Flow Cytometry Core Facility
Standard Operating Procedure
Attune NxT Setup and Operation

Created: April 17, 2020
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1. Purpose

The purpose of this standard operating procedure (SOP) is to outline the setup and operation practices used for the University of Alberta Faculty of Medicine and Dentistry (FoMD) Flow Core Facility Attune NxT analyzer. The procedures outlined here are to supplement the general laboratory procedure for the FoMD Flow Core Facility.

2. Biosafety level

The Attune NxT is classified as a Biosafety level 1 (BSL1) instrument. BSL1 instruments are designated for human or non-human cell lines that do not contain human or animal pathogens and are categorized as BSL1, Containment Level 1 (CL1) or Risk Group 1 (RG1). Live primary non-human primate cells not infected with a human pathogen or that have been virally transduced (if more than 24 hours since transduction) are also considered BSL1. Live primary human cells are BSL2; these and other BSL2 cells or cell lines can only be acquired on the Attune NxT if they are fixed with a suitable fixative, such as FACS buffer + 2% paraformaldehyde, which decreases their risk level to BSL1. Please refer to flow charts in Section 13 Appendix for further guidance.

3. Hazard Identification

Working on a BSL1 cell analyzer is considered low risk, scoring <5 on a scale of 1-25, where 1 is the lowest and 25 the highest risk.

4. Control/Protective Measures

Wash hands after working with samples and equipment. PPE is not required to use the Attune NxT.

5. Materials

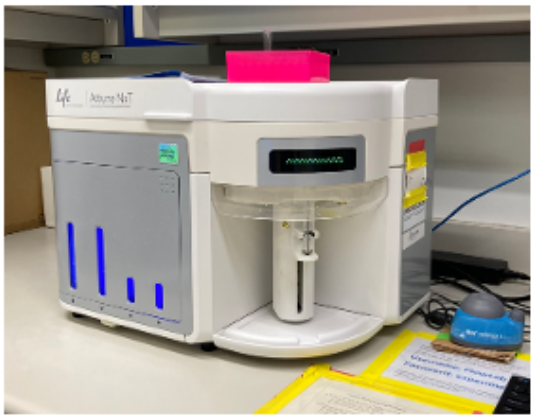
Attune Performance Tracking beads (cold room; ThermoFisher Cat#4449754)
Debubble Solution (top drawer labeled "Attune NxT", ThermoFisher Cat#A10496)
Flow Cell Cleaning Solution ("Attune NxT" drawer, ThermoFisher Cat#A43635)
1 x Focussing Fluid (near Attune NxT in glass bottle, refill from box in storage area)
10 x Focussing Fluid (ThermoFisher Cat#A24904)
Shutdown Solution (on shelf above Attune NxT, ThermoFisher Cat#A24975)
Wash Solution (on shelf above Attune NxT, ThermoFisher Cat#24974)

Samples in 1.7 ml Eppendorff or 5mL round bottom FACS tubes; suggested volume: 400-500 µl

6. Instrument setup

Attune NxT "OFF"

Attune NxT "ON"



1. Ensure instrument's Focusing fluid, Wash and Shutdown containers are full, and that waste is empty
    a. To disconnect Waste: unplug black cable on the right first, then the other cable. Waste can be poured down the sink. **Do not** fill with bleach. When reattaching cables, do so in reverse order to unplugging. Be careful disconnecting the fluid and sensor lines.
2. Log into the computer under your MED IT account
3. Start Attune NxT software on desktop
4. Log into software account
5. Lower arm that holds tubes and remove tube
6. If the instrument is off, select *Startup* from the *Instrument* tab in the Tools Bar and follow software prompts
7. If daily Performance test has not be done:
    a. Load tube of calibration beads (3 drops of Attune Performance Tracking beads with ~3mL of focusing fluid)
    b. Run Performance Test, follow prompts
    c. Once finished, all channels listed should have green check marks
        i. If not, rerun test or run *Debubble*, *Sanitize SIP*
    d. Lower tube for rinsing

7. Experiment setup

To analyze samples:

1. Click *New Experiment* every time you are running a new type of experiment.
    a. If a window is missing/ has been accidently removed, reset the view layout by selecting *View* and then *Reset window layout*. The instrument settings will be on the bottom left corner – click and drag them to the right-side pane.
    b. You can create # groups # tube samples.
2. In *Instrument Settings*, you can unclick channels you do not need. Keep area, height and width for all. Otherwise, in the right panel, you may click and drag tiny boxes from previous experiments (WS – worksheet settings; IS – instrument settings). This will populate the same settings into the new experiment.
3. Click on *Compensation* tab in the toolbar along the top of the screen. Click on *Compensation Setup*. Select the appropriate colours/channels for your experiment in the pop-up window, select "use unstained control" if using a universal negative control. Click OK and this will populate the compensation controls for this experiment. You may type in the fluorophore name into the labels column in each channel you are using; this will then populate the axis titles in the workspace. **You must do this every time you have new samples.**
4. You may also right click on an experiment name that you wish to duplicate, but check to make sure parameters are still okay.
5. Set flow options: choose a low speed and low volume for set-up (e.g. 50 µl volume at 25 µl/min). Be sure to have brought enough unstained sample for setting up, keeping in mind the dead volume for each acquisition is 50 - 75 µl depending on the volume set to acquire; this is indicated by the software prior to sample acquisition.
6. Note that the flow rate cannot be changed during sample acquisition. Maximum flow rate is 1000 µl/min; 30,000/sec max event acquisition rate.
7. Remove the water and allow the machine to rinse. Manually lower and raise the lifter; there's no need to wait for the pump to shut off.
8. Then vortex and load first compensation control.
9. Make sure to click on sample name in the right pane.
10. Adjust FSC and SSC voltages to ensure your population of interest is on the SSC vs FSC plot and not near the axes. Click and drag the gate box on this plot so it captures the cluster of cells. Right click on the box and apply to all controls. Recover unused sample if not enough volume left in tube for subsequent acquisition: click on *Recover Volume* and then *next* to use the same tube or supply a fresh empty tube if desired.
11. Vortex and load your fully stained sample.
12. Fix the gates on fluorophore histograms so that they capture the negative and positive peaks. Adjust voltages in relevant channels if needed such that populations remain on scale. Recover unused sample as noted in step 10.
13. Once you are satisfied with voltage settings, set your acquisition volume, acquisition speed and stop option. Click on *apply to group* to apply these conditions to all compensation tubes.
14. Vortex and load compensation controls one by one. Click on *run* and then *record*.

15. Adjust gates. When setting gates for compensation controls: the negative gate should extend all the way to the Y axis. Note that you may use fluorescence Area or Height; however, some feel Height is better on this instrument.
16. Click *View Matrix* in the compensation tab after all samples are complete to ensure all values look appropriate.
17. To toggle between compensation workspace and experiment workspace, just click on sample name in the Experiment Explorer panel (right side of screen).

8. Acquisition

1. In the Experiment Workspace, add plots and gates as needed using options in the Workspace tab on the main toolbar.
2. Right click on the first sample and rename.
3. On the left side pane, under 'Run Protocol', set acquisition volume, acquisition rate and stop option (events/gate, time, or volume). This can be modified from sample to sample, or applied to the entire experiment (latter is recommended).
4. Click *Apply to experiment*.
5. Option: on statistics bar, click on events/µl and see concentration in table; click on volume and calculate absolute numbers from count and volume values.
6. Take first sample, vortex, and load onto the SIP.
7. Ensure the correct sample tube is highlighted in the experiment explorer panel on the right. The current sample name appears under the run and record icons in the left panel. Advance to the next sample by clicking on > next to the sample name.
8. Click on run to start sample acquisition and then record; if you forget to record, click on *save* and data will not be lost. You will see a checkmark beside the sample name if saved.
9. If acquisition stops prematurely (stopping condition has been reached and there is still cell suspension left over), recover unused sample: click on "Recover Volume" and then "next" to use the same tube or supply a fresh empty tube if desired.
10. Click the small > on the left side pane to create a new tube, and right-click on it in the right pane to rename it for the next sample
11. Repeat steps 4-6 until all samples have been acquired

9. Cleaning

1. To clean machine select *Instrument* tab in the toolbar, select *Sanitize SIP* and follow software prompts, use 3mL of fresh 10% bleach; if the bleach tube has liquid in it, please <u>dump it out and refill with fresh 10% bleach</u>.
   *If you are the last user of the day or if it is after 4pm, complete the shutdown procedure in section 9 instead.
2. Place tube of water on the SIP and lift the arm.
3. Close Attune program and log-out of MED IT account (stops the billing clock); you may do this while clean is in progress.

10. Exporting data

1. Right click on Experiment.
2. Click on Export, then choose FCS Files, update keywords, save to desktop in a folder.

3. To print to PDF, click on the experiment name, choose *Print*, select options, then *okay*.
4. To zip multiple files and send by email: right click on folder, save as zip file and send as attachment.

11. Instrument shutdown

1. Fill tube with ~3mL fresh bleach and load. If the bleach tube has liquid in it, please dump it out and refill with fresh 10% bleach.
2. Select *Shutdown* from the *Instrument* tab in the Tools Bar and follow software prompts
3. Log out of software and windows account but **leave computer on**. Instrument and computer will shut off by themselves.
4. Shutdown takes ~45 minutes. Bleach tube can be replaced with water tube after shutdown if in the vicinity.

12. Review of SOP

SOP was established on April 17, 2020. The SOP will be reviewed annually.

Revision	Date	Description of changes	Reviewed by
0.0	04/17/20	Initial release	KB
1.0	10/25/21	Update	AR
1.1	04/23/23	Review	AR
1.2	04/30/24	Review	AR
1.3	04/09/25	Updated, added sections 2-5 plus Appendix, images of “OFF” and “ON” states and more detail in existing sections.	RN, GS, LX
1.4	04/11/25	Added more detail to sections 7-10.	GS

13. Appendix

Guide to choosing flow cytometer based on sample type: For non-human samples, use upper flow chart; for human samples, use lower flow chart. Refer to ATCC designation to determine classification of commercial cell lines. Follow arrows across to determine whether your samples are BSL1 or BSL2 then check the colour-coded sorter and analyzer lists below to select the appropriate instrument.