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Planning your cell sorting experiment

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How does cell sorting work?

Cell sorting is a technology used for separating cells or particles based on their light-scattering and fluorescence properties.

Seven sorters are operated in the Flow Cytometry lab at CHOP: **Aurora CS-1**, **Aurora CS-2**, **FACSAria Fusion**, **FACSAria II**, **FACSJazz**, **FACSMelody,** and **BioSorter**. All of them operate by encapsulating each particle of interest into a droplet, which is then directed into a collection device (a tube or a well of a plate)

Aurora CS, **FACSAria, FACSJazz** and **FACSMelody** are **electrostatic droplet sorters**. They are used for separating particles with diameters up to 30µm. These devices work by shooting a thin stream of sheath fluid (PBS) down through a small orifice (nozzle). The cells or particles contained in the sample are "injected" into the center of this stream and arranged in a straight single file (hydrodynamic focusing). The nozzle vibrates with very high frequency (10 to 100 kHz), causing the liquid stream to break down into tiny droplets that will contain the cells to be sorted. The droplets that contain desired cells are electrically charged and then deflected sideways (left or right) in an electric field, adjusted so that the deflected droplet falls into a collection device. The droplets containing unwanted cells are not deflected and go vertically into the waste tank.

The **BioSorter** can be used for separating particles with diameters larger than 40µm. Typical mammalian cells cannot be sorted on our BioSorter because they are too small. This instrument operates on a different principle: it generates a continuous stream of sheath fluid which is pushed sideways by an air blower into a waste tray. For each desired particle, the air blower stops very briefly (a few milliseconds), causing a droplet containing the particle of interest to fall vertically into a collection device. The unwanted particles are sent into the waste tank.

Unavoidably, some droplets will not contain only one, but two or more cells. Some droplets will contain one or more "good" cells and one or more "bad" cells. Almost always we set up the sorters to collect the droplets that contain one or more desired cells, but no undesired cells ("**purity mode**"). However, a sorter can be also set to collect all the droplets that contain at least one desired cell, including those droplets that contain both desired and undesired cells ("**enrich mode**"). The enrich mode will be used only if you specifically request it. In "**single cell**" mode, the sorter sends into the collection device the droplets that contain only one desired cell. Single cell mode is used for depositing one single cell into each well of a plate.

We often mention the term "**sort efficiency**", which is the ratio between the number of desired cells that are detected by the sorter and the number of desired cells that are deflected into the collection tube. In enrich mode, the sort efficiency is practically always 100%. In purity mode, we aim to keep the sort efficiency higher than 75%. The sort efficiency can be improved by preventing the cells from sticking to each other and clumping (see below the *Sample preparation* section), by minimizing the amount of debris in your sample, by diluting the sample and by keeping event rate low.

*For an in depth introduction to cell sorting watch **The Art of Sorting** webinar by Beckman-Coulter.*

https://www.beckman.com/resources/videos/webinars/the-art-of-sorting-for-advancing-cytometry

Guidelines on how to plan a cell sorting experiment

The reliable identification of the target populations is critical for the success of a cell sorting project. If your cells need to be dissociated from solid tissues, begin with practicing and fine-tuning cell prepping. Use a microscope to confirm that the cells are well dissociated and the amount of debris is reasonably low.

Next, we advise to schedule **a pilot experiment**, which should have the following aims:

- Confirm that the cell preparation procedure and cell staining are reliable.
- Confirm that the populations to be sorted can be identified and sorted with acceptable purity.
- Estimate how much time is needed for sorting sufficient cells for the actual experiment.

High-speed vs. regular-speed sorting

Aurora CS and **FACSAria** can be operated in **high-speed** mode (using **70µm nozzle**, sheath fluid pressure 60 PSI) or in standard-speed mode (100µm nozzle, sheath fluid pressure 18 to 30 PSI).

Generally, the diameter of the cells to be sorted should be at least 5-fold smaller than the nozzle size. Thus, cells with diameter smaller than 14µm (e.g., lymphocytes) can be efficiently sorted in high-speed mode; cells with diameters below 20µm can be sorted using the 100µm nozzle. Larger cells will cause inconsistent deflection of the droplets, and some sorted cells won't reach the collection device.

Please do not request high-speed sorting unless the diameter of your cells in suspension is 14µm or smaller AND the total number of cells in your samples is larger than 35 million.

FACSJazz and **FACSMelody** can be operated in **regular-speed mode only** (100µm nozzle).

Sample preparation

- Before bringing the cells to the Flow Lab, consider taking a small droplet of sample on a microscope slide and place it under a microscope to check the quality of your cells: typically, the cells should be round, abundant, with very few aggregates, and a reasonably low amount of debris.
- Adjust the concentration of the cells to be sorted to max. 10 to 20 million/ml for regular-speed mode and max. 30 million/ml for high-speed sorting mode. Note that we can dilute the cells if they are too concentrated, but for practical reasons we will not concentrate them. If the number of cells in each sample is low, adjust the volume to 0.5ml. In principle, any physiologic buffer may be used to resuspend the cells to be sorted. However, it is essential to have the cells **well-dissociated** and to prevent their clumping to achieve good recovery rates, high sort efficiency and to **prevent clogging of the nozzle**.
- For cells that tend to clump, we recommend suspending them in **Ca^{2+}/Mg^{2+}-FREE buffer**, such as **PBS** (Phosphate Buffered Saline) or **HBSS** (Hank's Balanced Salt Solution) with low protein content (≤ 2% BSA). **EDTA** (0.5 to 5 mM) and/or **DNase** (20 – 100µg/ml) may be added to further prevent cell clumping. For pH-sensitive cells, **HEPES** (25 mM) may be added to the buffer. Note that standard cell culture media contain Ca^{2+}, $Mg^{2+}$and proteins from serum that favor cell clumping.
- It is highly recommended to pass the cells through a cell strainer with 35µm mesh size (*Falcon* cat. 352235) immediately before sorting. Some cells tend to clump after being filtered.

Collection tubes

The table below summarizes the collection devices that can be used on each electrostatic droplet sorter.

Collection Device	Sorter			
	Aurora CS	**FACSAria**	**FACSJazz**	**FACSMelody**
1.5ml Eppendorf	6 ways	4 ways	2 ways	4 ways
5ml FACS	4 ways	4 ways	2 ways	4 ways
15ml conical	2 ways	2 ways	2 ways	Unavailable
50ml conical	1 way only	Unavailable	Unavailable	Unavailable
Multi-well plates	1 way	1 way	1 way	1 way

Example on how to read the table above: The FACSAria sorters can be used to sort 1, 2, 3 or 4 populations at a time in 1.5 ml Eppendorf tubes or in 5 ml FACS tubes, up to 2 populations in 15 ml tubes, or one population at a time in 96 well plates.

Please bring **labeled** collection tubes containing cell culture medium or buffer of your choice, in sufficient volume to cover the bottom of the tube. The sorted droplets should fall into liquid, not on hard plastic and we also want to have the cells sorted into media that contains some nutrients. FBS 100% may also be used as collection media if desired.

Note that all our sorters are capable of collecting **cells in multi-well plates (96 well plates)**.

Sorting time

The amount of time needed for sorting depends mainly on how many cells need to be run through the instrument.

- In high-speed mode (**70µm nozzle**), the sort speed is up to **80 - 90 million events/hour**.
- In standard-speed mode (**100µm nozzle**), the sort speed is up to **30 million events/hour**.

Most sorts are performed on the 100µm nozzle. All other nozzle sizes require additional setup which may take up to 30 minutes to complete.

The **sample volume** is also a limiting factor: up to 5ml of sample can be processed in one hour.

Some of the reserved time will be used for **setting up the sorter** for your project. For a one-color experiment, 15 minutes or less are typically sufficient for setup. For multicolor experiments, consider reserving at least 4 extra minutes for each additional color. (For instance, for a five-color sort consider reserving about 35 minutes of time for setup, running the single-color controls, and compensation/ unmixing.) On our spectral sorters (Aurora CS), the setup for large panels that have been thoroughly tested ahead of time may take less time (with a very well-designed and tested 20 color panel, it takes about an hour to record the reference controls and run the unmixing).

Purity check

Checking the purity of sorted cells can be done by taking an aliquot of sorted cells and re-running them on the sorter or on another cytometer. Approximately 5,000 sorted cells are necessary for a purity check. We recommend you ask the operator of the sorter to check the purity of sorted cells, but we do not always do it by default because sometimes the number of sorted cells is very low and because it takes additional time. The purity of sorted cells is typically above 95%.

Maximizing the chances of success of a cell sorting experiment

- Unfortunately, the Flow Lab at CHOP cannot guarantee that every cell sorting experiment will be successful. Cell sorters are complex devices that can break down unexpectedly and it is impossible to have them repaired instantly. Unfortunately, we get only one shot at sorting the cells right: if something goes wrong, the sample will be lost.
- If there is a problem with a sort, let us know as soon as possible. It is always better to try figuring out what went wrong at the at the time when the problem happens, rather than days later.
- Whenever possible, bring more cells than can be sorted in the reserved time, and we will focus on sorting the number of cells needed for the actual experiment, which ultimately is the most important. When a minimum number of cells cannot be defined, we will run the samples in the order they are presented to us, and we will stop when we run out of time. Tell the operator how much time should be allocated to each sample or if some samples should be prioritized. Also keep in mind that, for multiple reasons, samples prepared identically may yield fewer sorted cells (e.g., small differences in cell prepping procedure, biological differences between samples).
- When calculating the time necessary for a cell sorting experiment, keep in mind that the sorter will need to analyze the debris particles, which takes as much time as regular cells. We often sort samples in which 25% to 50% of particles are debris or non-viable cells.
- Some cells may be brought to us in cell culture media, and they can be sorted without any problem. However, when sort efficiency is low or when the nozzle tends to clog, resuspending cells in a cation-free buffer with low protein content can have a dramatic effect on improving the sort outcome.
- Researchers are encouraged to observe their sorts, at minimum at the beginning of the sort when the gates and the sort logic is set. Then watch the sort for at least several minutes to get an estimate of the sort rate and sort efficiency and decide whether any adjustments need to be made.
- We encourage researchers to diligently keep track of the details of the cell prepping workflow and the cell sorting outcome. Many of our users had great success improving their sort outcome by using this approach to optimize the cell prepping procedure.

Types of cell sorting services

We offer three types of cell sorting services: **full-service**, **semi-assisted**, and **unassisted**.

End-users should schedule reservations directly in iLab by logging in, navigating to our core on the "**Reserve instruments**" page and opening the calendar of the desired cell sorter. A questionnaire must be filled out and a form of payment needs to be available in iLab at the time the reservation is being set. Please contact our administrative team at coresadmin@chop.edu for assistance with setting up a form of payment (grant number for CHOP researchers, PO or credit card for researchers from Penn or from other institutions).

Important: When scheduling reservations for cell sorting, make sure you click "**Pricing details**" and select from the drop-down list the appropriate type of service (**full-service**, **semi-assisted**, **unassisted,** or **training)**.

Full-Service Cell Sorting

- **No training is required** before scheduling full-service cell sorting.
- A member of the Flow Core Lab will be available to operate the sorter during this type of service.
- This type of service should be also selected by trained users who are not yet confident using the sorter without assistance from our staff.
- Full-service is available during business hours (Mon – Fri 9AM to 5PM).
  Important note: This type of service depends on our staff availability.
- On some sorters, it may not be possible to set up reservations in iLab during lunch time (12 to 1 PM).
- We expect that the researcher who schedules a full-time reservation will bring the cells to our lab and will be present during the setup of the sort, to confirm the gating strategy and the sort logic. We welcome users to watch their sorts, at least during the first 5 - 10 minutes after the effective sorting starts, to check the sort efficiency and the sort rate for each target population.
- The sorted cells should be picked up by the researcher when the sort is completed.

Semi-assisted Cell Sorting

- Only **trained end-users** may schedule semi-assisted sorting.
- This service is available only **during business hours** (9AM to 5PM, during working days).
- Our staff will set up the sorter (check alignment and drop delay).
- The end-user will need to set up their experiment (compensation, gating strategy, sort logic etc.) and operate the sorter for the entire duration of the reservation.
- Assistance will be available from our staff for troubleshooting unexpected situations.
- Trained end-users who need extensive assistance should reserve the sorter in full-service mode.

Unassisted Cell Sorting

- This level of service is available only to **proficient end-users** that can set up the sorter and are able to troubleshoot unexpected events (clogs, air intake, etc.) without assistance from our staff.
- Unassisted sorting is available only **during after-hours** (Mon-Fri after 5PM and during weekends).

The hourly rates for semi-assisted or unassisted cell sorting are significantly lower than for full-service.

Training for cell sorting

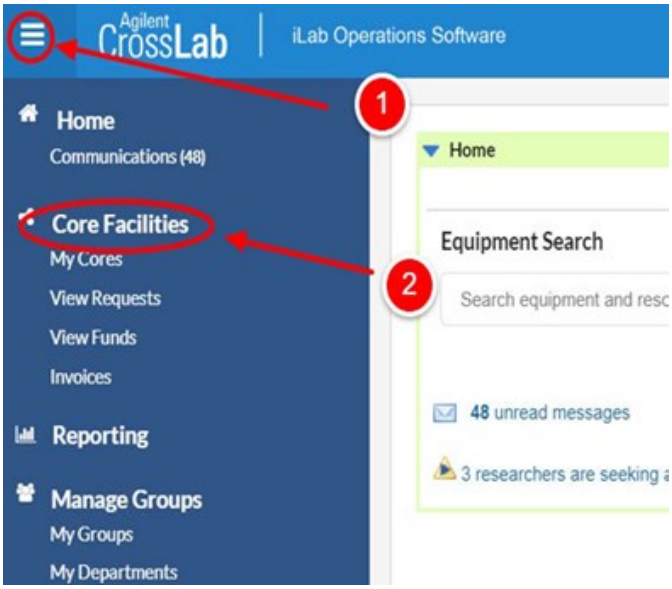
- All researchers who want to learn how to operate a sorter should start by watching ***The Art of Sorting*** webinar by Beckman-Coulter. This webinar explains multiple concepts that are essential to know before the hands-on training on instruments.
- Training sessions for cell sorting can be scheduled as iLab reservations and carry the same rates as full-service appointments.
- Basic cell sorting skills on **Aurora CS, FACSAria, FACSJazz** can be gained in **two training sessions** of approximately **2 hours each** (4 hours of training total).
- The training for Aurora CS and FACSAria may take only one session of two hours if the trainee is proficient in using the corresponding software package: SpectroFlo for Aurora and FACSDiva for FACSAria.
- **FACSMelody** is very user friendly. One session of two hours is typically sufficient to learn how to operate the FACSMelody sorter.
- FACSAria trainings must be scheduled on the FACSAria II. After the training on FACSAria II, you will be able to sort on both FACSAria instruments (FACSAria II and FACSAria Fusion).
- Typically, it takes one session of approximately two hours to learn how to use the **BioSorter** at basic level.
- **Additional training** will be needed for covering advanced features and for learning how to troubleshoot common technical problems. We suggest that the first cell sort after completing the training should be scheduled in full-service mode, and a member of our lab will be available to provide extensive guidance during that time.
- Our **written training guides** for each instrument can be downloaded in iLab, on the "**Reserve instruments**" page.

Guidance on setting up in iLab a reservation for cell sorting

1. ***Register in iLab if you do not have an account***. Inquiries regarding the iLab registration process should be sent to coresadmin@email.chop.edu or feel free to contact any member of the flow lab.

   Researchers not affiliated with CHOP should follow the guidance below to find our iLab site:

   - Use a web browser to log into **iLab**
   - Click the **menu icon** (1) in the top left corner
   - Select "**Core Facilities**" (2)
   - From the "**View**" drop-down list, select "**Cores at partner institutions**" or "**Cores at other institutions**"
   - Look below for the **Flow Cytometry Core at CHOP**.



2. **Set up a payment method:**.
   - **CHOP** researchers should ask their PI or business manager to give them access to iLab in an active payment account. Questions on enabling CHOP accounts in iLab should be sent to coresadmin@chop.edu.
   - **PENN** researchers will need to set up a PO as a form of payment.
   - For setting up the PO, the business manager may need the following information: BenBuys information: Phila 200, Site 49513 Children's Hospital of Philadelphia
   - **All other researchers** must set up a PO from their own institution or use a credit card for payments.
     Contact coresadmin@chop.edu for details on setting up the payments.

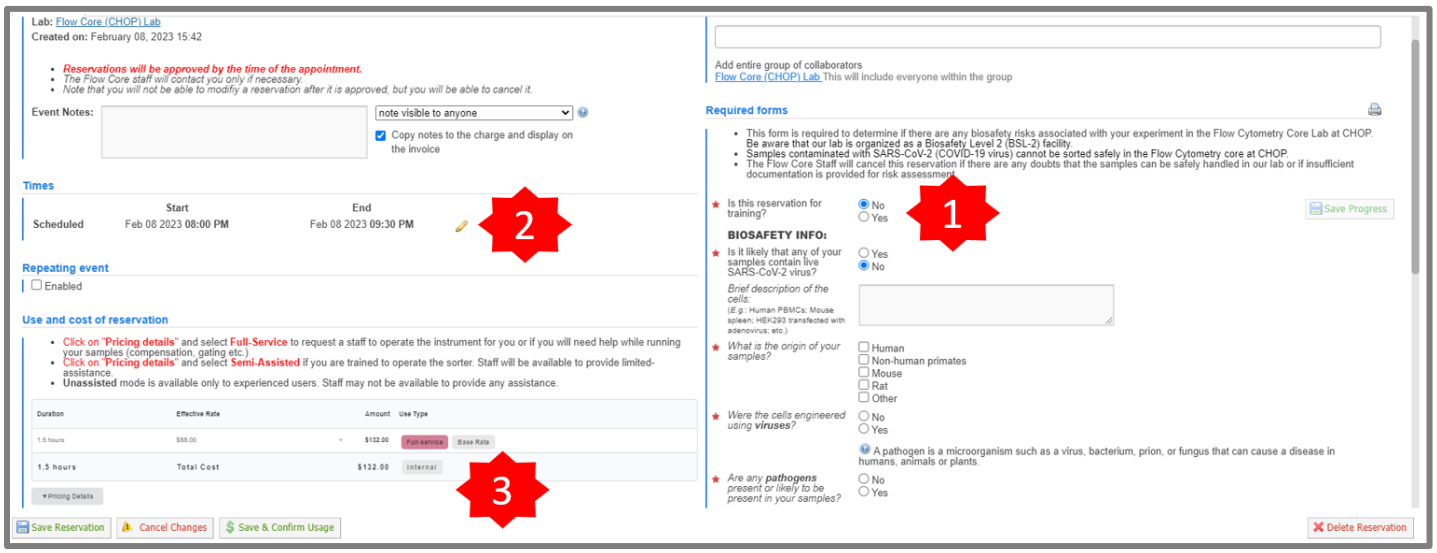
3. **Determine what instrument you should use for your project.** The optical configurations of our instruments can be downloaded in pdf format from our website. Please email flowcytometry@chop.edu if you need help with selecting the appropriate sorter for your project.

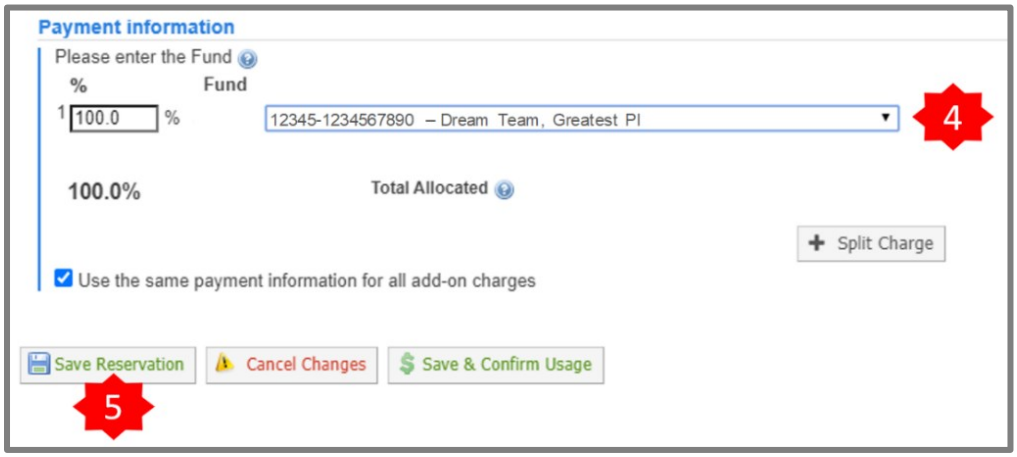
4. **Reserve sorter:**

   a) **Log in iLab**, click the "**Reserve instruments**" **tab** and scroll to the bottom of the page. Click on "**Sorters**" to open the instrument schedules.

b) Click "**View schedule**" to open the calendar. **Click-and-drag** over any available time slot in the calendar to initiate a reservation:

- If you need to change the **time**, **click on the pencil (2)** to the right of the End time, then click **green checkmark** to save when done.
  *Note: this does not save the reservation. The button "**Save Reservation**" at the bottom left saves it.*
- If you need to change the **Use Type**, click on **Pricing Details (3)** to select the **type of service** desired.
- **Full-service** is for all untrained users during the Core's operating hours;
  Semi-assisted is for trained users operating independently DURING operating hours;
  Unassisted is for trained users operating independently off-hours;
  Training is for all training reservations.





- Choose the account number or PO number **(4)** to be used with this reservation.
- Indicate whether the reservation is for **Training** or not. Fill out the **Questionnaire (1)** for regular reservations.
- Finally, click on the "**Save Reservation**" **(5)** button at the bottom left of the screen to save the changes.
- The reservation will be marked as "Needs approval" (this is normal!) and if we have any questions, we will contact you. Note that **reservations are approved by the staff**

immediately prior to the start time. After a reservation is approved, the iLab system does not allow the end-users to make any changes to the reservation.

5. **At the time of your appointment, bring the items listed below to the Flow Lab in ARC 1207:**
   - **Filtered cell suspensions in 5mL FACS tubes**, including single color compensation controls, unstained cells and FMO controls if needed.
   - **Labeled collection tubes containing cell culture medium or FBS.**
   - Approx. 10ml of **sample buffer** for diluting the cells if needed.
   - **Extra collection media and tubes** in case new collection tubes must be prepared.
6. Always check with the operator of the sorter to **make sure that all gates are properly set and populations to be sorted are clearly identified**.
7. You are welcome to watch the sort. At minimum, **do not leave the lab until you see how many cells were sorted during the first 5 or 10 minutes**. This should give you an estimate of how many cells you should expect to get. If necessary, ask the operator to make any adjustments. After a sort is completed, nothing can be done.

Biosafety

Please note that the Flow Core lab is organized as a BSL-2 facility. We cannot sort any agents that require containment above BSL-2.

The iLab questionnaire that must be filled out for each reservation includes biosafety questions that must be answered accurately.

- **FACSAria II** and the **BioSorter** may be used only for sorting samples that require **BSL-1** containment (animal samples that have very low risk of containing pathogens).
- **Aurora CS-1** and **CS-2**, **FACSAria Fusion**, **FACSMelody** and **FACSJazz** are encased in class 2 biosafety cabinets and may be used with samples that require containment up to **BSL-2**.
  Per current policies at CHOP, all cells of human origin, including established cell lines, must be contained at BSL-2 or higher.

Contact

Send your message to **flowcytometry@chop.edu** if you need to contact us by email. Messages are auto forwarded to all members of the Flow Core lab, and you will likely get an answer sooner than if you email only to one member of the lab.

For all **urgent matters** that require immediate action, please call us at **215-590-3402** or talk with a member of our lab.

Cell sorting table

The table below summarizes the main characteristics of the sorters existing in the Flow Cytometry lab at CHOP.

	Regular Speed Sorting	High Speed Sorting	Large Particles Sorting	
Sorters	**Aurora CS-1 / CS-2 FACSAria Fusion / II FACSJazz FACSMelody**	**Aurora CS-1 / CS-2 FACSAria Fusion / II**	**BioSorter**	
Nozzle size**	100 µm	70 µm	250 µm FOCA	1,000 µm FOCA
Nozzle frequency (equals the number of drops generated per second)	~25,000 – 45,000 sec.	~75,000 – 90,000 kHz	Not applicable	Not applicable
Droplet volume	~ 3 nl	~ 1 nl	1 µl	7 – 8 µl
Maximum cell diameter recommended	20µm	14µm	100 µm	500 µm
Sheath pressure	18 - 27 psi	~60 psi	< 5 psi	< 1 psi
Maximum sort rate	8,000 to 10,000 events/sec. (up to 36 million events/hour)	20,000 to 25,000 events/sec. (up to 90 million events/hour)	20 events/ second	20 events/ second
Minimum recommended sample volume	0.5 ml	0.5 ml	5 ml	5 ml
Recommended pre-sort cell concentration	10 – 15 million cells /ml	20 – 30 million cells /ml	0.1 – 1 million/ml	0.1 – 1 million/ml
Post-sort concentration	~0.3 million cells /ml	~1 million cells /ml	~1,000/ml	~150/ml
Typical sorting efficiency	70 – 90%	70 – 90%	60 – 90%	60 – 90%

* Sorting cells using a nozzle smaller than the recommended size may result in poor cell viability and loss of sorted cells.

** 85µm and 130µm nozzles are available on FACSAria.

Notes:

- **Replacing the 100µm nozzle takes up to 30 minutes** on **FACSAria** and **Aurora CS** sorters.
  On FACSJazz and FACSMelody, only the 100µm nozzle can be used.

- Keep in mind that the **BioSorter is very, very slow** (sort speed less than 20 particles per second) and it should be used only for large cells (hepatocytes, adipocytes, etc.), cell clusters or small organisms (*C. elegans*, zebrafish).