Sample Preparation for FACSAria Cell Sorter

For high speed tube sorts
The cell concentration should be about 10 to 20 x 10^6 cells/ml (sticky cells 7-8 x 10^6 cells/ml; large 4-7 x 10^6 cells/ml). This allows the cells to be sorted without having to apply excessive extra pressure to the sample tube in order to achieve a rate of 10 to 15'000 events per second. However, cells at lower concentrations can also be sorted without problems, for example when a relatively small number of cells are to be sorted.
I recommend resuspending the cells in PBS containing 2% FCS for very sticky cells add 1mM Na_2EDTA to prevent nozzle clogs.

For single cell sorts into plates (e.g. 96well plate)
The cell concentration of samples for a plate sort (single cell cloning) should not exceed $1.5*10^6$ cells/ml (because of the motion limit of the robotic automated cell cloner) and should not go below $0.5*10^6$ (however higher cell concentrations are better to handle than lower). Resuspend the cells in PBS containing 2% FCS for very sticky cells add 1mM Na_2EDTA to prevent nozzle clogs.

Cell straining / filtering
In order to minimize the possibility of nozzle clogs, it **is required** that the sample be put through a **40 micron cell strainer** before sorting. These are available **from Falcon (cat. no. 352235).** After filtering, the cells should be kept on ice and protected from light. For really sticky cells, it may be necessary to filter them again just prior to sorting because they can clump when sitting for longer periods of time.

In addition, with every sort, please supply
- A small preparation (~3-4 x10^5 in ~0.5ml) of the cells unstained (and/or with isotype control for negative population setup).
- A small preparation (~3-4 x10^5 in ~0.5ml) of the cells with a single stain for each fluorochrome (for spectral overlap compensation in multicolor assays).
- Tubes (or plates) with medium, PBS (see Table 2 for volume information) or what ever your cells like and/or what's necessary for your following examinations

Additional Information
- the survival rate or cloning efficiency largely depends on the cell type (one cell line has shown as few as 5 or 6 surviving cells ("wells") on a plate, and has never exceeded 20%, really hardy lines, usually suspension cells, have more than 90% survival)
- Serum concentration and pH-value do also affect survival. Increasing serum concentration from 10 to 20% in the collection medium the cloning efficiency increased from 40-50% to 80-85%. Furthermore a increment of cell survival can be achieved by reducing Antibody (or what ever) incubation time
- For single cell cloning it is recommend to use conditioned cell culture media with increased serum concentration
- the sorting process should be as prompt as possibly to the sample preparation
- one sorted drop (=sorted particle) is approximately 3.88nl ($1*10^6$ sorted particles approx 3.9ml (100micro meter nozzle)
- the purity and the yield of sorted samples are dependent on several factors. The most influencable one is the sample preparation process. A big source of decontaminations is the number of doublets ("cell clusters") (most of this could be excluded by software settings but not all). To minimize this it is essential to trypsinize, resuspend and filtering the sample accurately.

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Sort Buffer Selection

The proper design of sort buffer for both your pre-sort sample and your collected sample is crucial for a successful sort. The following will be a basic recipe and some suggestions for modifications that might be relevant to your particular experiment. Culture media is not an ideal sort buffer for two reasons: the pH regulation fails under normal atmosphere causing the media to become basic and the calcium chloride in most culture medias is not compatible with the phosphate component of the instrument sheath buffer (the Basic Sorting Buffer without additional protein) leading to precipitation of calcium phosphate crytsals. Following the suggested recipes below will help maximize the recovery and viability of your sorted cells.

Basic Sorting Buffer
1x Phosphate Buffered Saline (Ca/Mg++ free)
1mM EDTA
25mM HEPES pH 7.0
1% Fetal Bovine Serum (Heat-Inactivated)

For 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) 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.

Cell Size and Morphology

The cell size should not exceed one-fifth of the nozzle diameter
This rule helps ensure that stream stability can be maintained during the sort. If larger particles are entering the stream, there is a deleterious effect on the droplet breakoff. A drifting breakoff can disrupt the careful calibration of the droplet delay and lead to problems ranging from fanning of the side streams (desired sample ends up missing the tube) to changing of the breakoff (severely comprimises sample purity) to ultimately clogging the nozzle. Morphology is a trickier subject due to the variation between cell types. In general, the more the morphology deviates from an ideal spheroid shape, the more susceptible the cells become to shear induced damage. We typically find that the greater the deviation from the ideal sphere, the larger the nozzle required by the instrument. It is important that you communicate information relative to cell morphology prior to scheduling a sorting experiment.

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Sorting with the BD FACSAria

With the FACSAria, particle position is known to within 1/32 of a drop, and with extreme stability of the sort set-up, the maximum sort envelope is 2 drops, when the "Recovery Mask" is set to its maximum value of 32. Running the FACSAria sorting lymphocytes in High Sort mode at 70 psi and drop-drive frequency of 90 KHz, it is perfectly feasible to sort with high purity at analysis rates of 30,000 per second. (Sorting for yield rather than purity can be done significantly faster still.) The actual number of target cells that are sorted out of the mixture will be lower, proportional largely to their frequency, so for a 10% target population, collecting 2000 target cells per second is a reasonable number. Sorting cells >10um can be achieved by use of the 100um diameter nozzle at 20psi with sort rates of 10,000 cells per second are optimum at 30 KHz.

Sorting sub-populations of less than 0.5%

Low frequency sub-populations are sorted at only a few events per second. Recoveries tend to fall dramatically as smaller sub-populations are investigated. A useful strategy when attempting to isolate significant numbers of cells from sub-populations comprising less than 0.5% of the whole, is to adopt a two stage procedure. After a preliminary estimate of the frequency of the sub-population, the flow rate is adjusted to give, on average, one cell per droplet.. The required analytical rate would now be that of the drop drive frequency i.e. up to 50,000 per second which is well above the capabilities of conventional flow cytometers. However, if the required cells are fluorescently labeled it is then possible to trigger the machine such that it only detects positive cells at a frequency it can then process. The unwanted cells are then treated in the same way as debris below a conventional scatter threshold, that is to say ignored. If three droplets are charged and sorted we would expect a 33% purity and high recovery; the sort decisions will not be aborted as the unwanted cells are not detected. This strategy becomes more effective as the sorted population gets smaller.

The table below illustrates some of the sort times required for the collection of cells from different starting populations.

Assuming flow rate of 10’000 cells per second $3.6*10^7$ cells per hour					
No. of cells requested	0.1%	1%	5%	10%	20%
1000	5.5mins	33sec	6.5secs	3.3secs	17.secs
10,000	55mins	5.5mins	1.1mins	33secs	17secs
100,000	9.8hrs	55mins	11mins	5.5mins	2.8mins
1,000,000	3.8days	9.2hrs	1.8hrs	55mins	28mins
10,000,000	38days	3.8days	18hrs	9.2hrs	4.6hrs

Table 1. Recommend collection volumes in different collection devices

Flow sorting and magnetic cell sorting (MACS)

Clearly as shown in the table above there are situations in which flow sorting is not practicable to obtain the required number of cells. In such situations MACS should be used. The MACS microbeads are superparamagnetic particles that are coupled to highly specific monoclonal antibodies. They are used to magnetically label the target cell population. They are approximately 50 nm on size, biodegradable and do not affect the cells. Labelled cells are placed in a column within a magnet. By rinsing the column all unlabelled cells are washed from the column leaving the labelled cell fraction. By removing the column from the magnet the labelled cells can be recovered. The entire procedure can be performed in less than 30 minutes and the cells may immediately be used for experimentation.

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Collection device	Recommend “collection volume” to bring in before sorting
6-Well Plate	3 ml/Well
12-Well Plate	2 ml/Well
24-Well Plate	1 ml/Well
48-Well Plate	0.5 ml/Well
96-Well Plate	0.1-0.15 ml/Well
384 Well Plate	0.05 ml/Well
5ml Tube (FALCON)	1 ml
15ml Tube	3 ml

Table 2. Recommend collection volumes in different collection devices

Sort modes and characteristics

Mode	Characteristic	General Application
Yield	Tries to capture all positive events regardless of the presence of negative events	High Recovery
Purity	Sorts positives only in the absence of a negative event	High Purity
Single Cell	Same as Purity mode except will only accept one positive event per sort decision (only suggestive for plate sorts)	Single Cell Deposition

Table 3. Available sort modes