

## Aria Sample Preparation

The Aria is a very powerful cytometric tool and due to complexity of multi-fluorescent data analysis it is very important to plan ahead and prepare all the necessary controls. The Aria is capable of sorting at very high rates with exceptional purity of 4 simultaneous populations. In order to accomplish this, the sample must be prepped for optimal throughput. Conditions that can adversely impact your cell sample prior to sorting include cell media, buffering capacity, and protein concentration. Low cellular viability, autofluorescence, and cell aggregates will also impact the quality of your sort. Identification of these factors and taking the appropriate action to remedy the issues will improve sort purity, yield, and cell viability.

### Generalized Staining protocol:

Protocols for staining vary depending on the assay. This is a generalized cell surface staining protocol to use as a guide. Adjust as needed for your particular cells.

### Sorting Buffers:

#### *Hanks Sorting Buffer*

1X HBSS with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ; <2% FCS; 10mM HEPES\*, pH 7.2. *For some cell types it may be preferable to use HBSS without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ . Store at 4°C.*

#### *PBS Sorting Buffer*

1X PBS; <2% FCS; 10mM HEPES\*, pH 7.2; 10ug/ml DNase I\*\*

\*HEPES is optional, it helps to buffer the cells from acidic conditions under high pressure.

\*\*DNase is optional for cell types that are adherent or if there are dying cells releasing DNA causing clumping.

### Staining Buffer:

1X HBSS or 1X PBS w/ 2-5% serum.

#### Cultured cells:

Split the cells 1-2 days prior to sorting when possible. This will improve the health of the cells and reduce overgrown and dead cells. For adherent tissue culture cells, trypsinize to release from the growing surface. Stop with serum. Wash cells in media or PBS before staining. If the cells are sticky or an adherent line, resuspend in a final sorting buffer containing DNase I\*\* to keep the cells from clumping during the sorting process.

#### Primary cells:

Dissociate cells from tissue using the method that is most suitable for the cells type of interest. Process whole blood and spleen removing the RBC's when possible. If the cells are sticky resuspend in a final buffer containing 10ug/ml of DNase I\*\* to keep the cells from clumping during the sorting process.

1. Cells must be in a single cell suspension.
2. Wash cells with staining buffer.
3. Centrifuge cells to pellet. Remove supernatant.
4. Resuspend in blocking agent (serum or Fc block). *This is optional, determined by cell type and nonspecific binding.*
5. Add antibody ~50 $\mu$ l/1E6cells (concentration is dependent on the specific antibody) *Titration of antibodies is recommended to get proper concentration.*
6. Incubate cell in the dark at 4°C for 30-60 min.
7. Wash cells with staining buffer 3 times.
8. Add Secondary Antibody if necessary, repeat steps 5-7.
9. Resuspend cells to a final concentration of ~**20E6/ml** (*see notes/FAQ below*) in sorting buffer of choice.

10. Cells must be sent through a 40 $\mu$ m filter to remove large clumps prior to sorting. (Falcon snap cap cell strainer cat# 352235) If your cells are larger than 30 $\mu$ m the standard 70 $\mu$ m nozzle will not be appropriate for sorting. Please contact staff for further assistance.
11. Total starting cell #'s is dependent on how many cells are needed from each population to be sorted. Recommended minimum sort volume is 200ul. (*See notes/FAQ below*)
12. Four (4) populations can be sorted simultaneously and deposited into the following tube types; microcentrifuge tubes, 12X75 tubes, 15ml conical tubes can be used for 2- way sorts only.
13. One (1) population can be sorted down to one cell/well of a 96 well plate, other plate configurations available, 96 well PCR can also be used.
14. Cells should be sorted into growth medium with serum if going into culture. Sorting into a dry tube should be avoided.

## Notes and FAQ

**Transfected cells:** *If you are trying to isolate cells from a transfection using EGFP, EYFP or other fluorescent proteins, include a mock transfection as a control. This can be very valuable when the transfection efficiency and /or the expression of the protein is very low.*

**Limit the amount of serum** *the final suspension of sorting buffer. Ideally it should contain less than 2% FCS or .5% BSA. Excess serum causes optical interference resulting in dynamic distortion of the light scatter signals this ultimately means decreased resolution of your data. Too much serum also interferes with the stability of the stream and increases the potential clumping of cells, these factors affect purity and cell yield.*

**Test your antibodies and fluorochrome combinations** *before embarking on a large or critical sort, but really this is how you should approach every sort to avoid wasting time and money. Test new lots of antibodies and realize that some fluorochromes do have lot to lot variation and may need to be titrated every time a new vial is opened (i.e. PE-Cy5 or PE-Cy7).*

**Always bring controls.** *These include unstained cells of the same type that are being sorted. If you are sorting primary cells avoid bringing a cell line as the negative control, the ambient fluorescence rarely will be equivalent. You need single color positive controls for each fluorochrome used. This should ideally be done on the same population of target sort cells. The antibody can be irrelevant to the experiment as long as it is highly expressed on the cells. These controls are for compensating spectral overlap between fluorochromes for example, if you are sorting T-cells you can use CD3 conjugated to each fluorochrome as the single color controls. The other option is using beads that will bind to your directly conjugated antibody.*

**Cell size** *plays an important roll in how the Aria is set up. If you are sorting activated cells that are much larger than their non-activated counterparts, the set up will need to take that into consideration for the FSC Area Scaling and the gating scheme. The largest cell size for the default configuration is <30 $\mu$ m.*

**Sorting is not 100% efficient!** *There is cell loss before, during and after the sorting process. The healthier the cells at the beginning the better the results of the sort will be. There are key values that the sorter will display once the sort begins, soon after the sort has commenced, these values should be looked at and the forecasted yield can be estimated. \*\*Counting cells just prior to final resuspension will aid in accurately determining how many cells you are starting with, what volume to resuspend in, and estimate the maximum yield for each sorted population.*

*The quality of your sorted cells depends largely on the presorted population. Using a **viability marker** such as PI (Propidium Iodide), 7AAD or DAPI in the sorting buffer can provide useful data on the original health of the sample. The use of these markers eliminates sorting or analyzing non-specifically stained events as wells as dead cells that can give misleading total cell yield of a sorted population and analyzed data. Make sure that a viability stain does not interfere with any fluorochrome you are using for sorting before adding to your buffer. Sorting does stress the cells, some types more than others. Determine the frequency of you target populations and calculate how much **starting material** you will need to reach final cell counts needed for the experiment. This will also help estimate the **amount of time** to sign up for.*

*Generally, the Aria can sort at 25,000 events /sec (this includes debris, dead cells, platelet's and RBC's etc.) for most lymphocyte cells. Other cells are dependent on size and their general characteristics but a*

good estimator is 10,000events/second. This translates to 36E6 cells/hour that can run through the sorter. Events/hour X % of target = theoretical yield/ hour. Example: to calculate how many cell can be sorted per hour of a 2% target population at a rate of 10,000 cells/sec.

$36E6/\text{hour} \times .02 = 720,000$  cells this number is theoretical with no loss due to the following factors:

1. **Abort rate** = events that the cytometer can not identify, these events are essentially ignored and can potentially be sorted into your sample even if they are not a target event lowering purity. Since these events are not included in the data file high abort rates can also contribute to misleading data.
2. **Conflict events / efficiency** = This is calculated for each target population being sorted, target cells that have another event leading or following too closely for the sort mask (yield, purity, single cell, etc.) will not be sorted lowering yield.

Possible reasons:

- cell clumping
- high percentage of debris
- cell concentration too high

Possible resolutions:

- filter cells
  - DNase and filter
  - Dilute cells to lower concentration
  - Decrease serum concentration
  - decrease sample flow rate
3. **General cell population health** = your starting population dictates how well the sorted population will be. The healthier the cells are to start with the better they will hold up after the sorting process. Cells that are fragile or already in a compromised state may have lower viability after sorting. Some cell types experience higher cell death, decreased yield and decreased functionality post sort. If these are the type of cells you are sorting, keeping the sample pressure low helps to minimize these effects. This should be taken into consideration when calculating time needed to sort, and cell concentration. Some cell types need to sort at lower concentration and pressure resulting in flow rates of less than 5000cells/sec.
  4. **Sticky or dry tubes** = Some cells are more likely to stick to polystyrene tubes meaning some cells will not get sorted and remain in the starting tube. If your cells are sticky use polypropylene tubes instead. Sorting low % populations into tubes with inadequate media or dry sided tubes can lower yield. The cells end up stuck to the side of the tubes and the sheath fluid dries out resulting in dead cells.
  5. **Sort Volume/droplet estimation** = For each nozzle size the amount of sheath that is deposited in the collection tube can be estimated as the following keep in mind sort precision will dictate how many drops are included in a sort event packet e.g. single cell mode will contain one droplet/sorted cell, purity will contain 3 droplets/sorted cell.  
70um ~ 1.85nl/droplet  
85um ~ 3.43nl/droplet  
100um ~ 5.39nl/droplet