

# Operation of the ACEA NovoCyte Flow Cytometer

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Version "1.1" – 24 Oct 2017

Updates 10/24/2017: revised room access policy

Updates 2/7/2017: revised I. Room access; revised VIII – Threshold; added further instructions & illustration on how to copy template/paste to all samples; fixed broken link to online copy of NovoExpress User Guide; revised & added to section X – Gates.

- I. **For additional details about NovoExpress Software not discussed in this protocol, ACEA's [NovoExpress Software User Guide](#) is available online.**
- II. **Room access**
  - a. If you do not work on the 4<sup>th</sup> floor BSRB, you MUST use the NovoCyte between the hours of 8 am and 5 pm, Monday through Friday.
- III. **Startup, fluidics check, QC**
  - a. Fill sheath if 1/3 full or less using the Fisher "NERL" cubitainer located next to the sink. Users are responsible for filling sheath. If you fill the tank with sheath, run "prime" cycle. Takes 17 min.
  - b. Empty waste if 2/3 full or more into the provided 15 gal poly barrel beneath the table. It's on rollers. Do NOT dump waste down the sink. Users are responsible for emptying waste into barrel.
    - i. After emptying waste, add ~200 ml bleach to the container (beneath sink)
  - c. If instrument is OFF: push power button – instrument will be ready in **3 min.**
  - d. QC: done by Peter 1x per week.
    - i. Note: If QC fails, instrument will "lock up" and NOT allow anyone to run samples until QC passes.
  - e. Turn on computer
    - i. To unlock Windows, the password is "flowcyto."
    - ii. Launch "NovoExpress" software
    - iii. Choose your name from the list. If you don't see it and want your own profile, contact me ([pouillet@med.umich.edu](mailto:pouillet@med.umich.edu))
    - iv. No passwords for individuals.
- IV. **Hardware**
  - a. You can bring 12x75 mm tubes or 96-well plates.
  - b. For tubes, use the special rack designed to fit in the "NovoSampler." It can hold 24 tubes at a time.
  - c. 96-well plates are subject to the same volume limitations as the BD LSRII, meaning:

- i. Whatever volume you tell the instrument to aspirate, it will aspirate an **additional 20 ul**. (The NovoCyte is “greedy.”)
  - ii. A certain amount of each well in a 96-well plate is considered “dead volume,” i.e. you pipetted 200 ul into the well, but as far as the instrument is concerned, you only have ~170 ul. This dead volume is greater for flat-bottom plates. Don’t use them.
  - iii. Therefore if you pipetted 200 ul into your sample well and run it twice at 50 ul,
    1. The volumes removed are 70 & 70 = 140 ul (not 100!)
    2. The remaining volume is closer to 30 ul, not 60! You will suck air into the instrument even if you drop the volume aspirated to 25! (25+20 extra = 45 ul).
  - iv. Plan ahead so that you have “enough” cells. “Enough” cells is defined as the quantity and concentration that gets you enough information to effectively complete your experiment. While this is ultimately up to you and your lab, we recommend 30,000 events per sample.
  - v. You need to think in advance how many cells in your sample are contained in what volume. If you have 500,000 cells in 250 ul in the well, then you have 2000 cells per ul and in theory you should aspirate at least 15 ul to obtain 30,000 events. In practice I recommend taking twice that to begin with, particularly if this is a new experiment with cell types with which you are unfamiliar.
  - vi. The NovoSampler can move sample through the instrument at continuously variable rates measured in microliters per minute. However, there are three standard sample flow rate settings: Slow (14 ul/min), Medium (35 ul/min), and Fast (66 ul/min). Continuous adjustment of the sample flow rate from 5 ul/min to 120 ul/min is possible. NOTE that the core stream diameter will increase from 4.6 microns to 22.7 microns, respectively, under these settings.
  - vii. If you have a concentrated sample, use a speed that does not cause a measured event rate greater than **3000** events/sec. This is a “guesstimate.” Currently I cannot find a maximum recommended event rate from ACEA for the NovoCyte.
  - viii. If you have a dilute sample, increase the speed to save time.
  - ix. If you **suck air** into the instrument because you misjudged the amount of sample in your well, you must **prime** the instrument, and it takes **much longer** on the NovoCyte than it does on the LSRII (**17 min!**).
  - x. **Caution**: Note that ACEA does not recommend MIXING samples if you have >200 ul in a well of a 96-well plate. The method employed by the NovoCyte is to shake the plate at high frequency. At liquid volumes >200 ul, microdroplets can **cross-contaminate** other wells.
- d. **Nice feature** of the NovoCyte is that the dynamic range of the instrument’s plots is 7.2 log decades. What this means is that the range is so broad, **voltage adjustments of your populations are no longer necessary**. Just “load & go!”
- V. **Setting up your work area for a run**
- a. → NovoExpress operates a little differently if you’re used to BD’s FACSDiva software.

b. NovoExpress uses Experiments, Specimens, and Samples.

c.

#### VI. Fluorescence Compensation

a. Fluorescence Compensation is a **separate training module** that must be specifically requested, for the following reasons:

i. Many flow users elect to compensate their data back at their home lab using different software

ii. Avoid “information overload” during training

b. Nonetheless, compensation is a **critical** aspect of flow and it must be understood if you are going to do >1 color experiments!

#### VII. When you are done with your run:

a. If someone has an appointment after you, run the “Rinse” cycle. Takes 2 min.

b. If you are the last user of the day: press the power button. Button will start to flash, indicating that the shutdown cleaning cycle has begun. Takes about 30 min. Instrument will shut itself off when done. So, shut down computer, push instrument power button, walk away (lock door behind you).

#### VIII. Explanation of the various cleaning/unclogging commands

a. Debubble

i. Use the *Debubble* function in the Fluidics Maintenance panel if bubbles are suspected to exist in the fluidics system. To use *Debubble*, place a test tube containing at least 1 mL of 70% **ethanol** in the sample holder. Click the *Debubble* function, and the sample injection tube will aspirate the ethanol to initialize the debubble process. The *Debubble* process takes approximately 3 min. **Always use at least 1 ml ethanol!**

b. Cleaning

i. Uses “NovoClean” (bleach) and sheath fluid in sequence to flush the entire fluidics system. The *Cleaning* process takes about **14 minutes**.

c. Rinse

i. Use the *Rinse* function to clean the fluidics system. Uses sheath fluid only. Takes 2 min.

d. Extensive Rinse

i. Recommended that users run *Extensive Rinse* once a month. First flushes with NovoRinse (detergent), soaks the fluidic tubing for a certain period of time, then flushes the system with sheath fluid. Takes 12 min.

e. Priming

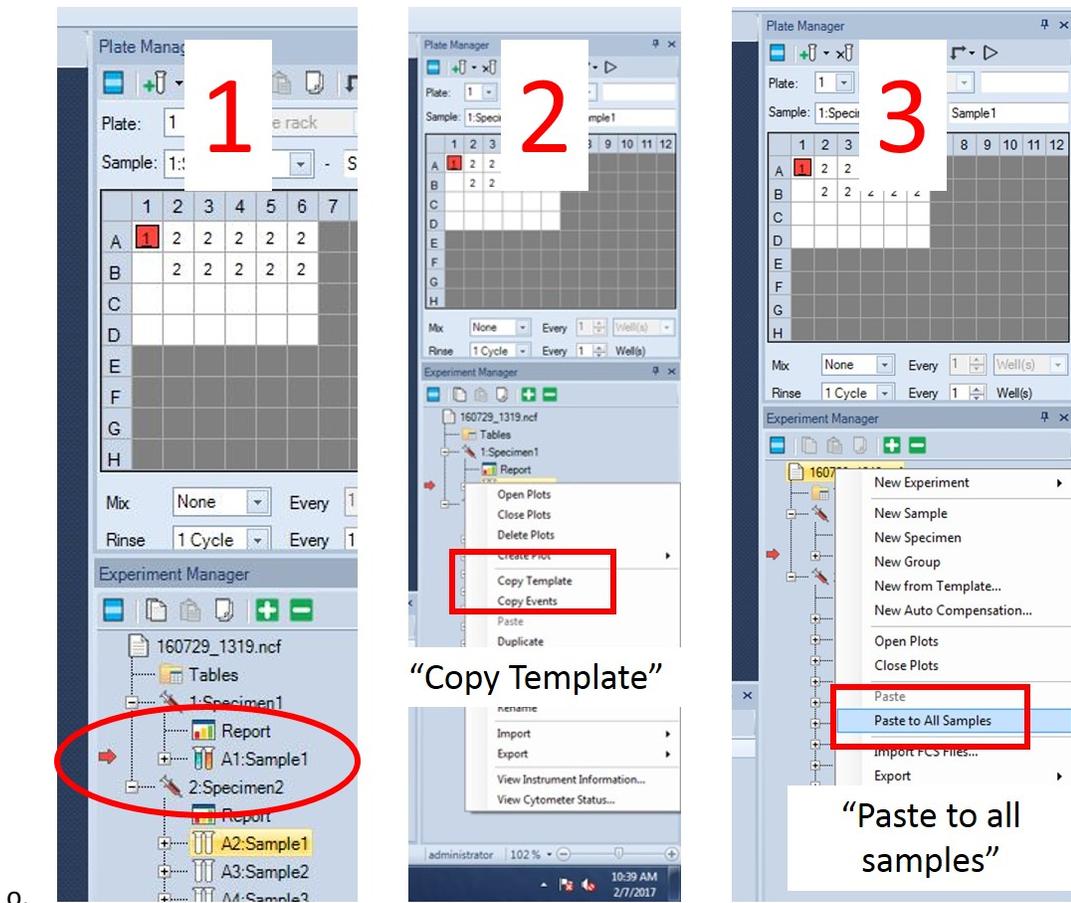
i. *Priming* function should be used whenever one of the following situations occurs:

- Non-operation of the instrument for more than two days.
- Running out of sheath fluid during run
- After adding reagent to the reagent container
- Running out of sample, sucking air into the fluidics system.
- **Takes 17 min!**

- f. Unclog
    - i. Run Unclog once a month as a preventive measure. Cleans out the **FLOW CELL**. *Unclog* uses “NovoClean” (bleach) to flush the flow cell under high pressure, then soaks the flow cell for a certain period of time, and then flushes with sheath fluid. Unclog takes **about 17 min**.
  - g. Backflush
    - i. When the sample injection probe is blocked, use *Backflush* to clear. This flushes the **sample injection probe** under high pressure using the sheath fluid in reverse of the normal sample flow direction, and the waste is aspirated by the SIP wash apparatus. *Backflush* takes approximately **3 min**.
- IX. **Setting up software for a run**
- a. Upon login you will by default be presented with a new, **blank** experiment document.
  - b. You may open previously saved experiments but it is unlike FACSDiva in that you do not see multiple experiments in the data tree. It is advisable to have a few experiments saved as “templates” with the file extension .nct, then open one of these upon starting a new day’s experiment.
  - c. Choose your sample source (24-well plate, 24-tube rack, 96-well plate, etc) – upper right corner
  - d. Set # mixing cycles (**caution** – see **III.d.x**, above) and number of wells between mixes
  - e. Set # rinsing cycles and number of wells between rinses (note: cannot be set to 0)
  - f. Choose the parameters for which you wish to collect data. Note that you will need to check both Height and Area if you wish to collect both. The NovoCyte will default to HEIGHT. Note that under “Parameters” in the Cytometer Setting window, note that the software calls the detector names column the “Alias” column. You can double-click on the alias for any detector and rename it as you see fit, e.g., “FITC” can become “GFP.”
    - i. To see which formal parameter label (BL1, VL2, RL1, etc) is associated with which filter, click the “instrument” tab, then the “configuration” button (icon = wrench)
  - g. Choose your Stop Conditions
    - i. # of events to collect
    - ii. Whether you wish have the # of events collected satisfy a gate to be counted
    - iii. # of microliters of sample to aspirate
    - iv. Either or both of the # events or # ul criteria can be checked.
  - h. Choose your desired starting Flow Rate
  - i. Choose your Threshold. Default is 10,000 on forward scatter (FSC), which is actually quite low. A value of ten thousand is low enough to enable a user to visualize platelets. For small lymphocytes, you may want to try 50,000. To analyze bacteria would require FSC threshold of 1000-10,000 AND SSC-H >5000 as a secondary threshold.
  - j. Draw plots. Draw gates and apply them.
  - k. Highlight wells in the plate icon and create tubes for all of these. Try to do it in such a way that you have only one **specimen** (syringe icon). This is my preferred approach; organizing your experiment into different specimens may be helpful to you. I have

always found it simpler to lump controls and all samples (tube icons) under one “specimen” icon (syringe icon).

- l. Click the floppy disk icon in upper left. The NovoExpress™ software automatically saves the data, along with the cytometer settings information, to the experiment file. However, data analysis, including all the plots and gates, fluorescence compensation, created Reports and Statistical Tables, etc., must be saved manually. To save, click *File* → *Save* from the menu or click the Save icon . Otherwise, when closing the NovoExpress™ software, a message will prompt users about saving the data.
- m. Now comes the rub: making all these choices made in c through j apply across your entire experiment. It’s different than in FACSDiva software. In FACSDiva the “worksheet” contained a set of plots and gates that was independent of your samples/tubes. **In NovoExpress each tube can have its own set of plots, gates, compensation matrix, Stop Conditions, Flow Rates, and analysis parameters.**
- n. Any time you make an addition or a change of *any kind* (add plot, draw gate, change flow speed, sample uptake volume, etc), your changes only apply to the well/tube at which you are currently “pointing.” For example, if you add a plot while well A1 is selected, ONLY A1 has that plot; none of the other wells will show this change, UNLESS you first copy the settings from well A1 and then paste them to the rest of the samples in your experiment. See illustration in “o,” below. Again, this applies to *any* kind of change you make, including changing the number of events to acquire, for example.



- p. In panel “O-1,” sample A1 is highlighted. This is indicated by the red arrow and by the red square in the plate schema at the top. In panel “O-2,” the user has right-clicked on sample A1. Click “Copy Template.” Finally, highlight the .ncf file at the top of Experiment Manager (Panel “O-3”), right-click and choose “Paste to all samples.”

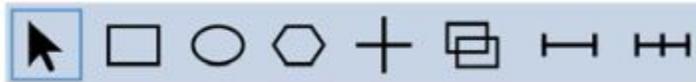
## X. Experiment Manager

- a. The NovoExpress Software uses a hierarchical structure including groups, specimen, and samples to organize and manage experimental data.
  - i. **Work List:** an optional way to view information on sample names and collection parameters. Anything entered in the work list is reflected in the Experiment Manager tree. This may be useful just because it seems easy in NovoExpress to end up with samples that have different collection params, i.e., you may have customized collection params in Sample 1 but you need to be careful that by Sample 7 you’re still trying to collect 20,000 events (not 10,000) at a threshold of 10,000 (not 100,000), etc.
  - ii. Similar to FACSDiva, the user has the option to organize his/her samples into Specimens, with each Specimen having Samples. Personally I don’t need such organization, so I stick with just one sample.

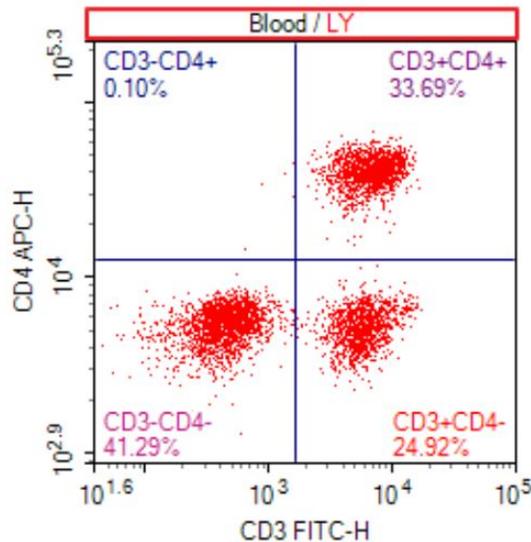
- iii. The Sample icon is represented by two tubes. The fact that there are two instead of one means nothing. The Sample is the most basic organizational unit and contains sample data collection parameters, instrument settings, fluorescence compensation settings, reports, analyses, and data. Tubes without data will appear white (“empty”). Tubes with data will appear “full.”
- iv. It is important to keep track of the various settings within each tube:
  1. Cytometer settings contain the sample parameters, the acquisition stop conditions, and the sample flow rate and threshold settings.
  2. Fluorescence compensation – the matrix for the sample.
  3. Report – found at both the specimen and sample level. Can include plots and statistical analysis for all samples under the specimen. Reports under sample nodes can include plots and statistical analysis only for the sample.
  4. Analysis – what plots and gates. This is one of the **most significant differences with FACSDiva**. Each sample tube has ITS OWN set of plots and gates, unless you carefully copy your set of plots’/gates to ALL Samples in your experiment.

## XI. Gating

- a. Gates allow for the analysis of subpopulations from the total population collected. The Workspace Toolbar includes icons for creating rectangular gates, elliptical gates, polygonal gates, quadrant gates, logic gates, range gates, and bi-range gates:



- b.
- c. All gate types can be created on dot plots and density plots. Range gates and bi-range gates can be created on histograms. Gates can also be combined to create a logic gate.
- d. To analyze subpopulations, plots can be set to only display events from within a specific gate. The new plot can then be used to analyze the subpopulation or to further gate for more specific populations.
- e. If plots are gated, the gate will be displayed on the header of the plot as shown below. The header will display the sample name and the gate. In the example below in f, the sample name is *Blood* and the gate is LY.



- f.
- g. There are multiple methods for gating a plot. Among these are:
- In the plot header, right-click to display a drop-down menu. In the drop-down menu, select the gate. If *Ungated* is selected, the plot is not gated, and all events are displayed in the plot.
  - Select a gate in the *Gate* tab of the *Menu Bar* and click *Gating*. Select the plot to be gated. If **All following plots** is selected, all the plots listed will be gated. **This is a handy feature** – it is most useful when setting the light scatter gate for the experiment, as normally all plots in an experiment are gated on light scatter.
  - Tip from experience:** always check your plots to verify what data you are looking at first before making an interpretation!

XII. **How to use the integrated cell cycle analysis feature**

- a. See Section 5.5.1 in the [ACEA NovoExpress Software user guide](#).

XIII. **How to perform absolute counts**

- a. Absolute counts are a determination of the number of cells or particles per unit volume. NovoCyte is a volumetric instrument; thus, exact volumes of acquired sample can be determined without the need for counting beads. After the dilution factor and unit of measure (default is # of events per microliter) are defined by the user, NovoExpress can display the number of events within specified gate per unit volume in the statistical information chart.



- b. This is the icon for setting up absolute counting conditions for the active sample. Once clicked, a window will appear titled "Abs. Count setting – Sample X" (depending on what sample is highlighted)
- Dilution factor – the conversion coefficient used to calculate the absolute counting results for the original sample. For instance, if the original sample is diluted 10 times and is then run on the NovoCyte, enter 1:10 in the Dilution

Factor. NovoExpress software will show the absolute counting results for the original sample by multiplying the concentration of the sample run on the NovoCyte by 10.

- ii. Abs. Count Unit – the parameter to set the unit for the absolute counting. It is based on counts of events per microliter. For instance, if events per milliliter is desired, enter 0.001 in the *Abs. Count Unit*. NovoExpress software will do the conversion based on this input and show the absolute counting results in the unit of Events/mL.
- iii. The concentration of events is defined as
  1.  $\text{Abs. Count} = \text{Count} / \text{Ve} / \text{DF} / \text{Abs. Unit}$ ,
  2. Where Count is the number of events in the gate, Ve is the sample acquisition volume, DF is the dilution factor, and Abs. Unit is the absolute number of units. To set the dilution factor and the absolute number of units, click on *Abs. Count Setting* from the *Sample* tab of the *Menu Bar*.