PHARMACOLOGY & TOXICOLOGY CORE FACILITIES

SORTING BEST PRACTICES

BD FACSAria-IIu: This sorter is the original model FACSAria-I cell sorter that has been upgraded in the field with a FACSAria-II flow cell. It is equipped with 3 lasers: a blue 488nm, a red 633nm, and a violet 405nm. The optical configuration allows multi-parameter detection of up to 13 fluorescent parameters and 2 light scatter parameters. The FACS Aria-IIu is capable of sorting through over 50 million cells per hour (dependent on cell type), with up to four populations simultaneously. The populations of interest can be sorted into 0.5 mL/1 mL Eppendorf tubes, 5 mL tubes, or 15 mL conical tubes. Populations can also be sorted directly into multi-well plates, including: 12-well, 24-well, 48-well, 96-well.

OPTIMIZING SORT EXPERIMENTS

On average, for well-behaved cells and clean cell preparations, our instrument can obtain a sort purity of >99.0% viable cells. There are many factors that affect sort purity, sort efficiency, yield, and viability. Instrument set-up, cell preparation, and maintaining cell viability are key components to successful sorts.

The FACSAria IIu has variable nozzle sizes that are used at specific pressure settings and drop drive frequencies to accommodate different cell types from different organs/sources. Nozzle sizes are chosen based on cell size, morphology, and fragility, and post-sort application to minimize stress on the cells. The table below describes the sorter specifications.

CELL SORTER SPECIFICATIONS

	FACSAria-IIu
Lasers (excitation λ)/# of detectors	1) 488 nm blue/6 detectors + FSC/SSC 2) 640 nm red/3 detectors 3) 405 nm violet/4 detectors
Max # of Fluorochromes	12 colors
Nozzle size: **ddf - kHz (drop drive frequency)/ Pressure (PSI)**	70 um high: 87-90kHz/ 61 PSI 70 um medium: 70-75 kHz / 40 PSI 85 um medium/low: 51-55/ kHz 45 PSI 100 um low: 30-32 kHz / 20 PSI
Average Rate (total # cells/hour)	70 um high: 54×10^6 70 um medium: 40×10^6 85 um medium/low: 36×10^6 100 um low: 21×10^6

Nozzle sizes: examples of cell types

70 um high: lymphocytes from spleen, thymus, lymph nodes, PBMCs
70 um medium: activated T cells, transduced T cells, T cell culture, monocytes, IELs
85 um: primary macrophages, b-cell culture, bone marrow
100 um: dendritic cells, fibroblasts, hybridomas, larger cell lines- B16, HeLa

A good rule of thumb is to choose the nozzle 5× larger than the diameter of the cell to be sorted.

CELL SORT BUFFER

Cells should be prepared in a way that maximizes recovery and optimizes viability. Once cells are harvested according to the appropriate protocol, they should then be re-suspended in the proper sort buffer. Higher protein concentrations in the sort buffer may create a refractive bias with protein-free sheath buffer due to the increased optical density and refractive index of the sort buffer containing protein. Laser light passing through buffers having different indices of refraction will be bent causing distortion of the light scatter signals. Best results will be obtained using just enough protein to keep your cells happy but at low enough concentrations to not significantly affect light scattering. The sort buffer contains a low concentration of protein – 1 to 2% FBS or 0.5% BSA, which will also help maintain viability during the sort. HEPES acts as an adequate pH buffer for cells exposed to high pressure. EDTA will help reduce cation-dependent cell-to-cell adhesion. Cell clumping can also affect sort purity and recovery. Addition of DNAse (if necessary) and filtration of samples using nylon mesh will help reduce cell aggregation.

Appropriate sort buffer is necessary for a successful sort. Culture media is not an ideal sort buffer for a number of reasons, including that the pH regulation fails under normal atmosphere (CO_2 evaporates), causing the media to become basic, the calcium chloride in most culture media is not compatible with the phosphate component of the sheath buffer used in the sorters leading to precipitation of calcium phosphate crystals, and also phenol red can increase background auto-fluorescence. In order to maintain optimal pH regulation, the sort buffer described below is recommended. Although cell conditions may vary, the suggestions below will help in increasing cell viability and recovery. **Please re-suspend sort samples at the recommended concentration below and FILTER your samples immediately prior to sorting.**

A good reference for cell sort buffer information:
http://expertcytometry.com/how-cell-culture-medium-can-decrease-cell-viability-during-a-flow-cytometry-cell-sorting-experiment/

Buffer Recipe
1X Phosphate Buffered Saline or HBSS (Ca/Mg++free)
1 mM EDTA
25 mM HEPES pH 7.0
1% Fetal Bovine Serum (heat-inactivated)
0.2 um filter sterilize, store at 4°C

Adherent cells: Adherent cells are typically dissociated using trypsin or some type of EDTA treatment. Adherent cells can be a problem when sorted. Debris-free, single cell suspensions sort best. Often when cells are trypsinized, the trypsin is inactivated by adding fetal calf serum. This addition of fetal calf serum adds back divalent cations enabling the cells to clump. Consider using soybean trypsin inhibitor, in place of serum/media. Products like Accutase, which gently detaches confluent cells from tissue culture plates/flasks and does not have to be neutralized like trypsin. Additionally, it preserves epitopes that that may be sensitive to tryspin. Cells can often be killed in the trypsinization process, and as debris-free single cell suspensions sort best, DNAse I (100 µg/ml, or 10 units/ml) or II can be added to prevent free DNA adhering to cells causing them to aggregate. To prevent cells from clumping, please centrifuge your cells at the slowest speed possible in round bottom tubes.

Sticky Cells: Doublets and higher order aggregates cannot be used for analysis or sorting, because the true status of each fluorescent marker cannot be determined. The presence of aggregates means there are fewer cells available for your analysis or sort. Additionally, aggregates will clog the instrument, shortening the available time to obtain your sorted population of interest. Issues with sticky cells can often be remedied by removing divalent cations. Concentration of EDTA should be raised to 5mM and the use of FBS that has been dialyzed against Ca/Mg++ free PBS. Not all cells may tolerate this concentration of EDTA, so test prior to using for a sort. Also note that DNAse will not work in the absence of divalent cations, so EGTA may be a better option, as it has a lower affinity for Mg++.

Samples w/ Low Viability: FBS concentration can be increased up to 5%. A higher concentration of FBS will cause a refractive mismatch and change the scatter profile.

With an increased number of dead cells in the sample, it is likely that elevated levels of soluble DNA will be released. DNA could coat cells and lead to increased clumping. DNAse I in the presence of magnesium chloride will help reduce cellular aggregation.

1) Treat cells for 15-30 minutes in a solution of 100 μg/mL DNAse I in the presence of 5 mM $MgCl_2$ in HBSS at room temperature.
2) Wash the cells once in the presence of 5 mM $MgCl_2$ in HBSS.
3) Gently resuspend the cells in buffer containing at least 1 mM $MgCl_2$ (although 5 mM may be optimal) and 25-50 μg/mL DNAse.

Sort Sample Concentration: Sample concentration will affect a few different variables including abort rate, throughput, and sort efficiency. Cells should be counted **AFTER** staining for a more accurate number. We would prefer you to bring samples more concentrated, and can be diluted as needed.

For larger cell lines – ~5-10 x 10^6/mL
For medium cells, e.g. dendritic cells, monocytes, activated t-cells – 10 to 20 x 10^6/mL
For smaller cells, e.g. lymphocytes – 20 to 30 X 10^6/mL

Tube Types for <u>PRE-SORT SAMPLE</u>: Polypropylene tubes are recommended (rather than polystyrene) and will reduce adherence of cells to the tube walls. Polystyrene 5 mL tubes are the type of tubes that are typically used on analyzers. Please do not bring controls or samples in 1 mL microtubes!

- For under 5 mL, use 5 mL polypropylene or polystyrene tubes
- For over 5 mL, use 15 mL conical tubes

<u>FOR COLLECTION OF SORTED CELLS</u>

Sorted cells are diluted out with sheath buffer, which is sterile phosphate-buffered saline and not conducive to keeping cells healthy for long periods of time. For better viability and recovery, it is best to sort into an appropriate buffer. **For most cell types, we recommend to sort into tubes pre-coated with 100% FBS, particularly for long sorts**. Pre-incubating tubes with a solution that contains protein will help reduce charge on the plastic (particularly important for polystyrene, which is why use of polypropylene is recommended) and will reduce the attraction of charged sort droplets to the sides of the tube. If tubes are not pre-coated, droplets can dry on the sides of the tube during the sort causing the cell to die if the liquid evaporates. If the cells of interest cannot be sorted into 100% FBS, then we recommend a buffer with at least 10% FBS. Cells can be also be sorted directly into lysis buffer (depending on sort population/sort volume). For RNA, please look at other alternatives to sorting directly into Trizol. Trizol should be used under a fume hood and the sorter is not maintained in a hood. **If cells are being sorted into multi-well culture plates, <u>media with antibiotics</u> should be used.** Centrifuge plates for 30-60 seconds at 1200 RPM (300 x g) to settle the cells in the wells immediately after sorting, as some cells can be deposited on the sides of the wells.

Up to 4 populations can be sorted simultaneously and can be sorted continuously or for a specific # of events. Based on nozzle size and pressure settings, the volume of sorted cells will vary. For 5 mL collection tubes, we recommend **POLYPROPYLENE** over polystyrene to reduce adherence of cells to the tube walls. Please bring tubes with the recommended volume of collection buffer (ie, 100% FBS, media containing 20% FBS,

etc.) listed in the table on the next page. For large populations, please bring multiple collection tubes filled with appropriate collection buffer.

Only one population at a time can be sorted into multi-well plates. For multi-well plates, a specified # of events can be sorted into each well (eg, single-cell sorting for clones or for single-cell PCR).

All collection manifolds can be cooled to 4°C to aid in maintaining cell viability. Please let us know what temperature you would prefer for your sorted cells, as different cell types may do better at different temperatures.

After sorting, invert tube and/or wash sides with extra buffer to recover all sorted cells. Sorted cells should be centrifuged and buffer discarded. Please re-suspend cells in the appropriate buffer for your endpoint.

COLLECTION FORMAT

	FACSAria-IIu
Up to 2 populations	15 mL conical tubes: 1-2 mL collection buffer 5 mL tubes: 500 µL collection buffer 2.0 mL eppendorf tubes: 200 µL collection buffer 0.5 mL eppendorf tubes: 50uL collection buffer
Up to 4 populations	5 mL tubes: 500 µL collection buffer 2.0 mL eppendorf tubes: 200 µL collection buffer 0.5 mL eppendorf tubes: 50 µL collection buffer
Plates	Tissue culture: 96-well 48-well 24-well 12-well 6-well chamber slides petri dishes

SORTING GUIDELINES & TIPS

1. **Test the antibody/fluorochrome panel before the sort experiment.** Antibodies should be properly titrated for any flow cytometry experiment. The recommended concentration is usually okay but could be optimized by doing a simple titration experiment. New antibody/fluorochrome combinations or a change in an existing panel should be tested in a pilot experiment first on an analyzer. Changing a few conjugates (marker to fluorochrome) can change the appearance of a population.

2. **Use a viability dye!** This is always a good idea regardless of whether you're doing analysis or sorting. Just do it!

3. **Take advantage of dump gates.** Good panel design is key to a good experiment and sort. In addition to using a viability dye to exclude dead cells, multiple markers with the same fluorochrome can be used in the same panel to exclude unwanted cells. This will free up other detectors to be used with markers/fluorochromes you are interested in using.

4. **Enrichment helps!** Enriching by positive/negative selection (eg, MACS) or by gradient before the sort usually helps sort efficiency and sort recovery. Starting with a higher percentage of the desired population will improve sort efficiency and decrease the amount of sort time needed. The sorter will not have to go through more unwanted cells to get the ones you're really interested in and the decreased sort time will keep the cells more viable.
    ex: spleen cells ➔ RBC lysis **=** eliminates unwanted RBCs
    ex: spleen cells after RBC lysis ➔ CD4+ enrichment by negative selection using MACS (depletion of CTLs, B cells, NK cells, DCs, Macs, granulocytes, erythroid cells) ➔+80% CD4+ cells **=** increase in Naïve T cells

5. **AFTER STAINING, ALWAYS WASH TWICE!** Washing away unbound antibody is extremely important. One wash is inefficient and will affect population resolution, noticeably on sorters with a slightly increased flow rate. Washing twice will clean up staining patterns and ensure the purity of collected populations.

6. **The pre-sort health of the cells dictates how well they will withstand the sorting process.** Cells that are fragile or already in a compromised state may have lower viability after the sort. Some cell types experience higher cell death, decreased yield, and decreased functionality after sorting. Pressure settings can be lowered to minimize these negative effects.

7. **Count your cells after staining!** Sample concentration will affect variables including abort rate, throughput, sort efficiency, and purity. Cells should be counted **AFTER** staining for better accuracy. Do not rely on your pre-stained count.

8. **Expedite sample processing.** Sample preps can be staggered to minimize the amount of time that the cells are sitting. If sorting is taking a long time (e.g. one sort sample – 4+ hours), the post-sorted sample tubes can be taken and processed in increments (e.g. sorted tube processed and changed out with a new collection tube).

9. **Sorting is not 100% efficient.** Sorters are not exact cell counters. Keep in mind that there is cell loss before, during, and after the sorting process. Cells should be counted **AFTER** staining and before re-suspension in sort buffer. This will give an accurate estimate of how long the sort will take and how many cells to expect back.

10. **A cells journey:** Remember your cells are being exposed to pressures up to 70 psi, being accelerated rapidly to ~20 m/sec, rapidly returning to atmospheric pressure, passed through laser beams, exposed to a charge of significant voltage, and hitting a liquid surface traveling at ~20 m/sec. All cells will not survive this trip. Maintaining conditions to maximize viability is crucial.