Analysis and Sorting Protocols:

Sample Prep for BD FACSAria: The appropriate cell concentration for sorting or analysis on the Aria will vary from sample to sample and can range from $3x10^6$ up to $15x10^6$cells/ml. A good starting concentration is $7x10^6$cells/ml, which can be diluted as needed, so please bring extra cell buffer. The total number of cells required also depends upon the sample. For samples in which the desired population is rare, more starting cells and more sorting time will be needed than for samples in which the desired population is relatively abundant. Cells should be prepared in sorting buffer, and we recommend clear cell media with 1%FBS. Some cell types may need to be treated with DNAse to remove dead cell clumps and may also need to be suspended in DNAse buffer to maintain a single cell suspension or use Accumax from Innovative Cell Technologies. An alternate solution to prevent aggregates is 1 mM EDTA, but it's NOT compatible with DNAse!

Basic Sorting Buffer
1x Phosphate Buffered Saline (Ca/Mg^{++} free)
1mM EDTA
25mM HEPES pH 7.0
1% Fetal Bovine Serum (Heat-Inactivated)
0.2um filter sterilize, store a 4^oC
For Clean Lymphoid Cells: The buffer can be simplified to HBSS with 1% FBS. The additional cations in the recipe promote better viability. Since these cells are not prone to clump, the lack of EDTA is not a problem.
For Sticky Cells: Raise the concentration of the EDTA to 5mM and use FBS that has been dialyzed against Ca/Mg^{++} free PBS. Some activated cells become clumpy and the chelators (EDTA) help reduce cation-dependent cell to cell adhesion.
For Adherent Cells: In order achieve good single cell preparations, one must start at the moment of detaching your cells from the plate. Typically, the trypsin (or other detachment buffer such as Accutase) is quenched with culture media or a PBS/FBS buffer. This is problematic because it reintroduces the cations that facilitate the cells reattaching to the plate (or each other). One must use a cation-free FBS buffer in order to stop the detachment. Additionally, the level of EDTA can be increased if necessary (but too much EDTA can be deleterious).
For Samples with High Percentage of Dead Cells: If there are a large number of dead cells in the prep, it is likely that there is soluble DNA from the dead cells that will come out of solution. This DNA will start to coat the cells and and lead to severe clumping. The addition of 10U/mL DNAase II to the buffer recipe will help reduce DNA associated clumpiness.

These are basic suggestions and should be used as the starting point to help optimize sample preparation for both enhanced viability and enhanced recovery. We suggest more comprehensive modifications evolving from these simplistic guidelines to arrive at the proper buffer for your cell type. To ensure that viable cells are analyzed or collected, a viability dye should be used. For live cells and depending on the fluorescent markers panel, propidium iodide (PI), 7-AAD, or DAPI may be added to cells just prior to sorting. For cells that will require fixation, there are many options available in fixed/viability dyes.

Sample can be provided in 12 x 75 mm polystyrene or polypropylene, 1.5ml Eppendorf, or 1.2ml microtiter tubes. **Sample must be filtered prior to sorting or analyzing on the FACSAria with a 30-40um filter.** We recommend CellTrics, PARTEC, #04-0042-2316, for analysis and sterile, #04-004-2326, for live cell sorting.

Sample Collection for Sorting:

Samples may be collected in any of the following:

1-2 Different Populations Simultaneously:

15ml conical with 7ml collection media (holds roughly 3x106sorted cells)
12x75 mm with 1ml collection media (holds roughly 1x06 ells sorted cells)
1.5ml Eppendorf with .5ml collection media (holds roughly 120K sorted cells)

3-4 Different Populations Simultaneously:

12x75 mm with 1ml collection media (holds roughly 1x06 ells sorted cells)
1.5ml Eppendorf with 600ul collection media (holds roughly 120K sorted cells)

Automated Cell Deposition Unit (ACDU)

6- to 96-well plates (collected populations may vary from well-to-well, but wells are collected one at a time)
Single cells can be sorted at 1 - 50,000 particles into microtiter wells (6,12,24,48,96)
Plates should be pre-filled (100 microliters conditioned media in 96 well plates)
Or use 50% serum in PBS and change buffer when you return to your lab.
Can also directly sort into PCR extraction buffer or other non-culture buffer (i.e. serum)
Cell cloning works best in phenol red-free media and/or on feeder cells

When using tubes for collection, it is a good idea to coat them with BSA or FBS, especially the 15 ml conical tubes. This will help keep the cell droplets from sticking to the sides of the tube and drying out. To coat the tubes, fill them with 4% BSA (bovine serum albumin) in 1X PBS (filter-sterilized) or pure FBS and incubate at 4^{o}C for at least 1 hour prior to sorting. Just prior to bringing the tubes for sorting, pour out the BSA or FBS (this may be reused for up to 1 month), and then add the media.
The collection vessel should contain media rich with FBS or some source of nutrients for the cells to keep them happy just after sorting and to keep the cell droplets from directly hitting the collection vessel.
Cells will be sorted in tiny droplets of sheath fluid, and drop size depends on nozzle size (100um nozzle generates relatively large drops, so for 100K sorted cell about 400ul of sheath fluid is added, while 70um nozzle generates small drops, so the volume will not increase as much)
* Polypropylene tubes are recommended for collection from the FACSAria. The Aria charges the stream to sort cells and a charge may build around polystyrene tubes as the cells are sorted. Initial samples may be in either polystyrene tubes or polypropylene tubes.

Compensation Controls:

Compensation is necessary whenever there is emission overlap between fluorochromes in the panel used.
Controls need to be at least as bright as any sample you will apply the compensation to.
The autofluorescence should be the same for the positive and negative control populations for any given parameter
Your compensation color MUST be matched to your experimental color (use exactly the same Ab-fluorochrome on beads that you used on your cells)
We recommend antibody capture beads for compensation, and they can be purchased from most companies that specialize in flow cytometry products. Please make sure you have both positive (beads that bind your Abs) and

negative beads (beads that do not bind Abs), and please make sure that you have the right beads for the right species of your Abs.
In some instances beads can not be used: viability dyes, proliferation dyes, Annexin V, membrane potential dyes, … For these controls make sure you use cells that will give you the brightest signal: stimulated cells, treated cells, mixture of live and dead cells…
We also recommend that you have a sample of unstained cells.
Compensation Controls needed:

1) Unstained cells
2) Unstained cells or beads + Stained cells or beads with Ab Fluorochrome 1
3) Unstained cells or beads + Stained cells or beads with Ab Fluorochrome 2
4) Unstained cells or beads + Stained cells or beads with Ab Fluorochrome 3
5) Unstained cells or beads + Stained cells or beads with Ab Fluorochrome 4
6) …

Gating Controls: FMO

The use of FMO (fluorescence minus one) is a very useful tool in gating strategy. FMO controls are critical to use when accurate discrimination is essential, and when antigen expression is low or variable. It helps address the spread of data and the contribution of error measurements in channel of interest from all other fluorochromes in the panel.
FMO Controls (have to be made with cells and not beads):

1) Cells stained with Abs Fluorochrome 2, 3, 4, ….
2) Cells stained with Abs Fluorochrome 1, 3, 4, ….
3) Cells stained with Abs Fluorochrome 1, 2, 4, ….
4) Cells stained with Abs Fluorochrome 1, 2, 3, ….
5) …