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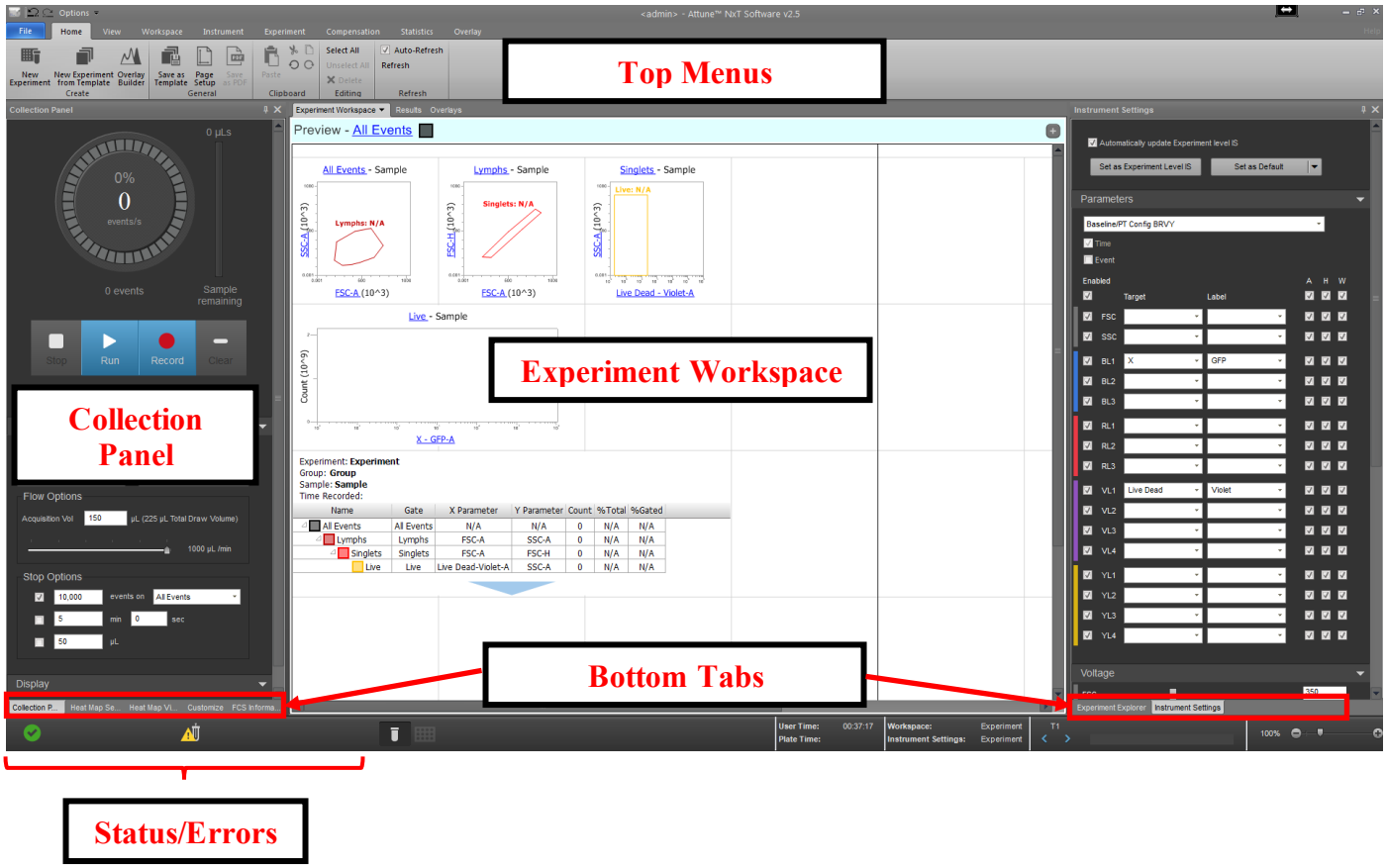
1. Startup

1. Check **Sheath, Waste, and Cleaning** containers:
   - Empty Waste container in the sink – Add bleach to the first mark (~10% final concentration).
   - Fill Sheath container with Attune 1X Focusing Fluid (on top of the Attune instrument).
   - Fill Wash container with Attune Wash Solution (in Attune fluidics drawer).
   - Fill Shutdown container with Attune 1X Shutdown Solution (in Attune fluidics drawer).
2. Turn on **PC**, **Autosampler**, and then **Attune NxT** (power switch in the back – follow orange label).

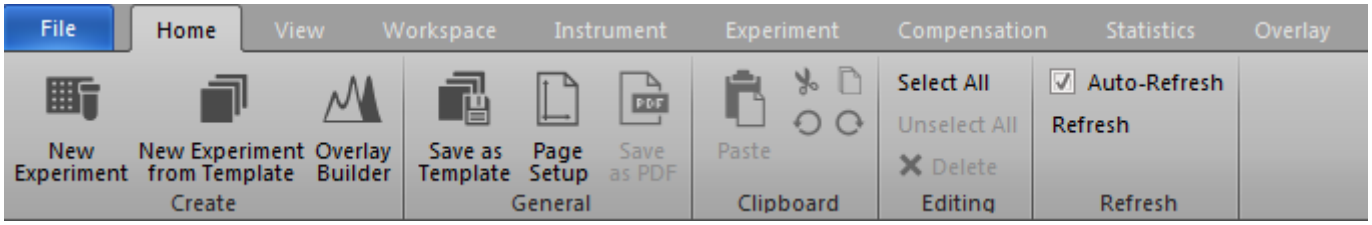
3. Log in to Windows (Onyen + Onyen password).
4. Launch **Attune NxT Software** (Username + password).
5. Check that the Performance Test (PT) has been performed for the day. If PT has not been performed, proceed to Section 3. If it's already been completed for the day, proceed to Section 4 or Section 5.

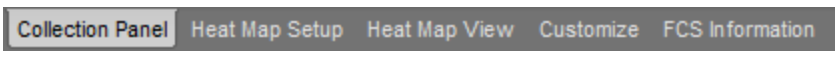
2. Attune NxT Software

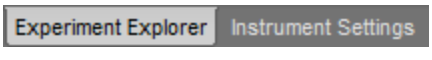
Menus Overview



Top and Bottom Menus







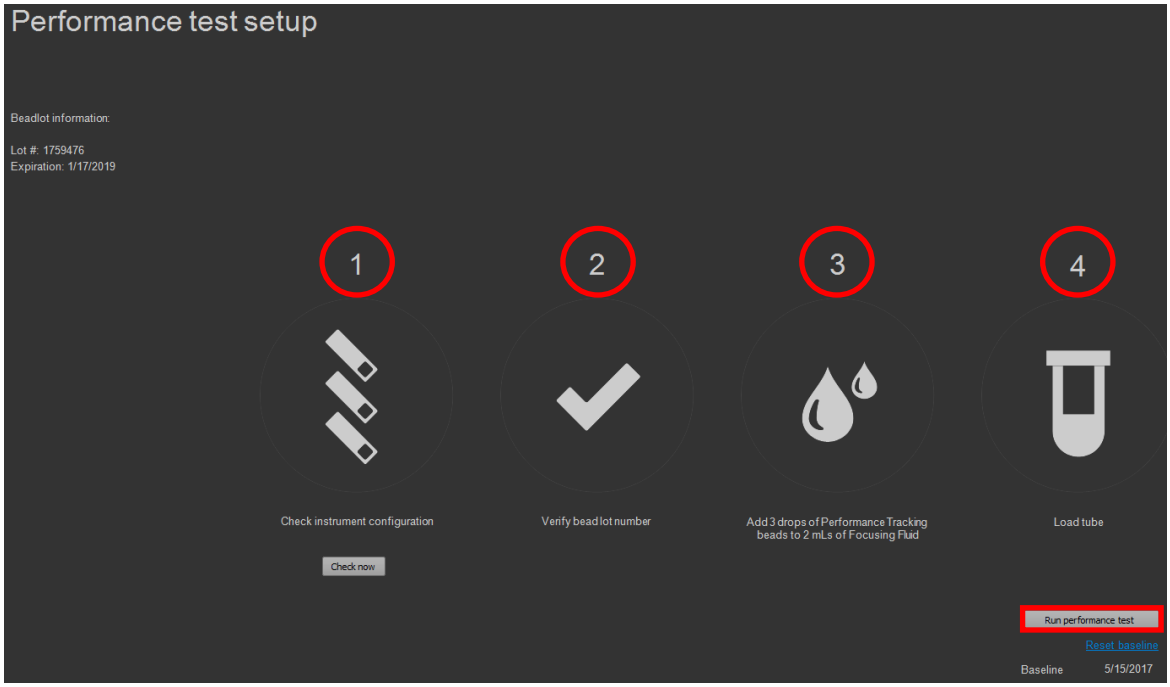
3. Quality Control – Performance Test

After the baseline values are defined, the Attune Performance Tracking Beads are used to run daily performance measurements to track the daily performance of the cytometer. The intensity level 4 bead is placed in the target channel and the PMT voltage is recorded, and then compared to the previous PMT voltage to calculate the delta. The %HPCV (half-peak coefficient of variation) of the bead is recorded. Using assigned MESF values for each fluorescent bead, the relative quantum efficiency (rQ) and relative background (rB) is calculated for each channel, and the linear regression is calculated and recorded. The laser delay setting is also automatically calculated. Levey-Jennings charts provide a visual to track the %HPCV and PMT voltage to check for shifts and trends.

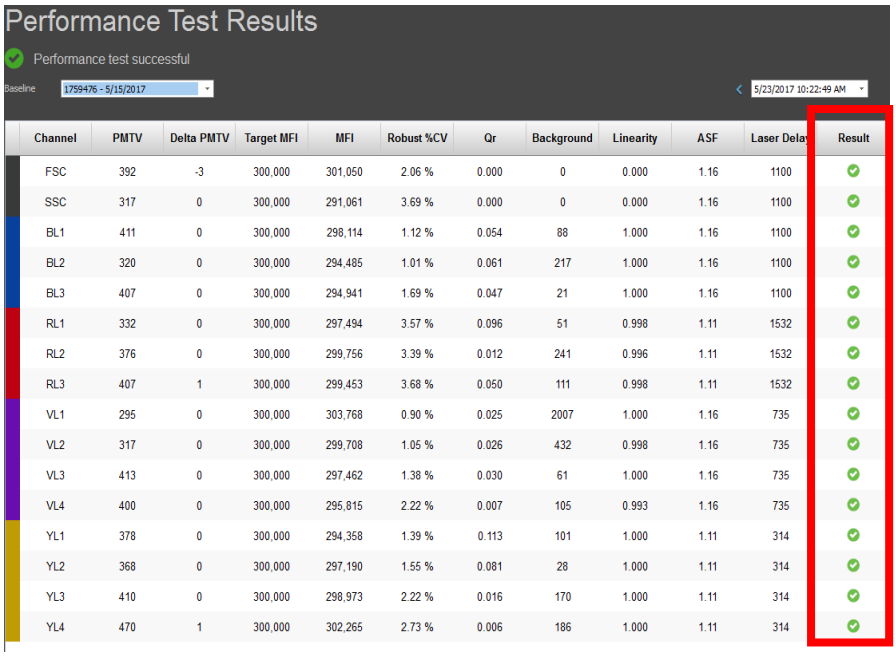
Launching the Performance Test

1. On the Main page, click on the **Performance Test** button.

2. Follow the four steps and click the **Run Performance test** button. Find the tube used to make beads in the drawer labelled ‘Attune QC’.



3. Upon completion of the Performance Test, the results are presented in a table. If one or more parameters fail, try the following troubleshooting:
    - Ensure the beads are diluted fresh and at the appropriate concentration.
    - Perform the Debubble function
    - Perform a Unclog function



Channel	PMTV	Delta PMTV	Target MFI	MFI	Robust %CV	Qr	Background	Linearity	ASF	Laser Delay	Result
FSC	392	-3	300,000	301,050	2.06 %	0.000	0	0.000	1.16	1100	✓
SSC	317	0	300,000	291,061	3.69 %	0.000	0	0.000	1.16	1100	✓
BL1	411	0	300,000	298,114	1.12 %	0.054	88	1.000	1.16	1100	✓
BL2	320	0	300,000	294,485	1.01 %	0.061	217	1.000	1.16	1100	✓
BL3	407	0	300,000	294,941	1.69 %	0.047	21	1.000	1.16	1100	✓
RL1	332	0	300,000	297,494	3.57 %	0.096	51	0.998	1.11	1532	✓
RL2	376	0	300,000	299,756	3.39 %	0.012	241	0.996	1.11	1532	✓
RL3	407	1	300,000	299,453	3.68 %	0.050	111	0.998	1.11	1532	✓
VL1	295	0	300,000	303,768	0.90 %	0.025	2007	1.000	1.16	735	✓
VL2	317	0	300,000	299,708	1.05 %	0.026	432	0.998	1.16	735	✓
VL3	413	0	300,000	297,462	1.38 %	0.030	61	1.000	1.16	735	✓
VL4	400	0	300,000	295,815	2.22 %	0.007	105	0.993	1.16	735	✓
YL1	378	0	300,000	294,358	1.39 %	0.113	101	1.000	1.11	314	✓
YL2	368	0	300,000	297,190	1.55 %	0.081	28	1.000	1.11	314	✓
YL3	410	0	300,000	298,973	2.22 %	0.016	170	1.000	1.11	314	✓
YL4	470	1	300,000	302,265	2.73 %	0.006	186	1.000	1.11	314	✓

4. Experiment Setup (TUBE)

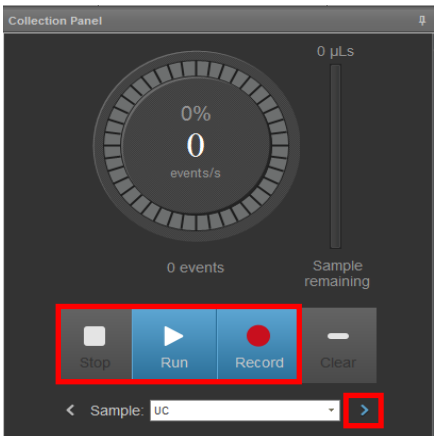
5. Click **New Experiment** or **Templates** (New Experiment from Template) > Experiment type = tube.

6. Name the experiment (optional: select the number of groups and the number of tubes). Click OK.
7. Click the **Instrument Setting** tab>**Parameters**.
    - Select the parameters used in the experiment.
    - Name the Target (i.e. CD3) and the Label (i.e. BV421).
    - Select the Height and Width parameters for aggregate discrimination.
8. Set Optimal Voltages
    - Use the Configuration at the instrument for staff generated voltages based on a voltration using beads and lymphocytes.
        - Note: The particles used to voltage walk the instrument have very low autofluorescence; therefore, will most likely be the highest the voltages need to be set. They may not be ideal for all experiments.
    - Ideally, voltages will be set after performing a voltage walk of the instrument with user materials.
    - In lieu of a voltage walk, the following procedure can be used to sub-optimally set voltages:
        - Click the **Experiment Explorer** tab and R-click **Compensation**>**Compensation Setup**.
        - Check the **Use Unstained Control (UC)** box and click OK.
        - On the **Collection Panel** tab, adjust the **Flow Options** (50µL @ 12.5µL/min) and click **Apply to Experiment**.
        - Select (double-click) the UC tube (highlighted in orange) under Compensation Controls.
        - Mix and load a fully unstained tube and click **Run** (Collection Panel tab).
        - Click the **Instrument Setting** tab>Voltage.
        - Adjust the FSC and SSC voltages.
        - Adjust the fluorescent parameter voltages to ensure all signals are on scale (optimal voltage ranges should have already been defined with a voltage walk).
        - Click **Stop** then **Recover** (leave the tube in place).
        - Click **Next** to return any unused sample to the fully stained tube. Do not remove tube before the prompt or software will crash.
        - Lower the tube lifter (catch the drop on the SIP using the lip of the tube) and click **Next** to initiate the **Rinse** cycle.
        - Load and **Run** each single stained compensation control in the UC tube to ensure:
            1. The positive particle is on scale (not too bright)

2. The fluorophore being viewed is at least half of a log brighter in the primary detector compared to any secondary detectors it may spill into.

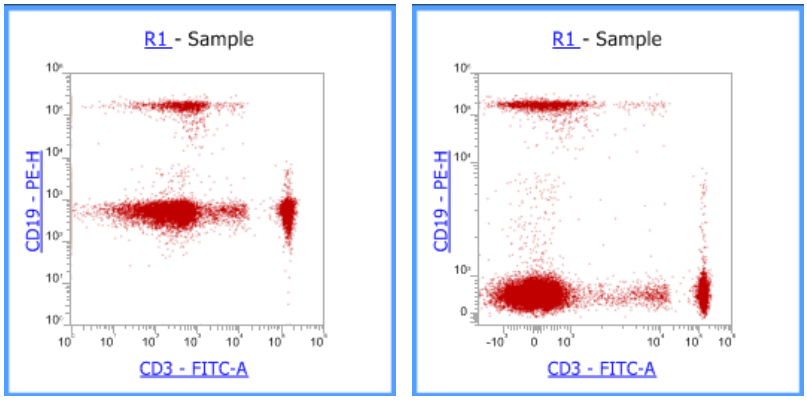
Note: if you are using compensation controls with positives and negatives within the same tube, return to the Compensation Setup and check the Use Negative Gate box.

9. Record Compensation Controls
   - Modify the **Flow Options** (100µL @ 100µL/min, or a speed that does not exceed 20,000 events per second) and **Stop Options** (at least 5,000 for compensation beads; more if using cells depending on the positive and negative frequencies), and click **Apply to Group**.
   - Load and **Record** the UC and each single stained control, using the right arrow to navigate to the next tube.



   - Adjust the gates for each control.
   - When all compensation controls have been recorded, the software automatically calculates the compensation matrix. To view the matrix, go to the **Compensation** menu>**View Matrix**.
10. Record experimental samples.
   - Use the **Workspace** menu to create plots, gates, and statistics (can be customized in the **Statistics** menu).
   - Modify the **Flow Options** and **Stop Options** and click **Apply to Experiment**.
   - Mix, load, and **Record** the sample.

Note: if a menu is missing, go to the View tab to select it.
Note: modify the fluorescent parameter scaling from log to HyperLog (Biexponential) using the Customize tab.



Exporting Data

- R-click Experiment>Export>FCS Files [3.0 or 3.1].
- Select Location (path).
- When prompted, update FCS keywords for all files.
- R-click Experiment>Save As Template (rename if desired), click OK.
- **R-click Experiment>Delete.**

5. Experiment Setup (PLATE)

1. Click **New Experiment** or **Templates** (New Experiment from Template) > Experiment type = plate.

2. Name the experiment and select Plate type (optional: select the number of groups and the number of tubes). Click OK.

Note: it is best to bring compensation controls in tubes while the remaining samples can be in a plate format.

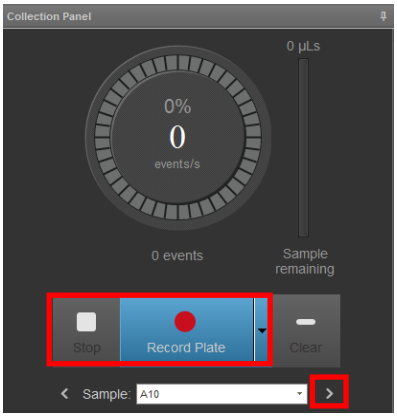
3. Click the **Instrument Setting** tab>**Parameters**.
   - Select the parameters used in the experiment.
   - Name the Target (i.e. CD3) and the Label (i.e. BV421).
   - Select the Height and Width parameters for aggregate discrimination.
4. Set Optimal Voltages

- Use the Configuration at the instrument for staff generated voltages based on a voltration using beads and lymphocytes.
  - Note: The particles used to voltage walk the instrument have very low autofluorescence; therefore, will most likely be the highest the voltages need to be set. They may not be ideal for all experiments.
- Ideally, voltages will be set after performing a voltage walk of the instrument with user materials.
- In lieu of a voltage walk, the following procedure can be used to sub optimally set voltages:
  - Click the **Experiment Explorer** tab and R-click **Compensation>Compensation Setup**.
  - Check the Use Unstained Control (UC) box and click OK.
  - On the **Collection Panel** tab, adjust the **Flow Options** (50µL @ 12.5µL/min) and click **Apply to Experiment**.
  - Select (double-click) the UC (highlighted in orange).
  - Mix and load a fully stained tube and click **Run** (Collection Panel tab).
  - Click the **Instrument Setting** tab>Voltage.
  - Adjust the FSC and SSC voltages.
  - Adjust the fluorescent parameter voltages to ensure all signals are on scale (optimal voltage ranges should have already been defined with a voltage walk).
  - Click **Stop** then **Recover** (leave the tube in place).
  - Click **Next** to return any unused sample to the fully stained tube.
  - Lower the tube lifter (catch the drop on the SIP using the lip of the tube) and click **Next** to initiate the **Rinse** cycle.
  - Load and **Run** each single stained compensation control to ensure:
    1. The positive particle is on scale (not too bright)
    2. The fluorophore being viewed is at least half of a log brighter in the primary detector compared to any secondary detectors it may spill into.

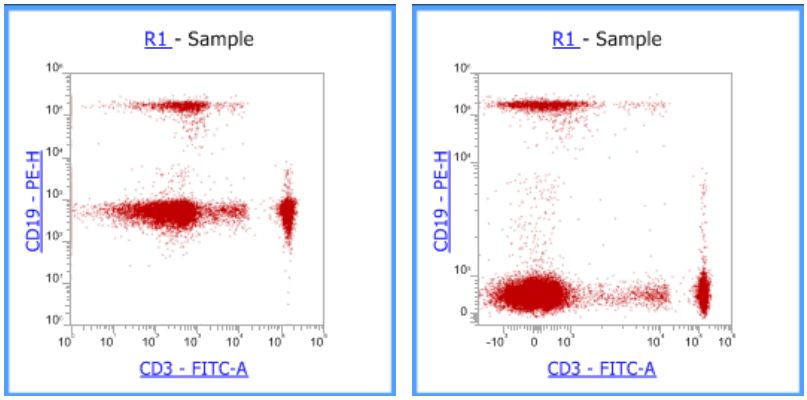
Note: if you are using compensation controls with positives and negatives within the same tube, return to the Compensation Setup and check the Use Negative Gate box.
Note: If compensation controls are in Plate, use Manual Well to adjust voltages prior. This is not recommended.

5. Record Compensation Controls
   - Modify the **Flow Options** (100µL @ 100µL/min, or a speed that does not exceed 20,000 events per second) and **Stop Options** (at least 5,000 for compensation beads; more if using cells depending on the positive and negative frequencies), and click **Apply to Group**.
   - Load and **Record** the UC and each single stained control, using the right arrow to navigate to the next tube.
6. Set up Samples
   - Open the **Heat Map View** tab and R-click well(s) to be acquired>New Sample>Add to New Group.
   - Select the experimental sample (Experiment Explorer tab or Heat Map View tab).
   - Use the **Workspace** menu to create plots, gates, and statistics (can be customized in the **Statistics** menu).
   - Modify the **Flow Options** and **Stop Options** and click **Apply to Experiment/Group.**

7. Load plate and click **Record Plate**. Double-check settings have applied to all wells prior to clicking Record Plate.



Note: if a bottom menu is missing, go to the View tab to select it.
Note: adding a sample as a 'Manual Well' allows to Run a sample from the plate without Recording the sample.
Note: modify the fluorescent parameter scaling from log to HyperLog (Biexponential) using the Customize tab.



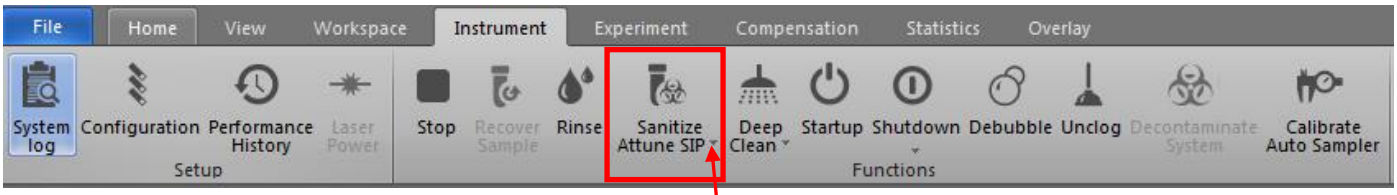
Exporting Data

- R-click Experiment>Export>FCS Files [3.0 or 3.1].
- Select Location (path).
- When prompted, update FCS keywords for all files.
- R-click Experiment>Save As Template (rename if desired), click OK.
- **R-click Experiment>Delete.**

6. Cleaning / Shutdown

End of Experiment Cleaning (~2:30min.)

1. Empty the Flow Cell Clean tube and fill with 2 ml of *Flow Cell Cleaning* solution located in a yellow-labelled bottle on top of the instrument.
2. On the Instrument menu, click **Sanitize Attune SIP** and follow the onscreen instructions (use Flow Cell Clean instead of Bleach).



3. If the Autosampler was used, click on the small triangle and select **Auto sampler SIP**.
4. Rinse instrument when cleaning is completed. Do not leave cleaning solution on SIP.

Shutdown

Last user of the day is responsible for starting the Shutdown of the instrument

1. Navigate to the Instrument menu, select **Shutdown> Thorough**
2. Follow the onscreen instructions (use FACSClean in place of Bleach)
3. Once the protocol has started, you may log off the computer. Make sure the progress bar shows at least 3% completed before logging off. Do not turn the instrument off.

7. References

- Attune™ NxT Acoustic Focusing Cytometer 100024235
- Attune™ NxT Software User Guide 100024236

8. Revisions

SOP Revision #	Date	Tracked Changes	Primary Reviewer/Editor
UT SOP007.2	06/26/2023	Updates throughout to align with modern training	Ayrianna Woody
UT SOP007.3	11/29/2023	Cleaning protocol updates	Ayrianna Woody
UT SOP007.4	08/21/2025	Update and Training Notes Page	Bob Immormino

9. Training Notes

Start-up: Check Fluid bottle levels in the Instrument and Autosampler, Login to workstation with Onyen, Open instrument software.
Performance test: Performed weekdays by staff
Create an Experiment: Open a new Experiment or a previously saved Experiment Template. **Rename!** Select the Instrument settings tab and update for your experiment; Add FSC and SSC area, height and width and remove un-used fluorescent detectors.
Create a Compensation Experiment: Once parameters are set create a compensation experiment (Compensation menu > Compensation Setup). Briefly acquire all the controls and adjust PMT voltages to 1. Confirm that fluorophores are brightest in the expected detectors, 2. Put parameters on-scale, 3. Balance spill-over with spreading.
Calculate Compensation: After finalizing PMT voltages, collect the compensation controls (need >500 negative and >500 positive events; more is better).

Generate a gating scheme and collect data: Switch to the Worksheet tab and add plots to set-up a gating scheme for your experiment. It is handy to take a picture of the PMT voltages with the snipping tool and keep digital notes in notepad. Doing this while on the cytometer will ensure that you have the notes if you need them later!

Export Data and Templates: Right-click on your experiment select Export > FCS files. Temporarily store files on the J:drive (\\ad.unc.edu\med\microbiology\Groups\Flow Facility). The J: drive will be cleared automatically of data over 3 months old.

Clean-up: Between Users (Run Sanitize SIP with Flowcell Clean), End of Day Shutdown (Run Shutdown > Thorough with FACSClean)