Cell Sorting

Cell Detachment/Isolation:

You can use **ACCUTASE or ACCUMAX** (Non-enzymatic cell dissociation buffers) for detaching cells, which are the best options to use. The samples can stay in either buffer throughout your entire sort. **Take note** that Accutase can alter some surface epitopes and this effect will need to be determined empirically for the epitopes being evaluated.

The two products, Accutase and Accumax, are from Innovative Cell Technologies for cell dissociation. Accutase gently detaches confluent cells from plastic ware, does not have to be neutralized like trypsin, and preserves epitopes that can be stained by flow cytometry. Accumax breaks up aggregates in suspension culture and can extend the sort time of a sample.
http://www.accutase.com/

For adherent cells:

Adherent cell lines can reaggregate when serum is used to inactivate trypsin. After trypsin treatment, for detaching adherent cells in the plate, **Soybean Trypsin Inhibitor** is recommended to stop trypsinization instead of using serum. When using trypsin to detach adherent cells, you can use FBS that has been dialyzed against Ca/Mg++ free PBS or increase the EDTA concentration to 5mM or higher (first make sure that your specific cell type is not sensitive to high concentrations of EDTA). Each cell type responds differently to SBTI and the ideal conditions must be determined empirically, but usually between 0.05%-0.25% SBTI in HBSS will work.

For samples with a high percentage of dead cells:

Cellular clumping can be caused by DNA released from dead cells. If you have extensive cell death in the sample, add DNAse (deoxyribonuclease). It will not only decrease clump formation in the sample, but also will decrease viscosity of the single cell suspension buffer, by digestion of the free DNA. Sort purity and yield could go substantially up after DNAase treatment.

DNAseI in the presence of magnesium chloride will help to reduce cellular aggregation.

- Treat cells for 15-30mins in a solution of 100ug/ml DNAse & 5mM MgCl2 in HBSS at room temperature
- Wash cells once in HBSS with 5mM MgCl2
- Gently resuspend cells in HBSS with 5mM MgCl2 & add 25-50ug/ml DNAse
- (as a maintenance dose) prior to & during the sort.
- Note: DNAse requires at least 1mM Mg to work effectively, although 5mM is optimal.

Actin released from dead cells irreversibly inhibits DNAse I so it is important to

minimize the presence of dead cells. (EDTA should be avoided for the DNase to work properly!)

Ideal Cell Suspension Concentrations:

Having the sample too concentrated, or too diluted can be problematic. There is no ideal concentration that works for all cell types and sort set-ups. It is a matter of understanding some of the issues and deciding what factors are most relevant to a given cell type and experimental design.

- Samples should be concentrated optimally, depending on cell type.
- For adherent cell lines and large cells, cell concentration should be approximately 3-5 million cells/mL, for the 100um nozzle tip.
- For lymphocytes or small cell types, samples can be concentrated up to 10+ million cells/mL, for the 70um nozzle tip.
- Cells must be at the proper concentration in order for the sorter to function optimally. Cells that are too concentrated will have lower recovery due to coincidence aborts (two cells that are too close together will be rejected by the machine in order to ensure purity) and cells that are too dilute will have a longer processing time or if they are run faster, an increased signal CV.
- You should bring extra sorting buffer in case the sample requires dilution.

Coincidence Aborts

While the sorter is evaluating which cell to sort, it must also determine whether it can do so it a manner that ensures the sorted material remains pure. The sorter makes this decision based on the proximity of events in time. If the desired event is too close to a potential undesired event, the machine will abort (not sort) the desired event to ensure purity. There are other modes of sorting that favor recovery over purity, so if total cell numbers are more important than purity, we can accommodate this. This should explain why we would not want the sample so concentrated that it becomes difficult to space the cells far enough apart while they are going through the sorter. If recovery is a prime concern, aborts tend to be the issue.

Signal CVs and Sensitivity

If the sample is too dilute, to get them to run at a reasonable rate can cause another set of problems. To get them to run at the appropriate rate, the sample differential must be increased. If the differential is too high, the CV's (coefficients of variation) start to become higher. This ultimately leads to less resolution and lower sensitivity. If you are trying to separate two very close populations, the CV becomes more important.

Cell Adhesion and Clumping

Adherent cells are trickier to sort than suspension cell types. Adherent cells typically like to stick to each other given the opportunity. Careful cell preparation and media can help avert this problem, but so can cell concentration. These sticky cell types typically like to be slightly more dilute to mediate clump formation. There is no absolute formula for this, it is determined by trial and error for a given cell type. The take home message is to consider running very sticky samples slightly more dilute.

Sticky cells Cell Sorting require cells in a single cell suspension. If the cells are clumped they cause several problems such as:

- A large clump will clog the cell sorter nozzle tip which causes a delay and may contaminate the collection tubes.
- Clumped cells will also reduce the sort yield due to the clumps failing the singlet discrimination gating
- Aggregated cells cause more coincidence (or software) aborts

Sorting Buffer:

This is probably one of the most important factors to achieve an ideal sort. A properly designed buffer recipe will help maintain a single cell suspension as well as keep the cells in a good physiological state. Culture media is typically a poor sort buffer.

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 crystals. Following the suggested recipe below will help maximize the recovery and viability of your sorted cells.

- 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 40C
- antibiotics (pen and strep)

Addition of **HEPES** will significantly increase the buffer capacity of the original sample buffer. It may aid with sample viability by buffering the pH of the sample while sorting. Buffer capacity of the common phosphate and carbonate buffers gets compromised by high pressure within the instrument during the cell sorting procedure. Using HEPES **is important** to sure up the cell membrane. It is even good to use HEPES during staining.

Do **NOT** use sorting buffers that contain **phenol red,** as this causes an increase in background fluorescence and decreases sensitivity. If using RPMI as your sorting buffer, which is not recommended, make sure there is **NO** phenol red (highly autofluorescent) and that 25mM of **HEPES** is added.

For sticky cells: Try increasing the concentration of EDTA to 5mM and use FBS that has been dialyzed against Ca/Mg++free PBS or 0.5% BSA. **Caution:** Some cell types can be sensitive to high concentrations of EDTA.

Dead Cell Exclusion:

Propidium Iodide, DAPI, or 7AAD can be added for excluding dead cells during sorting. 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.

Filtering

In order to minimize the possiblity of nozzle clogs, it is **required** that the cell samples be put through a 40 micron cell strainer before sorting. 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.

Collection Media:

You should bring collection media, that can range from PBS to enriched tissue culture media with antibiotics or 100% FBS to suit your cells. Using **100% serum** is recommended for collection if it works well for your protocol.

The sorted cells will be diluted with the sheath fluid. A very general rule of thumb is that 1×10^6 sorted cells will end up having a sheath fluid volume added of about 1 ml after sorting.

Extremely Important: After cells are sorted into collection tubes, centrifuge cells to remove diluted buffer and replenish with fresh culture media.

Coating Collection Vessels

We highly advise that you use coated tubes. It has been found that a significant number of cells can stick to uncoated tubes and drastically reduce the number of cells recovered after your sort. Please follow the simple protocol below to prepare coated tubes.

- Use polypropylene tubes, we recommend Sterile 5ml culture tubes (BD Falcon 352063).
- Fill tubes with PBS with 20% FBS or 5% BSA.
- Incubate 2h at RT or overnight at 4°C

- Just before sorting empty tubes and add your choice of collection media. This procedure works best if tubes are not allowed to dry prior to receiving cells.

Single Cell Sorting:

- Single cells can be sorted into 6 to 96 well microtiter plates or onto a slide.
- Plates must be pre-filled (100ul minimum of complete medium in 96 well plate).
- Keep in a sealed sandwich bag or keep a tiny chip of CO 2 (dry ice) in the plates container to keep bicarbonate-buffered pH correct

Control Samples:

Each control sample should contain a minimum of around 200,000 cells

For all multicolor sorts, a negative or unstained control and separate single stain control sample tubes for each stain to be used is required for protocol set-up. *(ie: if you are using FITC, PE, PECy7, APC, a control sample of each is required for calibration and compensation of instrument. In this case, 5 control samples total including unstained cell sample)*

Fluorescent Protein sorts require only a negative or not-transfected (not-transduced) control sample. Control samples should be prepared the same as the sort sample from the same type of cells as experiment samples.

More Tips:

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.

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.