler tubing is not kinked or twisted. Hold the coupler with one hand while you tighten the top nut with your other hand.
4. Make sure the HTS is turned on.
Notice: The HTS is usually kept on. The switch is located behind the HTS and will display a green LED light if on.
5. Put the instrument in “Run” and set flow rate to “low”.
6. **Run Prime once by selecting HTS menu in Diva software > Prime while on “Run” in low.**

7. Prepare a CST well in A1(top left corner) of a 96-well plate and load the plate. Prepare beads per protocol and pipette 200uL into well.
8. Replace the cover on the HTS.
9. Select Cytometer > CST.
10. Ensure that “96 Well U Bottom” plate is selected and the “Load Tube Manually” checkbox is **unchecked** prior to running CST.
11. The performance check takes approximately 5 min to complete.
12. Select File > Exit to close the CST window and connect back to the BD FACSDiva Interface. Click the **Use CST Settings** in the settings mismatch dialog box that appears. By selecting this, the laser delay, area scaling, and other cytometer settings will be updated to the latest optimized settings from the performance check.
13. In the “Experiment” tab, select “New Experiment”, and select Blank experiment with sample tube.
14. Click on the arrow next to the “New Plate” button in the browser toolbar and choose the plate type from the drop-down list.
15. A plate layout window will appear.
16. In the top right corner of the plate window, choose the mode you would like to use, high throughput or standard, and adjust loader settings as needed. Refer to HTS Loader Settings instructions at the end of this guide for more information.

HTS Experiment Set-Up

1. Add wells to the plate as needed: setup controls, compensation controls and samples.
2. In the “Parameters” tab of the Cytometer window, delete any unnecessary parameters.
3. In the “Plate” window, select the pink Add Set-Up Controls button to create 2-3 wells for full stained samples to set PMT and threshold values. 
4. Select a well in the next row, choose Experiment > Compensation Setup > Create Compensation Controls.
5. The software will auto-populate the compensation samples. Ensure there is an “Unstained” compensation well.
6. Click and drag to select the desired wells, and then click the Add Specimen Wells button to add specimens to the plate layout.
7. Change the specimen names under the “Specimen” tab in the Inspector window.
8. Go to Experiment > Experiment Layout > Labels tab to assign antibody marker labels, and set events to record according to the following suggested guidelines:

Name	Events to Record
setup controls	2500000
compensation controls	10000
specimens	10000

9. Set up Global Worksheet with plots and gates.
10. Remove the safety cover on the HTS unit and place the prepared plate on the plate holder.
11. Replace the HTS safety cover correctly, or the HTS will not run sample.
12. Select a setup control well.
13. Click “Acquire Data” in the Acquisition Dashboard.

14. Adjust FSC, SSC, and PMTs while running the Set-up Controls.
15. Stop acquiring once you have adjusted settings appropriately.
16. Highlight the compensation wells in the plate layout.
17. Ensure the cytometer is in “Run”, then click “Run Wells” in the Acquisition Dashboard (while still viewing the Global Worksheets). DO NOT put the instrument in “Standby” while samples are being acquired.
18. Once wells are completed, toggle to the Normal Worksheets to adjust gates for compensation controls.
19. Go to Experiment > Compensation Setup > Calculate Compensation, link and save the compensation.
20. Return to the Global Worksheets and select the wells that you wish to run.
21. Highlight sample wells, then click “Run Wells” in the Acquisition Dashboard. Wells are acquired in the order they were created. Select “Run Plate” when you wish to acquire and record all assigned wells on the plate.
22. When the run is complete, open the safety cover and remove the plate.
23. To export and save template for future use, in the browser select plate to be exported, right click > select Export.
24. In the prompt that appears, select Global Worksheet to export, hit Next > select template and name.

Data Export (Same as Page 4)

1. Right-click the title of your experiment and go to “Export > FCS Files”.
2. Save to “C:\FACSDATA”.
3. Go to the Desktop.
4. Open (double-click) the “Data_Upload” program.
5. A command terminal will open.
6. Follow the on-screen instructions:
 - a. Type your SUnetID, hit Enter;
 - b. Type “yes” when asked, if your SUnetID was typed correctly, then hit Enter;
 - c. Confirm you are uploading the correct experiment, then hit Enter when prompted.
7. Leave the program to run until it closes on its own. DO NOT CLOSE THE PROGRAM EARLY, OR TURN OFF THE COMPUTER. Otherwise your upload will be cancelled.
8. (First Upload Only) You will receive an email from a FACS Core staff member containing a link and password to your data. This information will be used to access all subsequent uploads.

Shutting Down/Preparing for Next User

1. Run a “Clean Plate” Protocol:
 - a. Choose “HTS > Clean”.
 - b. Select “Daily Clean 96-Well U-bottom” Template.
 - c. Click Ok.
 - d. Fill the wells according to the template. A1-A4: 200 ul BDFACS Clean. B1-B4: 200 ul DI Water.
 - e. Open the Safety cover and place the plate.
 - f. Return cover and click “Ok” to run cleaning plate.
2. The cleaning process can take up to 15 minutes. Click “Ok” when completed.
3. Remove the plate and set aside for future use.
4. Place the instrument into standby.
5. Logout of the DIVA software.

6. Return setting to “Tube Mode” by flipping the control switch from “Plate” > “Tube” on the instrument, and carefully remove the HTS coupler from the SIT and replace with a tube of DI water (1mL).
7. If you are the last user of the day, or more than 4 hours until the next user, you will turn the computer and cytometer off.