

# AMREP FLOW – Canto-II Users SOP

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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  $1 \times 10^6$  cells / ml ( $2 \times 10^5$  cells / 200µl)
- Users who continue to block the SIP through neglectful sample preparation may have their usage privileges withdrawn.
- Instrument specifications:
  - The Canto-II (Alfred Centre) has a 488nm and 633nm laser setup with the ability to detect signals in 6 channels
  - The Canto-II (Monash) has a 405nm, 488nm, and 633nm laser setup with the ability to detect signals in 8 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](http://www.amrepflow.org.au) > we have some additional filter combinations, please ask if your panel could be better optimized

## Fluidics

- The Canto-II strictly uses double distilled H<sub>2</sub>O, from the MilliQ purification system, as its sheath fluid. There are 20L containers available in the lab, located next to the sink.

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

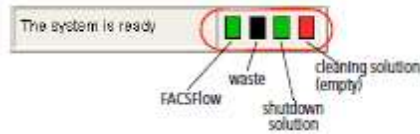
## Cytometer start-up

1. Turn on power to the cytometer (green switch) – system power button turns on lasers and power to the fluidics cart
2. Turn on the PC and both monitors. You will be prompted for a password which can be obtained from AMREP Flow Staff
3. When logged in, click the DIVA software icon  
You will be prompted for a username and password. Contact a member of the AMREP Flow staff for Canto-II training & licensing to access the Canto-II online booking system and DIVA log-in.

**\*\*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 LICENCED USERS ONLY\*\***

4. Make sure the software is connected to the cytometer  
If not connecting, from the main menu choose Cytometer > Connect  
If still not connecting, try turning everything OFF > and then ON again  
If still not connecting, contact a member of the AMREP Flow staff

- 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.
- Check fluid levels > Low fluid levels or a full waste container are indicated in red



- From a cold start, choose Cytometer > Fluidics Startup  
 Monash Canto-II (Lvl1) – Ensure there is no tube on the SIT at Start-up  
 Burnet Canto-II (Lvl7) – should be started in plate (HTS) mode > Sample Coupler attached
- When Fluidics Start-up finishes, choose Cytometer > Cleaning Modes > De-gas Flow Cell to ensure there are no air bubbles in the flow cell  
 Repeat this step once (and more times if necessary)
- Check that laser warmup has finished

### Running samples – manual tube loading

Ensure that the aspirator arm bar is vertical and there is no sample coupler attached

### Running samples – using High Throughput Sampler (HTS) mode

Ensure that the aspirator arm bar is horizontal and that the sample coupler is attached to the SIP

### Software and Sample Set-up

- (Optional) Create a folder > Rename the folder by right-click Rename or in the Inspector Window (Folder)
- Create a new Experiment (lab book icon)  
 An open (active) experiment appears as an open lab book icon, closed experiment is a closed lab book icon  
 Rename the experiment by right-click Rename or in the Inspector (Experiment)
- Select the experiment-level cytometer settings > Inspector Window select Parameters Tab  
 Change, add or delete parameters as needed  
 Deleting a parameter can reduce the size of data files by not collecting un-necessary parameter/s, it can also simplify compensation matrix tables ... BUT decide carefully if a parameter is not needed > no data is recorded in a deleted parameter
- Create a new Specimen (syringe icon)  
 Rename the specimen by right-click Rename or in the Inspector (Specimen)  
 Experiments can contain multiple specimens > AMREPFLOW recommend 10 – 16 specimens per experiment.  
 The processing power slows down with increased number of specimens and tubes.
- Click (+) left of the Specimen > Rename Tube\_001 to unstained control etc. by right-click Rename or in the Inspector (Tube)
- Activate the tube by clicking the current tube pointer (arrow to the left of the tube).  
 A tube needs to be “active” for the Acquisition Dashboard to be operational

16. In the Acquisition Dashboard, verify the SIT Flush checkbox is selected > this will minimise carryover between tubes by flushing the sample injection probe.
17. Optimise cytometer settings > adjust the FSC and SSC voltages to appropriately display the scatter properties of the unstained control
18. Click the Threshold tab and adjust the FSC Threshold  
Threshold is a cut-off point; below threshold events are not registered. Threshold off FSC enables the removal of debris and small events without cutting off populations of interest. In doing so, data files will be smaller because they will not be inundated with small particles.  
(Optional) To add another Threshold parameter click Or/And & Add > SSC is another commonly used Threshold criteria
19. Use either rectangle or polygon gate to select population of interest > automatically titled P1 (Population 1) > Click the P1 gate boundary to select it which will enable you to drag to move, or select an anchor point to re-size or re-shape.
20. Set baseline voltages using your negative control, confirming with single colour controls if you suspect a very bright fluorophore might be off scale
21. Record baseline voltages and start compensation controls
22. Click “next tube” > rename using the inspector > start with compensation controls
23. Build up compensation matrix, working through compensation controls
24. Define recording rules and limits (10,000 events is the default but this might not be a sufficient number of events for sufficient statistical significance.)
25. Run & record samples.
26. Export FCS3.0 files  
Right click on Specimen > Export > Save to lab designated Hard Drive  
**Do not delete experiments from the DIVA database. AMREPFLOW staff periodically backup the database and will export and delete experiments.**

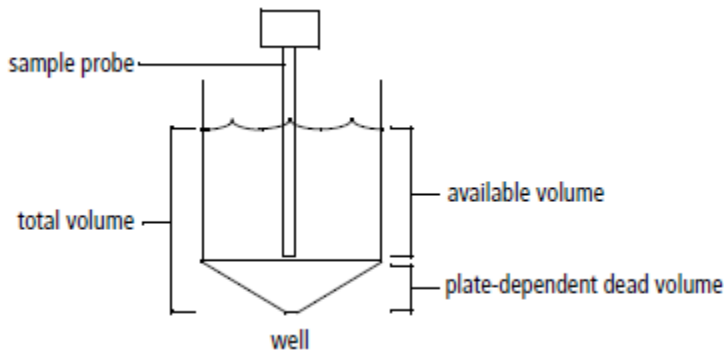
## Using the HTS

- 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 Canto-II 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	200	180	

(ul/sec)			
Number of Mixes (cycles)	2	2	1 – 5 (recommend $\geq 3$ )
Wash Volume (ul)	200	400	200 – 800 (recommend $\geq 400$ )
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

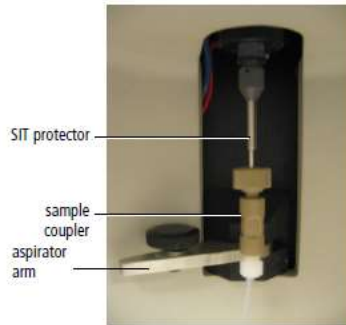
Knowing the components can help with troubleshooting when things go wrong.

- 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

### 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

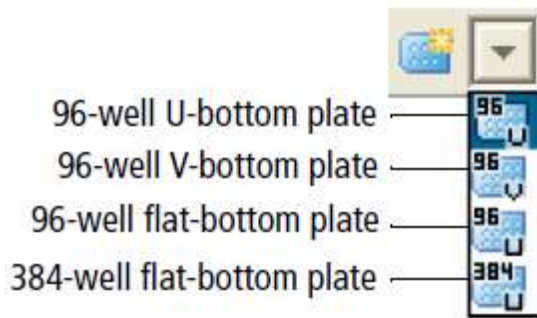
- Time it takes to complete a plate depend on acquisition time (sample volume/sample rate), mixing volume and speed, number of mixes and wash volume.
- To reduce time take – BD recommends concentrating the sample, reduce sample volume, increase the sample rate ( $\geq 1\text{ul/sec}$ ), reduce number of mixes ( $\leq 2$ ), 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

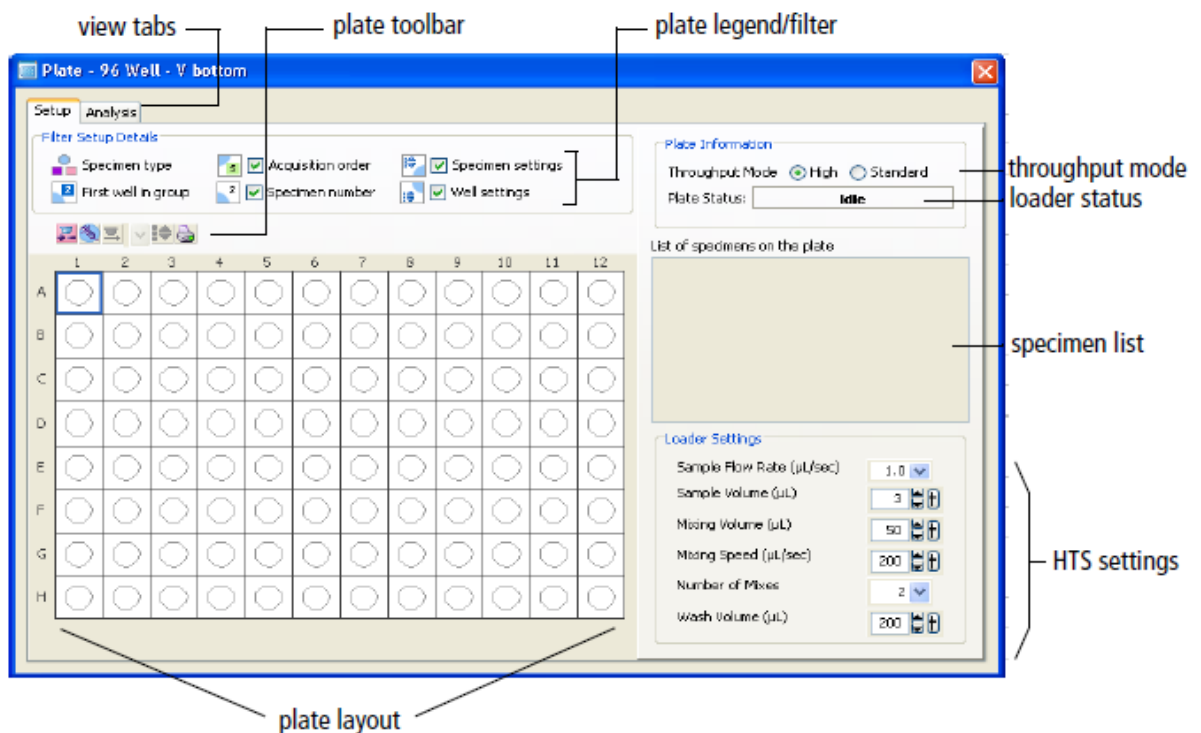
- Start DiVa and open a new or existing experiment





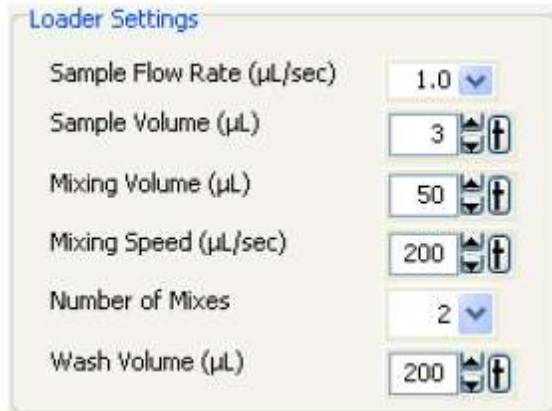
- Click new plate button and the arrow next to it to choose a the plate type



- 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



- 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. Place a tube of MilliQ water on the SIP and let it RUN at HI flow rate for 5 minutes
3. Place a tube of 5% hypochlorite (Bleach) solution for 5 mins between users, or 10 mins if you are the last user of the day.
4. Repeat step 1, run MilliQ water for 5 mins.
6. If there is no one booked after you, perform a Fluidics Shut-down.  
Choose Cytometer > Fluidics Shut-down (takes about 5 mins).  
Then 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 Canto-II 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  $\geq 250$  ul fresh 5%Bleach
  - b. Fill wells B1 – B4 with  $\geq 250$  ul MilliQ H<sub>2</sub>O
4. Close the safety door and run plate. It will take about 10-15 mins. If you are acquiring large cells, sticky cells or dying cells, consider a more rigorous cleaning procedure, by running the clean plate twice (refilling after the first time) or copy the cleaning plate loader settings onto a new plate and increase the number of water and bleach wells.
5. Leave the Canto-II (Alfred Centre) with the HTS attached – this is the default setting for this instrument as this is the most frequent form of sample acquisition.
6. If there is no one booked after you, perform a Fluidics Shut-down.  
Choose Cytometer > Fluidics Shut-down (takes about 5 mins).  
Then 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 Canto-II within an hour.

## Common Troubleshooting Solutions:

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 Canto-II off. Wait 10 seconds. Switch the Canto-II back on. If the Canto-II has not connected after 2 minutes (be patient, connection can take this long!), try:
2. Switch the Canto-II off and shutdown (not restart!) the computer. Wait a few minutes. Switch the Canto-II and the computer back on. Start DiVa as normal. If the cytometer will not connect after a few minutes, see AMREP Flow Staff.

### **My compensation matrix keeps reverting back to 0.00 when I create a new tube**

- A. Choose Edit > Users Preferences > Un-tick “*remove tube specific cytometer settings on duplication*”
- B. Choose “new tube” from the Acquisition Dashboard and not from the Browser Workspace Toolbar

### **I have accidentally exit my Browser, Cytometer, Inspector, Worksheet, Acquisition Dashboard, Bi-exponential Editor or Plate windows**

- A. Click the corresponding buttons/icons in the Workspace toolbar to display them again

### **I am not seeing any events ...**

- A. Have you eliminated the possibility of a blockage?  
Try De-gas flow cell