

AMREP FLOW – LSR-Fortessa Users SOP

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Lab Contact number: 9903 0601

Geza: paukovic@burnet.edu.au 9282 2246

Jeanne: jeanne@burnet.edu.au 8506 2363

Phil: phil@burnet.edu.au 8506 2332



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Important Points

- If problems arise which you are unsure how to fix please do not try and fix them yourself – address any questions to a member of the AMREPFLOW Staff (put a note on the instrument and email us if things go wrong on the weekend)
- All users must be licensed to use the instrument and be familiar with the AMREP Flow Department policies. Disregard of SOPs or abuse of systems may result in suspended use of instrumentation. To become licensed please contact AMREP Flow Staff.
- Correct shutdown procedure must be followed. Failure to follow thoroughly can result in instrument blockages and malfunction and can affect the work of the next user.

Sample Preparation

- ALL samples MUST be filtered through a 70um mesh filter before use – NO EXCEPTIONS.
- All samples must be at an appropriate cell concentration in order not to block the Sample Injection Probe (SIP).
 - Recommended cell concentration is 1×10^6 cells / ml (2×10^5 cells / 200µl)
- Users who continue to block the SIP through neglectful sample preparation may have their usage privileges withdrawn.
- The Fortessa has a 405nm, 488nm, 561nm and 633nm laser setup with the ability to detect signals in 16 channels. Before designing an experiment or purchasing an antibody, please ensure it is compatible with this instrument. Instrument capabilities are available at www.amrepflow.org.au > we have some additional filter combinations, please ask if your panel could be better optimized

Instrument Start-up

1. Switch on the LSR-Fortessa at the side of the instrument. This will warm up the lasers and pressurize the fluidics.
2. Switch on the Computer and login to the DiVa software. Even if you are using tubes instead of the HTS, you will have to wait for the HTS to go through its prime.
3. Check the fluidics.

Fluidics

- The Fortessa strictly uses MilliQ water as its sheath fluid. There are 20L containers available in the lab, located next to the sink.
- The Sheath Tank is the stainless steel container. It must only be filled with MilliQ water to ¾ of the tanks total volume (to the seam line) (enough fluid to run the instrument for approximately 4 hrs). **There is no sensor to indicate the volume remaining; users must be vigilant to check the level before each session by opening it and looking.**

**** Fill up Fluidics tank for the next person at the end of your session ****

To fill the sheath tank:



1. Unscrew the lid at the top of the tank and pull up the pressure valve (3) for a few seconds until pressure has been relieved
 - a. You do not need to remove the pressure (1) or fluid (2) lines from the sheath tank
2. You may need to push down on the lid to be able to overcome the pressure seal and remove the lid. If the LSR-Fortessa is on and the pressure line connected, the tank will try to re-pressurise.
3. Remove the lid and fill the sheath tank with MilliQ water to the metal seam line in the tank (4). This will ensure the tank is roughly $\frac{3}{4}$ full.
4. Replace the lid - it will only fit in one way – and tighten screw ensuring that the metal handle is in line with the sheath port (2) as shown in the first diagram. Do not force the lid or handle in any other way.
5. **Wait 30 seconds until the sheath tank is pressurised, then bleed any air bubbles by removing the bung on the bubble filter and drain the water into a bucket provided (5). This will expel sheath fluid at pressure, so ensure that the fluid line is facing into the bucket and any spills are wiped up. FAILURE TO COMPLETE THIS STEP WILL RESULT IN POOR OR NON-EXISTENT SIGNALS FROM THE 405, 561 AND 640 LINES.**

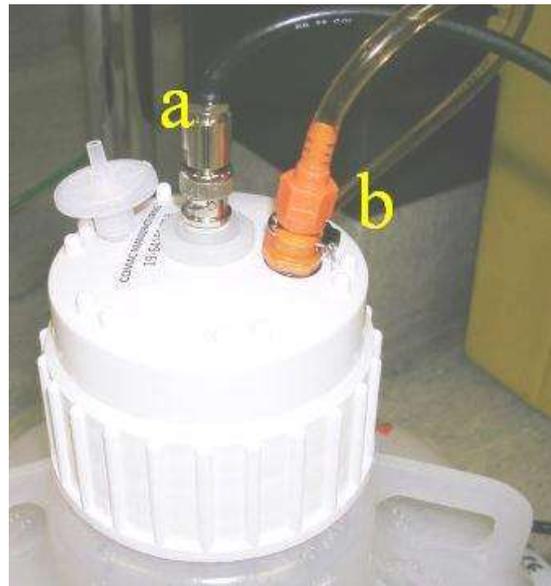
Waste Tank:

PLEASE NOTE: The waste tank holds a much higher volume of fluid than the sheath tank. Do not use the waste tank volume as an indicator of sheath remaining. Periodically check the sheath tank volume to ensure the sheath does not run dry.

At the end of your session please empty the waste and add 10% bleach to fill the bottom of the tank.

To empty the waste tank:

1. Remove the black coaxial cable (a) by turning it anti-clockwise.
2. Remove the orange waste tube (b)
3. Pour the contents of the waste tank down the sink whilst running tap water at the same time to flush and dilute any excess bleach down the sink.
4. Once the tank is empty, add 10% Sodium Hypochlorite to the tank. Pour between 100ml and 200ml. Please ensure you are wearing gloves, safety glasses and a lab coat for this step (all are provided, please ask if you don't know where to find them) – Sodium Hypochlorite is hazardous and could damage your eyes, irritate skin and will irreversibly damage your clothing should you splash or spill it on yourself (be familiar with the MSDS in case of spillages).
5. Reconnect the tubing and (b) coaxial cable (c).



Software and sample setup:

1. Turn on the PC and both monitors. You will be prompted for a password which can be obtained from AMREP Flow Staff
2. When logged in, click the BD FACS DIVA software icon.
3. You will be prompted for a username and password. When licensed, you will be given an individual login and password to use DIVA.

****UNDER NO CIRCUMSTANCES ARE LOGIN DETAILS TO BE SHARED. SHARING OF THESE DETAILS OR LOGGING IN FOR ANOTHER USER WILL NOT BE TOLERATED AND WILL RESULT IN YOUR ACCESS BEING LIMITED. STRICTLY LICENSED USERS ONLY****

4. Should you be prompted to use CST settings, select YES as these settings will use the most up to date time delay calibrations for the instrument. Should you not be asked to use CST settings, the instrument will already contain these settings.
5. From the SIP, remove the tube and prime the instrument twice using the button on the LSRII control panel. Place a tube of water on the SIP and press RUN from the panel with the speed setting on LOW
6. Adjust the SAMPLE FINE ADJUSTMENT knob to the middle setting. The knob is designed to make 10 complete rotations, thus in order to have it in the middle turn it all the way anticlockwise until it meets resistance, then turn it clockwise 5 complete rotations.

FACS DIVA Software guide is available on LSR-II computer desktop. Please refer to this document for comprehensive guidelines on how to use the software. If further assistance is required please contact a member of the AMREP Flow staff.

Using Tubes



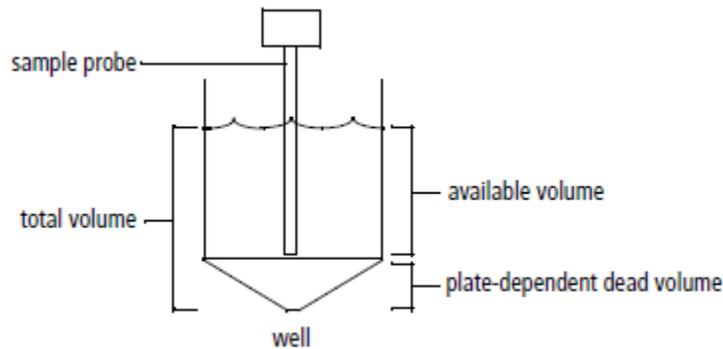
- Ensure that the switch at the side of the instrument is enabled for tubes (ie up!)
- Ensure that the O-ring is tight enough for your tubes to remain on the SIP hands free without the tube support arm
- Double check that there are no air-bubbles in the sheath line or bubble filter.
- Run a tube of water for 5 mins after priming the instrument to clear air bubbles from the waste-line. 'Wiggle' the orange tubing to move the air along.
- Run your tubes

Using the HTS

- Acquires samples from 96- and 384-well plates (standard depth)
- BD default is High-Throughput Mode – Always choose to run your plate in STANDARD mode, because you can optimise your Acquisition settings. On High-Throughput Mode, regardless of the volume entered into the software, the Fortessa will aspirate a fixed volume of 22ul (20ul delivery volume + 2ul sampled volume of your sample even if you enter another volume – and there is NO warning of smaller acquisition volume).

Setting	High-Throughput Mode	Standard	User defined (Ranges)
	Throughput Mode <input checked="" type="radio"/> High <input type="radio"/> Standard		
Sample Flow Rate (ul/sec)	1	1	0.5 – 3.0
Sample Volume (ul)	2 (+20ul)	10 (+20ul)	2 – 200ul (+20ul) (limited by max. capacity of well)
Mixing Volume (ul)	50	100	Recommend $\leq \frac{1}{2}$ the volume contained in well
Mixing Speed (ul/sec)	200	180	
Number of Mixes (cycles)	2	2	1 – 5 (recommend ≥ 3)
Wash Volume (ul)	200	400	200 – 800 (recommend ≥ 400)
Approx. Acquisition Time (mins)	44	15	Depends entirely on settings chosen

Getting volumes right ... to prevent air bubble being introduced into the system



When preparing your plate ... re-suspend samples in a minimum volume of 50ul.

Components

Knowing the components can help with troubleshooting when things go wrong.

- Plate holder (stage) – remove plate lid and orient the plate carefully so the probe doesn't get damaged hitting the plate top instead of sampling your well.
- Probe – it moves back and forth to the wash station transferring sample ... Ensure the sample line tubing is not going to be crushed when replacing the safety HTS cover!
- Primary pump and valve – enables mixing and aspiration of sample and delivers sample to the flow cell (in std mode) – if you notice condensation or suspect anything is wrong with this component, please find an AMREPFlow staff member
- Fluidics tubing – sheath (clear) and waste (orange) ... Sheath line has a filter which filters incoming sheath fluid to HTS unit
- Injection port/wash station – interface for sample injection and probe washing (extra wash volume will minimise carry over between samples)
- Sample coupler – connector between HTS unit to cytometer SIP

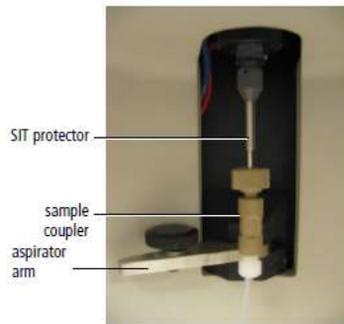
Cytometer Connections

Cytometer Acquisition Modes ...		
In tube mode ...		<ul style="list-style-type: none"> • The droplet containment module (DCM) vacuum functions normally – the vacuum is ON when the support arm is positioned to the side and OFF when the support arm is centred. • The RUN button will go orange if there is a problem with pressure in the system (ie loose fitting or cracked tube, damaged o-ring). You can only run tubes in tube mode.
In plate mode ...		<ul style="list-style-type: none"> • The DCM vacuum does not function • IMPORTANT ... you can run plates OR tubes in plate mode however the RUN button will NOT turn orange if your tube doesn't seal properly. Ensure tube fits so sheath backdripping doesn't lyse fresh cell samples. Even a centred support arm will not stop sheath backdripping

Sample Coupler

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

- You do not need to completely undo the nut on the sample coupler – just loosen it sufficiently to attach/remove it from the SIP
- Do not over-tighten the tightening nut!!!



Optimizing Acquisition Settings

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.
- Mixing efficiency is impacted by sample viscosity.
- 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.
- TROUBLESHOOTING: Sporadic event rates and high carryover? ... The mixing speed might be too high

Carryover

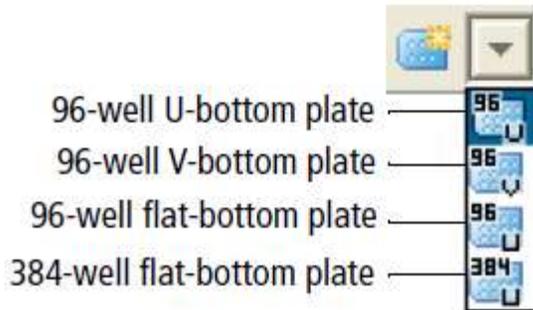
- A greater wash volume results in less carryover but slower throughput time
- Minimum wash volume - 400ul wash: 10ul sample volume (maximum wash volume is 800ul)

Throughput

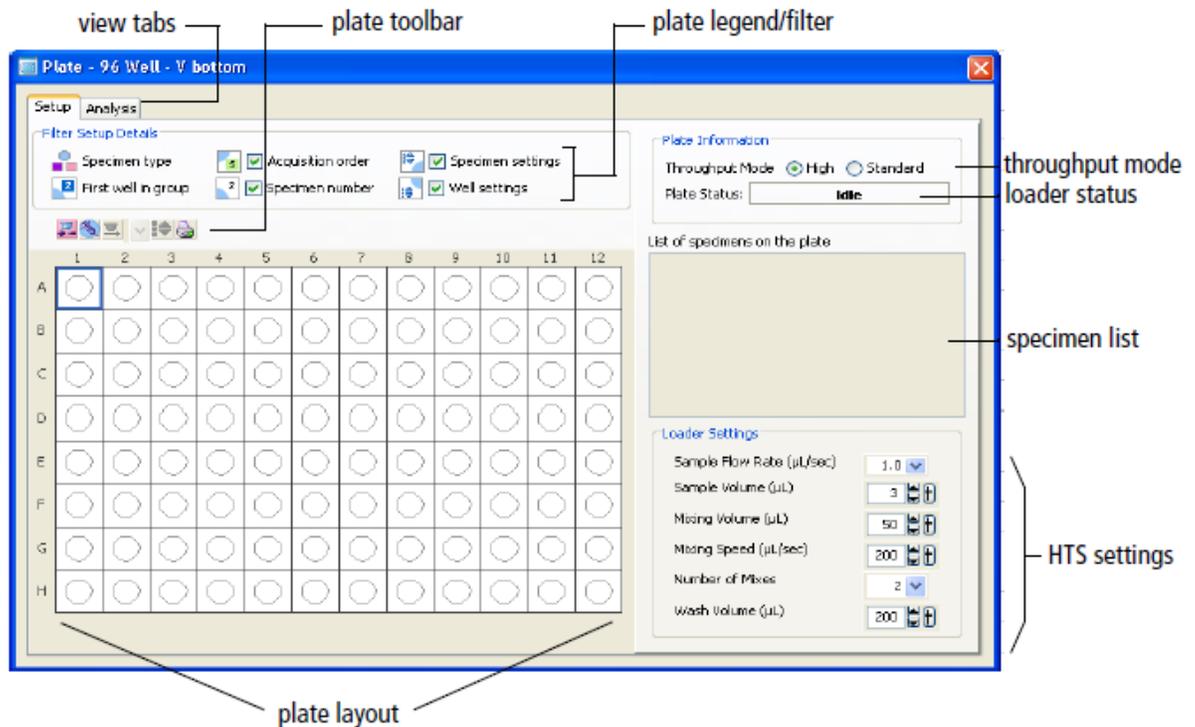
- Time it takes to complete a plate depend on acquisition time (sample volume/sample rate), mixing volume and speed, number of mixes and wash volume.
- To reduce time take – BD recommends concentrating the sample, reduce sample volume, increase the sample rate ($\geq 1\text{ul/sec}$), reduce number of mixes (≤ 2), decrease wash volume ($\leq 400\text{ul}$)
- Warning to those looking at sticky, large or cultured cells ... to prevent blockages, reduce the sample flow rate and increase the wash volume between samples.

Setting up plate and running samples

- Start DiVa and open a new or existing experiment 
- Click new plate button and the arrow next to it to choose a the plate type



- Use the Plate window to select the throughput mode (Standard mode is recommended) and this is also where you can adjust loader settings for wells



- Highlight wells corresponding to:
 - 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
 - To create compensation wells > select well > choose Experiment > Compensation setup > create compensation controls
 - Specimen (samples) 

- For every well/group of wells assign loader settings

Loader Settings

Sample Flow Rate ($\mu\text{L}/\text{sec}$)	1.0
Sample Volume (μL)	3
Mixing Volume (μL)	50
Mixing Speed ($\mu\text{L}/\text{sec}$)	200
Number of Mixes	2
Wash Volume (μL)	200

- If multiple wells are selected and they have different loader settings a red box will appear around the loader settings that are different
- Ensure each well contains sufficient sample for the entered volume + dead volume. Not having enough volume or having a mixing volume that is larger than the available volume will introduce air bubbles into the sample and may result in poor, fluctuating or no signals.
- Use the acquisition tab and set the number of events to record.
- 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 after running tubes:

1. After running of your final tube sample, move the support arm to the side, press RUN and allow at between 5-10 drops of sheath to fall from the SIP.
2. Place a tube of MilliQ water on the SIP and let it RUN at HI flow rate for 5 minutes
3. Repeat step 2 using 10% hypochlorite (Bleach) solution or 10 mins if you are the last user of the day.
4. Repeat step 2 using MilliQ water.
5. Leave a tube containing ≥ 1 ml of MilliQ water on the SIP and set to STANDBY
6. Turn off cytometer and shut down the computer – the only time you should leave them on is if you have contacted the next user and you know they will be using the LSR-Fortessa within an hour.

Shut-down procedure if the HTS was used:

1. Always run a cleaning plate if you use the HTS, even if you run tubes as well, as you need to clean the sample line tubing from the HTS to the SIP.
2. Under HTS menu > select “clean”> choose “cleaning plate”
3. You will be prompted to have a cleaning plate with wells of cleaning and rinse solutions
 - a. Fill wells A1 – A4 with ≥ 250 ul fresh 10%Bleach
 - b. Fill wells B1 – B4 with ≥ 250 ul MilliQ H₂O
4. Replace the safety cover and run plate. It will take about 10-15 mins. If you are acquiring large, sticky or dying cells, copy the cleaning plate loader settings onto a new plate and run > 4 wells each of 10%Bleach and MilliQH₂O.
5. 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 MilliQH₂O on the SIP and returning the support (aspirator) arm to its central location.
6. Turn off the cytometer and shut-down the computer – the only time you should leave them on is if you have contacted the next user and you know they will be using the LSR-Fortessa within an hour.

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. 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 APC/PE/V450 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 “Fine Tune Adjustment” knob is not turned all the way down. Turn it clockwise several time if you are able to.
6. With no tube on the SIP, move the supporting arm to the side and PRIME the instrument 3 times.
7. Run bleach (available from the squeeze bottle next to the ARIA) for a few minutes on HI followed by water on HI.



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