SOP for HTS
Getting started
Switch the cytometer and the computer on. The HTS initialises when cytometer is switched on.
Login to DIVA software.
Before proceeding with the HTS, clean the instrument according to standard AMREPFLOW SOPs:

- Check fluidics: empty waste and fill sheath.

- Ensure instrument is in tube acquisition mode (switch is located on RHS of instrument)

- Move aspirator support arm to the left,

- allow 3-4 drops to fall from SIP,

- prime X3

- run a tube of bleach followed by water on high flow rate (2-5min each). The counts should average around 20-50 events/second.
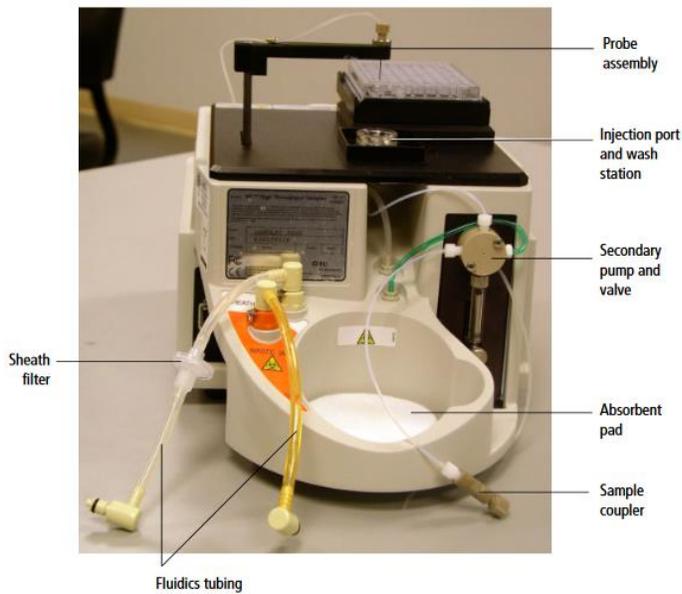

Now proceed with HTS:
- Flick switch to HTS acquisition mode (RHS of instrument)

HTS unit

- This is where the multi-well tray sits and sample is aspirated from individual well in tray to flow cytometer. HTS enables you to acquire samples from 96- and 384-well plates

The HTS unit is comprised of:

- Sample coupler
- Plate holder
- probe assembly: transfers sample from wells of a plate to Injection port/wash station
- Primary and secondary pumps

Probe assembly

Injection port and wash station

Secondary pump and valve

Sheath filter

Absorbent pad

Sample coupler

Fluidics tubing

The sample coupler connects the injection port tubing on the HTS unit to the cytometer sample injection probe (SIP).

- Move the aspirator support arm to the left and remove the tube of DI water from the SIP.

Attach the HTS sample coupler to the SIT.
- Loosen the top nut on the coupler (no need to completely remove),
- Slide the sample coupler with loosened top nut onto the SIT until it reaches a hard stop.
- Make sure the sample coupler tubing is not kinked or twisted.
- Hold the sample coupler with one hand and tighten the top nut with the other hand (do not overtighten).

A1

SIT protector

sample coupler aspirator arm
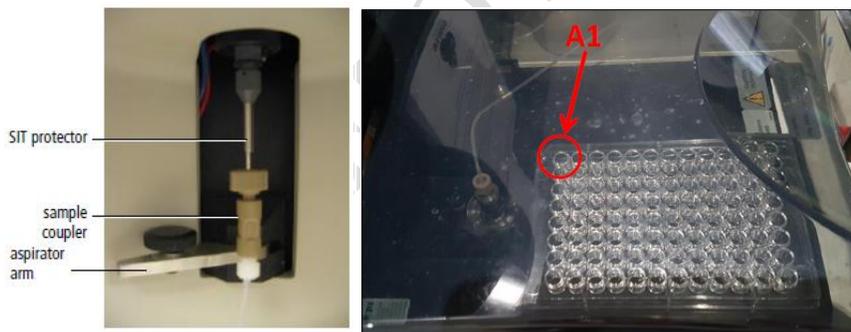
- Remove HTS safety cover (not shown in image above) and orient the plate carefully onto the plate holder.
- Remove the multi-well plate lid and replace the HTS cover (failure to do so can damage probe). Ensure the sample line tubing is not caught.

Setting up plate and running samples

BD FACSDiva workspace is comprised of 5 frames: Browser, Acquisition Dashboard, Cytometer, Inspector and Worksheet.

- Start DiVa (if haven't already) and open a new or existing experiment
- Click the arrow next to the new plate button located in the Browser Frame and select a plate type
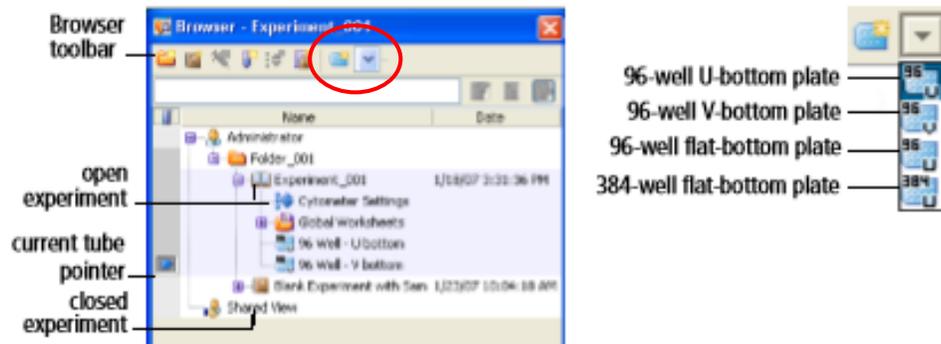
The Plate Window, a representation of the multi-well tray then becomes available (6th frame). The Plate window is also available when an experiment is open and you double-click on the plate in the Browser.
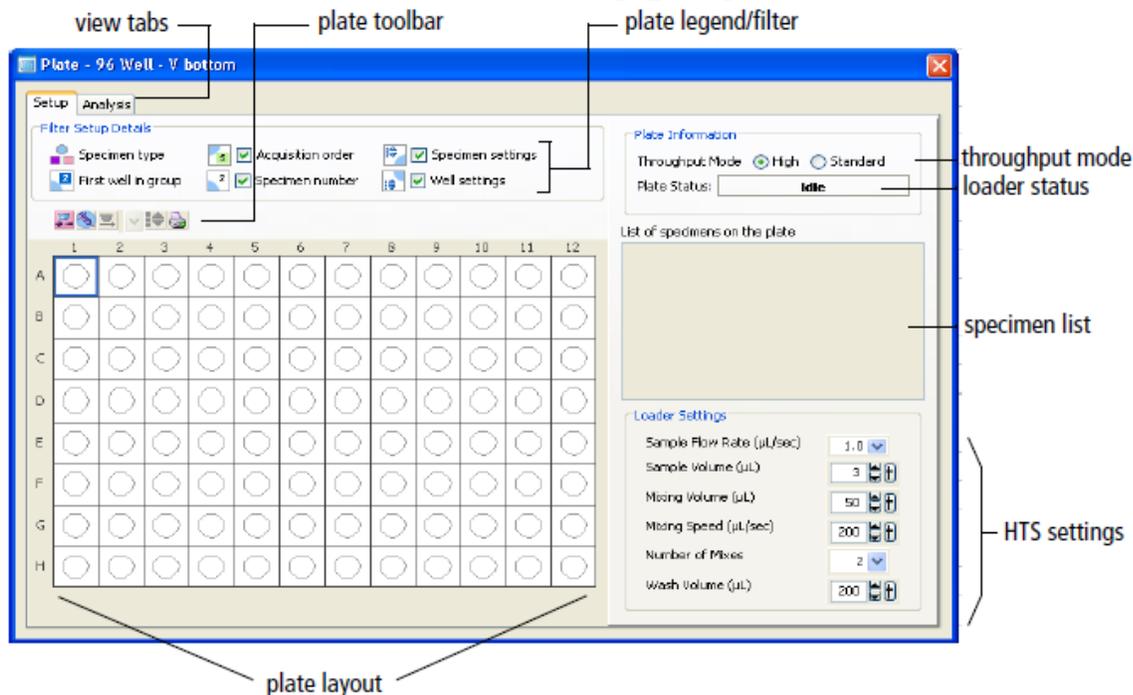
Use the Plate window (or inspector) to select the throughput mode:

High-Throughput Mode, the DIVA default mode. A 96-well plate can be processed in 15 min. Regardless of whether you define sample volume, the Fortessa will aspirate a fixed volume of 22µl (20µl delivery volume + 2µl sample).

Standard Mode is the recommended mode. Using default settings, a 96-well plate can be processed in 44 minutes (primary pump is used). Adjusting loader settings will affect run time.

Setting	High-Throughput Mode	Standard	User defined (Ranges)
		Throughput Mode ⦿ High ○ Standard	
Sample Flow Rate (µl/sec)	1	1	0.5 – 3.0
Sample Volume (µl)	2 (+20ul)	10 (+20ul)	2 – 200µl (+20µl) (limited by max. capacity of well)
Mixing Volume (µl)	50	100	Recommend ≤ ½ the volume contained in well, less 40µl
Mixing Speed (µl/sec)	200	180	
Number of Mixes (cycles)	2	2	1 – 5 (recommend starting with 3 as a guide)
Wash Volume (µl)	200	400	200 – 800 (recommend ≥ 400)
Approx. Acquisition Time (mins)	44	15	Depends entirely on settings chosen

Optimizing Loader Settings
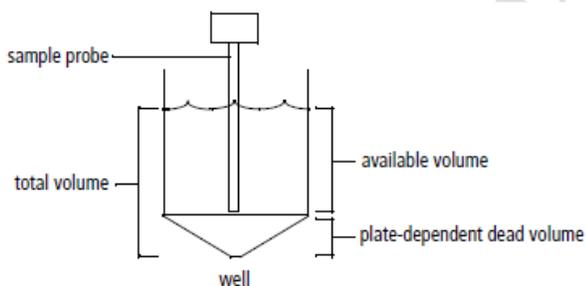
Sample volumes

When adjusting loader settings, it is important that sample volumes are taken into consideration so as to avoid the introduction of air bubbles which may result in poor, fluctuating or no signals. Each well must contain sufficient volume to cover:

1. the user defined sample volume (Standard Mode) or 22µl (High Throughput mode)
2. +20µl dead volume (standard mode only). In standard mode, the defined sample volume is taken up, together with 20 µl dead volume (required by system) followed by a separator bubble.
3. **+20µl (plate-dependent dead volume).**

Taken together, in standard mode, each well should contain at least 40µl more than sample volume. For example, if sample volume is 100µl, then the well should contain at least 140µl.

Mixing

- ALWAYS resuspend samples and do not rely on the HTS mixing setting to fully resuspend pelleted samples. Samples are mixed less effectively in flat-bottom wells than U-and V-bottom wells. Acquire samples before they settle.
- Sample viscosity will affect mixing efficiency.
- Ensure that the mixing volume is no more than half the sample volume. For example, if sample volume is 100μl, then mixing volume should not exceed 50μl.
- Increased number of mixes allows decreased mixing volume and speed but will lengthen time taken to run plate. Increased mixing speed and volume may improve throughput and mixing efficiency but could introduce air bubbles.

- Ensure the system is acquiring enough wash volume to minimise carry over between samples. The wash volume is the volume of sheath fluid dispensed for rinsing between wells. A greater wash volume results in less carryover but slower throughput time. **As a guide,** minimum wash volume should be approx 400μl for a 10μl sample volume (maximum wash volume is 800μl).

- Time it takes to complete a plate depends on acquisition time (sample volume/sample flow rate), mixing volume and speed, number of mixes and wash volume.
- To reduce time–concentrate the sample ie reduce sample volume, increase the sample rate (≥1μl/sec), reduce number of mixes (≤2) and/or decrease wash volume (≤400μl)
- Warning to those using HTS for cytotoxic drug screens or looking at sticky, large, apoptotic or cultured cells: to prevent blockages, reduce the sample flow rate and increase the wash volume between samples. We also highly recommend SIP Prime X3 and wash using a tube on SIP between plates.

Setting up a plate

The Setup view tab is used to adjust cytometer settings and plate layout.
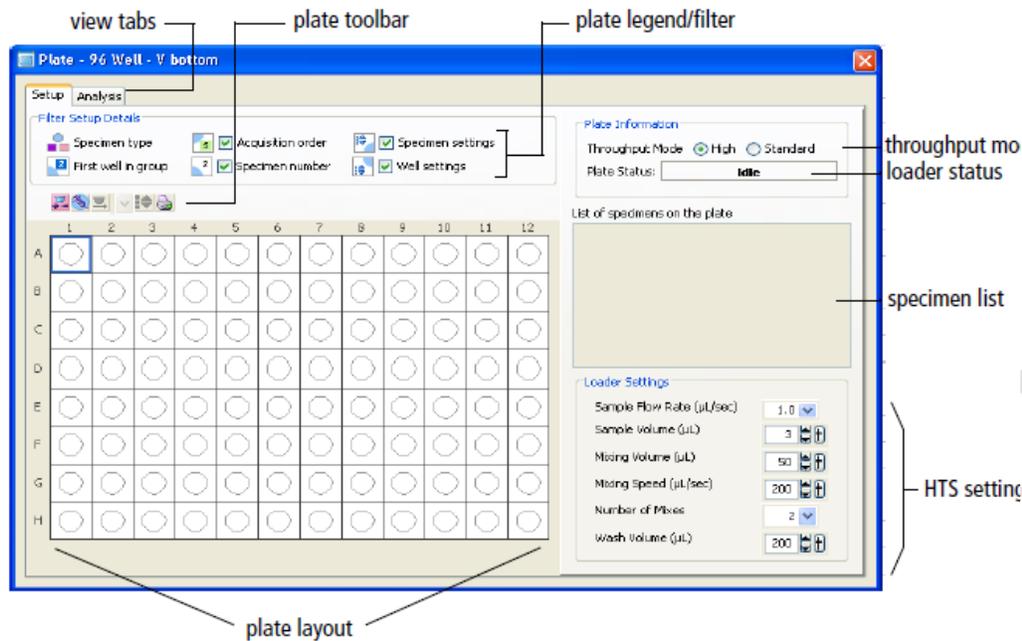
- Highlight wells corresponding to:

  o Setup Controls (unstained cells) - Recommend at least two wells of unstained cells to set threshold, FSC, SSC and fluorescent parameter PMTs so that you do not run out of sample while optimising cytometer settings.

  o To create compensation wells > select well > choose Experiment > Compensation setup > create compensation controls

  o Setup controls are not saved, therefore It is recommended that you have duplicate wells that can be labelled under specimen and saved.

  o Specimen (samples)

- For every well/group of wells assign loader settings
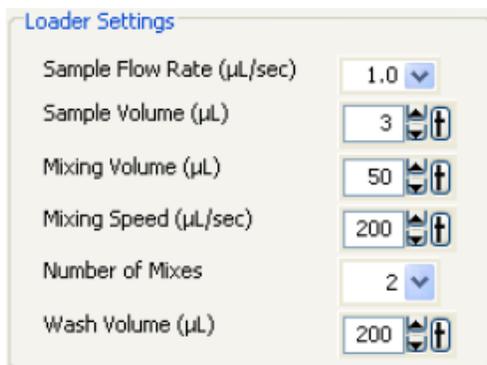
- If multiple wells are selected and they have different loader settings a red box will appear around the loader settings that are different

- Use the acquisition tab and set the number of events to record (or define timeframe of acquisition, eg 60 seconds).
- Select the set-up control wells in the plate layout to adjust and optimise FSC, SSC, threshold and PMTs (no data file is saved for set-up control wells, so if you want an FCS file remember to select a well containing cells only among your specimen wells)
- Once your voltages have been optimised, run compensation controls
Note - It may be easier to optimise FCS, SSC, baseline voltages and compensation using tubes and then apply the final cytometer settings to the plate (you can copy and paste cytometer settings to/from unrecorded wells only)
- Select the specimen well and click Run plate, or select the wells you want to run and click Run wells
- At the end of the acquisition a box will appear highlighting that run is finished

Instrument shutdown:

Shut-down procedure if the HTS was used:

Always run a cleaning plate after using the HTS to clean the sample line tubing from the HTS to the SIP.

1. Under HTS menu > select "clean"> choose "cleaning plate"
2. You will be prompted to have a cleaning plate with wells of cleaning and rinse solutions
   a. Fill wells A1 – A4 with ≥ 250 μl fresh 10%Bleach
   b. Fill wells B1 – B4 with ≥ 250 μl MilliQ H_2O
3. If you are acquiring large, sticky or dying cells, repeat the cleaning plate procedure
4. Return the LSR-Fortessa to tube settings by:
   a. Flicking the switch on the side of the instrument from plate to tube
   b. Putting the machine into STANDBY
   c. Loosening the HTS sample coupler nut and placing a tube of MilliQH_2O on the SIP and returning the support (aspirator) arm to its central location.
5. If someone is scheduled to use the instrument within the hour after you, then just logout of DIVA and leave the cytometer on standby. However it is important that you communicate with the next user and inform them that you have left the instrument on standby for them. Remember it is research, sometimes experiments don't work and the person booked after you may not honour their booking. It is important that the instrument is not left on for extended periods of time or overnight. If you can't get a hold of the next user, then better to be cautious and shut cytometer down.

Troubleshooting:

The following is a list of the most frequently encountered problems. Please be familiar with these and attempt to recertify them yourself before contacting a member of the AMREPFlow staff.

"The Cytometer will not connect"
This will be evident by everything being switched "on", but DiVa software stating the cytometer is disconnected.
1. While leaving DiVa on, switch the LSR-Fortessa off. Wait 10 seconds. Switch the LSR-Fortessa back on. If the LSR-Fortessa has not connected after 2 minutes (be patient, connection can take this long!), try:
2. Switch the LSR-Fortessa off and shutdown (not restart!) the computer. Wait a few minutes. Switch the LSR-Fortessa and the computer back on. Start DiVa as normal. If the cytometer will not connect after a few minutes, see AMREP Flow Staff.

"I'm not seeing events when I load my tube" / "There are no drops coming from the SIP when there is no tube on" / "My signals look funny"

This can be due to a number of factors. In this order try:
1. Adjust the O-ring at the top of the SIP so that it is tight enough to hold you tube independently of the supporting arm. (See Diagram).
2. Ensure your tube has no cracks – any breach in the O-ring seal will result in loss of pressure and your sample not being taken up by the SIP
3. Ensure that the sheath tank is properly sealed; the tubing is correctly connected and has no kinks. Bleed all air out of the bubble filter.
4. Ensure there are no air bubbles in the waste line – jiggle the orange tube to move the air along.
5. Ensure the waste lid connection is loose, to avoid waste line back pressure issues which will affect time delays.
6. Ensure the "Fine Tune Adjustment" knob is not turned all the way down. Turn it clockwise several times if you are able to.
7. With no tube on the SIP, move the supporting arm to the side and PRIME the instrument 3 times.
8. Run bleach (available from the squeeze bottle next to the instrument) for a few minutes on HI followed by water on HI.
9. Loss of signal during a run could be due to
   a. Dirty Flow Cell
   b. Turbulence in Flow Cell affecting hydrodynamic focusing
   Both of these can affect LASER Time Delays hence signal.

Still having issues? Please see a member of the AMREPFlow Staff