Sony ID7000™ Standard Operating Procedure

1. Startup

1) Empty the waste tank and fill the sheath tank.
2) Turn on the two power switches on the left side of the ID7000.
3) Press the power button on the front of the instrument.
4) Launch the ID7000 software and log in with your username and password.

2. Priming and QC

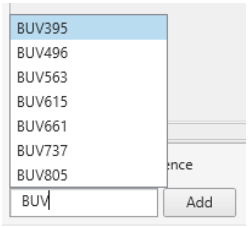
1) When prompted, confirm the initiation of priming by clicking *Start*.
2) Perform daily QC (approx. 12 minutes) using *Sony Align Check* beads.
   a. *QC* tab → *Daily QC* → *Start Daily QC*.
   b. Vortex the beads and follow the instructions in the *Daily QC* wizard. Confirm the correct bead lot number.

Refer to the *Sony ID7000 SOP* for instructions on performance QC, optics alignment and flow rate calibration.

3. Data Acquisition

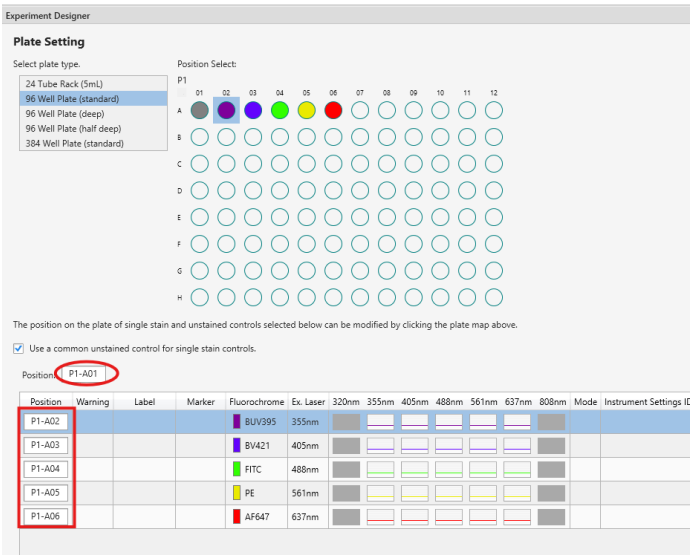
- Always ensure the *Standardization* mode is selected as the default mode under *Experiment* in *Preference Settings*.
- The steps below describe how to create experiments from scratch. To create experiments using templates or to continue acquisition in previously run experiments, refer to Part C, sections 3 and 4 of the *Sony ID7000 SOP*.

1) Create Experiment
   a. *Experiment* tab → *Experiment Designer*.
   b. Check the boxes at the bottom of the screen to ensure that all lasers of your ID7000 are ON.
   c. Type the fluorochrome names and click *Add*. If your fluorochrome is not present in the database, enter the fluorochrome information as prompted.
      - Refer to Part C, Sections 1.1.1e and 2 for instructions on importing colour panels from CSV files and using previously recorded spectral reference controls, respectively.



   d. Add marker names in the *Marker* field. Optionally, add a *Label* (panel name, fixation methods etc). Click *Next*.

   e. Select the plate type. Uncheck the box *"Use a common unstained control for single stain controls"* if you do not have a separate unstained control. To move the position of unstained control or change the order of fluorochromes, type new positions into the boxes under *Position*. Click *Next*.



f. Modify the default *Acquisition Settings* as needed. *Acquisition Offset Time* of 3-5 seconds recommended. Other settings such as *Low Dead Volume* mode and *Agitation* may be modified as appropriate.
g. Change the default experiment *Name* and enter other information as needed.
h. Review the experiment details. Go back if you need to make changes. Click *Create Experiment*.

2) Single colour controls

a. Place the tube rack or the plates in the auto-sampler stage. Go to the *Status* panel and click the *Load* button.

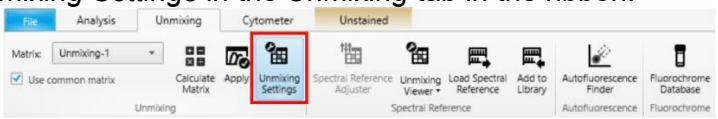
b. Go to the rack/plate map (lower left). Ensure that the current position (black arrow) is on the desired sample. To change the current position, right click on the new well and click *Set Current Position*.
c. Open the *Instrument Settings* window by clicking on the *Instrument* button in the *Status* panel. Ensure that the PMT voltages for different detector arrays are all set to the value of 4.7. This gain is recommended for compensation beads. If using other materials (e.g. cells) for controls, adjust the gain as needed.
d. Select your unstained control well/tube, or one of the other controls if you do not have an unstained, and preview it by clicking the *Preview* button.
e. Adjust the axes scaling (refer to Part C, Section 1.2e of the *Sony ID7000 SOP* for instructions), and size and shape of the pre-drawn *Gate A* to gate on the main population of the unstained control, e.g. the main bead population.
f. Click on the whole plot and then *Sync Scale and Gate* in the ribbon to apply the scale and gate settings across the entire single colour control sample group.
g. Click the *Stopping Condition* button beside the *Instrument* button. Set *Gate A* as your *Saturation Gate*, and define a *Stopping Condition* (e.g. total events, or gated events, or acquisition time) for your single colour controls. Click *Sync Stopping Condition* to apply these settings across the entire single colour control sample

group. **Note:** if you select more than one type of stopping condition, the acquisition will stop when the first one is reached.

h. Preview your brightest fluorochromes (e.g. BV421, PE, BB515, PE-Dazzle 594), while monitoring the *Saturation Rate* on the *Status* panel and ensuring that it never exceeds 2-3%.
i. Click *Auto Acquire* to initiate acquisition.
j. Once auto-acquisition is complete, refine the positive (and if relevant, negative) gates for each fluorochrome and obtain clean spectra for all controls.

3) Unmixing calculation

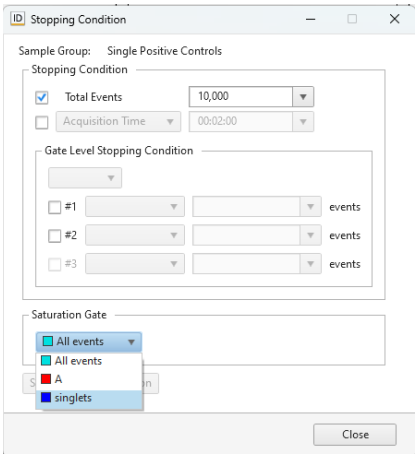
a. Open the *Unmixing Settings* in the *Unmixing* tab in the ribbon.



b. Ensure that all the positive and negative populations for each fluorochrome are correctly selected.
c. Press *Calculate* at the bottom of the *Unmixing Settings* window. If you see a warning about saturated events, tick the box "*Calculate Spectral Reference without saturated event(s)*".
d. Click *Apply* when prompted to apply these settings to other sample groups. You have now generated a spectral reference (SR) for each one of your fluorochromes. Refer to Part C, Section 1.1.3e of the *Sony ID7000 SOP* for troubleshooting of SR with error messages.
e. (optional) To add SR to the *Library*, click *Add to Library* in the *Unmixing* tab to open the *Add to Library* tool. Select the SRs you wish to save in the *Library* and click *Add to Library* to finish.

4) Unstained and fully stained samples

a. In the rack/plate map, select all sample wells to be acquired (click wells while holding [Ctrl] on your keyboard), then right-click the selection, navigate to *Move to Sample Group* and select the desired sample group if it already exists. To create a new sample group, click an existing sample group and then select *Duplicate* from the *Acquisition* tab at the top of the screen. Ensure that the fully stained and their corresponding unstained controls are in the same sample group. Refer to Part C, Section 1.2a of the *Sony ID7000 SOP* for more information on sample groups.
b. *Instrument Settings* → *Detector & Threshold*. Leave this window open while performing the following steps.
c. Right-click the well to be previewed and click *Set Current Position*.
d. Click *Preview* in the *Status* panel.
e. Once events begin to appear, click the FSC/SSC plot. Adjust the axes scaling as needed (refer to Part C, Section 1.2e of the *Sony ID7000 SOP* for instructions). If necessary, adjust the FSC gain and SSC voltage settings on the *Detector & Threshold* tab.
f. Click on the *Restart* button as needed to refresh the data.
g. Draw *Gate A* for your cell population (e.g. PBMC gate), then double-click on the gate to open a subplot and draw *Gate B* for your singlets (e.g. on a FSC-H vs FSC-A plot). Refer to Part C, Section 1.2h of the *Sony ID7000 SOP* for instructions on how to draw gates.
h. Open the *Stopping Condition* window and define the *Singlets* gate as your *Saturation Gate*. Additionally, select *Stopping Condition* and specify the time or number of events to be collected before data acquisition stops.



i. Adjust PMT voltage settings using One Max or All Max method. Refer to Part C, Sections 1.2.1 and 1.2.2 of the *Sony ID7000 SOP* for how to set voltages using One Max or All Max method.
j. Right-click on all wells to be acquired and select *Add to Auto Acquisition Target*. Click the *Auto Acquire* button to initiate acquisition.

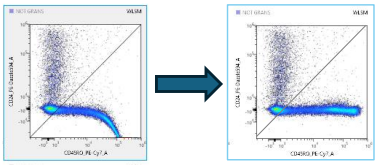
4. Data analysis

1) Autofluorescence extraction
   a. In the rack/plate map, select unstained control well/tube. Right-click → *Change Worksheet Mode* → *Individual* to change it to an individual worksheet. Refer to Part D, Section 1a of the *Sony ID7000 SOP* for information on worksheet modes.
   b. Select an unstained sample well and click on the *Autofluorescence Finder* tool in the *Unmixing* tab.
   c. Follow the autofluorescence finder wizard to identify autofluorescent (AF) populations.
      Step 1: Create a gate around the populations you will be analyzing.
      Step 2:
      - Identify all AF populations that can be distinguished as individual populations. Rescale the axes and scroll virtual filters left and right as needed to achieve better separation of the populations.
      - Create a gate around each population.

      Step 3: Exit the wizard by clicking *OK*. If you see a warning about saturated events, check the box "*Calculate Spectral Reference without saturated event(s)*".
   e. In the unstained sample worksheet, prepare a few subplots of the AF-Root gate and visualize parameters with high autofluorescence background on the x- and y-axes of each.
   f. Turn ON unmixing and adjust the scaling of the plots created in step e. Open the *Unmixing Settings* window.
   g. Untick all boxes in the *Use as AF* column in the *Autofluorescence* box. Tick one box at a time to test how efficiently each AF parameter extracts autofluorescent signals by observing the shape of the unstained cell populations on the plots.
   h. Remove unnecessary AF parameter(s), if any, by right-clicking on the parameter and selecting *Remove Fluorochrome*. Close the *Unmixing Settings* window.

2) Unmixing QC
   a. Perform hierarchical gating on your fully stained sample to include a cell gate, singlet gate, live/dead gate and if present, CD45+/- gate.
   b. Go to the *Unmixing* tab, click on the downward arrow under the *Unmixing Viewer* tool and select the population you wish to view (recommendation: start with singlets). The *Unmixing Viewer* opens to display NxN plots of one fluorochrome against every other fluorochrome in the panel.

c. Click on a plot and press [Ctrl] + [A]. Use the *Auto Adjust XY* button to automatically adjust the axes to your populations, then manually adjust all axes further so that the maximum fluorescence intensity displayed on all plots is 10^6.
d. Turn ON the spectral reference *Adjuster* at the top of the *Unmixing Viewer* screen, then change *Display Events* on the right to 50,000 or 100,000 events. Assess whether there is an unmixing error on any of the plots, indicated by populations bending towards the negative fluorescence values ("banana shaped" populations). Correct the error by dragging the population up/down or left/right with your mouse until the mean of the positive population is approximately aligned with the mean of the negative population, as shown in the screenshot.



e. Click *Next* to go through each fluorochrome in turn until you have looked at all NxN plot combinations and correct any unmixing errors you can spot. Ensure to monitor the spectral references on the right-hand panel of the *Unmixing Viewer* so the spectra do not dip below x-axis. It is not recommended to correct any AF spectra.
f. Once all unmixing errors have been corrected, switch the *Adjuster* OFF. When prompted, click *Save* and assign a new name to save it as a new matrix. **Note**: It is recommended to keep the original unmixing matrix in case incorrect unmixing adjustments have been made.
g. Click *Close* to exit the *Unmixing Viewer*.

5. Data export

1) Exporting SONY experiment files (.exdat files)

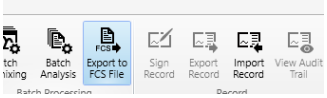
It is highly recommended to export and keep the original ID7000 experiment files, so you can go back and make any corrections if needed in the future. **In the event of experiment deletion or data loss from the ID7000 software, it is not possible to re-import FCS files into it, so please ensure to keep the .exdat files of your experiments as described below.**

a. *File* → *Database* → *Export*.
b. Select the experiment(s) you want to export and click on the *Move* button in the middle of the window.
c. Browse the location where you want to save your files and click *Export* to start exporting the data. It is recommended to export to *Desktop* in the first instance, then move your .exdat files to the desired location from there.
d. Once the export is completed, close the window.

2) Exporting FCS files

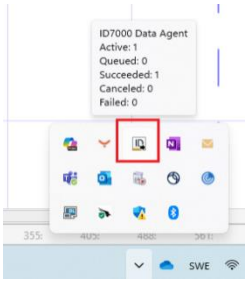
ID7000 data can also be exported in FCS format for analysis using third-party software.

a. Select an experiment in the *Analysis* tab. Click *Export to FCS file* in the ribbon.



b. Deselect the parameters you do not wish to include (e.g. Height, Width for fluorescence parameters) and check that all sample groups you want to export are selected. Browse the location where you want to save your files and click *Export*. As above, it is recommended to export to *Desktop* in the first instance, then move your FCS files to the desired location from there.
If several unmixing matrices have been created, a separate FCS is generated for each unmixing matrix. To deselect the unwanted matrices, click on the specific sample group in the *Select Samples* window, and untick the WLSM box.

c. A message will pop up once the export has started. Click *OK*, then *Cancel* to close the window. The data export will continue in the background. The export status can be viewed by hovering the mouse over the *ID7000 Data Agent* icon in the task bar.



For information on database backup, restoration and reset, please refer to Appendix 1) of the *Sony ID7000 SOP*.

6. Cleaning and Shutdown

1) Cleaning without hardware shutdown (between users)
   a. *Cytometer* tab → *Decontamination* → *Bleach Cleaning and Rinse*. Follow the instructions in the wizard.
      - Bleach: 1-3% sodium hypochlorite
      - Rinse: MilliQ water
   b. Leave the software logged in between users.
      - The air tank of the ID7000 unit is not pressurized while the software is logged out. If the system remains logged out for an extended period, the air tank pressure will gradually decrease and may fall below the lower limit, triggering a pressurization error. Therefore, to monitor and maintain proper pressurization of the instrument in between users, the software needs to remain logged in.

2) Cleaning and hardware shutdown
   a. *Cytometer* tab → *Hardware Shutdown* → *Bleach Cleaning and Rinse*. Follow the instructions in the wizard.
      - See above for recommendations on bleach and rinse solutions.
   b. Once the cleaning is complete, click *Shutdown*. The instrument will be turned off. Close the software and turn off the two power switches on the left side of the ID7000.

7. Troubleshooting

1) QC failure

   If QC fails, perform the following:
   a. Check the preparation, storage, lot number of the beads. Ensure that there was enough beads and the tube was loaded in the correct slot.
   b. *Cytometer* → *Flow Cell Purge*.
   c. *Cytometer* → *Decontamination* → *Bleach and Rinse Cleaning*.
   d. *Cytometer* → *Optics Alignment*.
   e. *Cytometer* → *Flow Rate*.
   f. Run both *Daily and Performance QC*.
   g. If the QC is still failing, *QC* tab → *Export QC Results to XML*. Send the resulting zipped folder to the Sony FAS Europe team for further troubleshooting (refer to Part 8 below for contact details).

2) Clogs

   If the event rate suddenly drops to 0 during acquisition and you suspect a clog in the system, perform the following:
   a. *Cytometer* → *Flow Cell Purge* and follow instructions.
   b. If the problem persists, *Cytometer* → *Priming* and initiate priming.
   c. If the above do not resolve the issue, run a cleaning cycle by clicking on *Cytometer* tab → *Decontamination* → *Bleach and Rinse Cleaning*. Follow the instructions in the wizard.
      - See Section 6, 1a for recommendations on bleach and rinse solutions.

d. If the issue still persists, contact the Sony FAS Europe team for further troubleshooting (refer to Part 8 below for contact details).

For any further information on maintenance and troubleshooting procedures, please refer to *Sony ID7000 SOP*.

8. Resources

- For more detail information on the ID7000 SOP, please refer to *Sony ID7000 SOP*.
- For additional information and guidance on any of the procedures, please consult *Sony ID7000 Spectral Cell Analyzer Operator's Guide* (File → Help → Open Manual).
- For additional help, get in touch with the Sony Field Applications Team by emailing to SBTE helpdesk at sbte.helpdesk@sony.com.