

The purpose of this document is to provide general guidelines and best practices for preparing samples for cell sorting. Be mindful that every sample type is different and modifications may be needed. This document contains hyperlinks so is best read electronically. New clients must schedule a consultation with the core manager (Monica DeLay) to discuss experimental objectives. She can be reached at [monica.delay@cchmc.org](mailto:monica.delay@cchmc.org) or 636-3575.

***A successful sort results in good purity and yield of the target population and is dependent on these five things:***

- 1. Cell harvesting and preparation***
- 2. Cell staining and fluorochrome choice***
- 3. Proper controls***
- 4. Sample and sorting conditions***
- 5. Sample collection conditions***

#### **1. CELL HARVESTING AND PREPARATION:**

***A successful sort is dependent on good sample preparation!***

When harvesting cells prior to staining and/or cell sorting, two important factors need to be minimized for the best sort purity and yield.

1. Cell death
2. Cell aggregation

Following are preparation tips that can help to achieve this goal.

##### Preparing Cells from Tissues:

The goal of preparing tissues for cell sorting is to maximize the yield of functionally viable, dissociated cells. Unfortunately, these types of samples often contain a high percentage of dead cells and debris as a byproduct of the dissociation process, which interferes with the quality of the sort and the resolution of the target population. A good resource for tissue dissociation can be found on the [Worthington Biochemical](#) or the [Sigma website](#).

##### Preparing Adherent Cultured Cells:

Adherent cultured cells are most commonly removed from the culture substrate by treatment with trypsin. Trypsin formulations and conditions vary depending on the cell type but incubating cells with too high a trypsin concentration for too long will damage cell membranes and kill the cells. In addition, trypsin can alter cell surface antigens and therefore alter binding of detection antibodies used to identify target populations. To inactivate trypsin use of a trypsin inhibitor such as Soybean trypsin inhibitor is better than serum, as serum adds back divalent cations that facilitate cell adhesion/aggregation. To inhibit cell aggregation, EDTA can be added as a divalent cation chelator as described in Section 4 (Sample conditions for sorting). An alternative for dissociation of adherent cultured cells is [Accutase](#) or [Accumax](#), which can aid in dissociating cell clumps especially for use in flow cytometry applications.

### Cell Enrichment/Depletion and Red Blood Cell (RBC Lysis):

Whether to perform enrichment or depletion depends on the frequency of the target population and the specimen type. Depletion of unwanted cells that constitute a larger population in your sample and RBC lysis cut down the sorting time and increase the efficiency of rare event sorting. Depletion/Enrichment can be done by using either magnetic bead based technology (such as AutoMACS) or by using density gradient centrifugation. Reagents like Lympholyte (Cedarlane) can be used for enriching lymphocytes from non-lymphoid organs by density gradient.

Samples abundant with RBCs should be treated with ACK (Ammonium-Chloride-Potassium) lysis buffer that can be made or purchased from a variety of companies.

### Cell Filtration:

Cell clumping can also be reduced by filtering samples during processing and just prior to analysis or cell sorting. Depending on the size of your cells, a 70µm cell strainer insert (Falcon 352350) or a 35-µm cell strainer tube (Falcon 352235) can be used to collect the filtered sample. Filter the sample immediately prior to sorting or analysis (even if you filtered before staining).

### Dead Cells:

Cell death should be minimized at every step of sample preparation. Hank's Balanced Salt Solution (HBSS) or a culture medium such as RPMI-1640 without phenol red is a better choice to maintain live cells than PBS. The addition of 2% BSA or 2% FBS and 25mM HEPES buffer pH 7.0 comprises the Basic Cell Sorting Buffer that has been found to maintain pH and cell viability. Dead cells typically have high auto-fluorescence and bind non-specifically to antibodies resulting in high background and/or false positives. Dead cells release nucleic acids and other intracellular material that cause intact cells to clump. Dead cells can be removed using a ficoll gradient and/or cell clumping minimized by DNase I treatment for 15 to 30 minutes in a solution of 100 µg/mL DNase and 5 mM MgCl<sub>2</sub> in HBSS at room temperature. Wash the cells once in the presence of 5 mM MgCl<sub>2</sub> in HBSS. Gently suspend the cells in Staining Buffer (or PBS) containing MgCl<sub>2</sub> and 25-50 µg/mL DNase (as a maintenance dose) prior to and during the sort. A dead cell discriminator such as 7AAD or PI should be included to eliminate dead cells from the sorted population. For an extensive listing of viability dyes, visit the [Molecular Probes Handbook](#).

### Prepare Enough Cells:

Cell loss occurs during every manipulation at each stage of preparation. A realistic estimate of the number of cells needed will be based on these losses and sorting efficiency. Sorting efficiency is an electronic calculation made by the cell sorter. Sorter-specific algorithms and rate of flow interact with sample characteristics such as cell size, type, cell death, cell population distribution and cell concentration to affect sorting efficiency. Empirically, dim and/or cultured cells tend to have lower sorting efficiencies than bright, primary cells such as mouse bone marrow cells. Typical sorting efficiencies for cultured fibroblasts may be as low as 60%, while a clean, bright preparation of primary lymphocytes may sort at 99% depending on relative abundance of target population and gating strategies. Preliminary experiments with cell

preparation and staining are recommended on the cell analyzers so that the number of cells needed for the sort will be available.

## 2. CELL STAINING AND FLUOROCHROME CHOICE:

### ***A successful sort is dependent on good resolution of sorted populations!***

If there are any questions about fluorochrome choice or cell staining procedures, please consult our staff. You can find instrument configurations (lasers and filter sets) on our website at [www.cchmc.org/rfcc](http://www.cchmc.org/rfcc).

#### Fluorochrome Selection:

Consider doing a preliminary analysis of your experiment before bringing your cells for sorting. Generally, “positive” populations that are dim and only minimally separated from a slightly dimmer “negative” population will lead to a poor analysis and/or a poor sort.

A thoughtful balance of fluorochrome brightness with cellular marker abundance is important for optimal resolution of cell populations. Please refer to the brightness chart on [our website](#) and the [BioLegend website](#) for more details on multicolor panel design.

#### Spectral Overlap:

Another important consideration in panel design is the amount of spectral overlap between fluorochromes. Spectral spill-over from a very brightly stained channel into a detector that requires high-sensitivity can be a real problem. This problem of overlapping emission spectra can be minimized with the use of one of the many “Spectra Viewers” available online. Please see the links for fluorochrome panel design software and tools in the “[Related Resources](#)” tab on our website.

#### Blocking Non-specific Binding:

An ideal antibody would have a high affinity to only one, specific cellular epitope. Unfortunately, non-specific binding can be a problem even when using a correctly-titrated antibody. In these cases, a blocking reagent is needed. Usually, a blocking reagent contains a high concentration of species-specific immunoglobulin that can bind to the Fc-receptors that are often responsible for the non-specific binding of the staining antibody. Specific antibodies to Fc receptors can also be purchased and have been used successfully, see the [eBioscience website](#) or the [Innovex website](#) for more information.

#### Titration Antibodies:

The optimal concentration for antibody labeling is when the ratio of antibody to antigen reaches a point of saturation. Too low and there will not be enough antibody to saturate all of the high-affinity binding sites; this will limit brightness, make small pipetting errors significant and make quantitative conclusions about cytometric data suspect. Too high an antibody concentration will waste reagent, and could actually lower the signal-to-noise ratio due to high non-specific binding to low-affinity sites. Antibody titration can reveal the proper range where small changes in antibody concentration or cell number have little effect, but non-specific binding is minimized. For more specific information about antibody titration see [this protocol](#) from Current Protocols in Cytometry.

#### Using Viability Dyes:

Most investigators are interested in sorting live cells – even when the end application of the sorted cells may be for DNA and RNA analysis. Dead cells may exhibit high non-specific binding and can then contaminate sorted populations and can lower RNA quality by releasing RNases. Careful cell preparation to produce single, viable cells and reduce clumping will help, but viability dyes such as propidium iodide, 7AAD or the various Live/Dead dyes are able to distinguish cells with intact membranes (live) from those with leaky membranes (dead or dying cells). For an extensive listing of viability dyes, visit the [Molecular Probes Handbook](#).

### 3. CONTROLS:

#### ***A successful sort is dependent on proper controls!***

Proper controls are **essential** for setting voltages, compensation and in identifying the gates for the population of interest. This is true for ANY fluorescent marker (e.g. GFP, viability dye).

#### Unstained and Single Stained Control(s):

Unstained controls are essential for determining background fluorescence and single stained controls are used to calculate the correct compensation values before a sort. Incorrect compensation can result in the wrong cells being sorted. Please bring an unstained/non-fluorescent control AND single stained controls for each fluorochrome/dye/fluorescent protein being used in your experiment to each and every sort appointment.

#### Fluorescence Minus One Controls or FMO's:

A fluorescence minus one (FMO) control contains all fluorochromes of the multicolor cocktail except one. They represent the combinatorial background fluorescence from other channels into the channel of interest and aid in setting sort gates properly. Many researchers are resistant to using FMO controls on a routine basis because they consume cells, reagents and time, but in many cases they are needed to validate the gating strategy of a reagent panel particularly if you are using the panel for the first time. For more information on FMO's, see Nature Reviews Immunology 4, 648-655 or the Daily Dongle [article](#) by TreeStar.

### 4. SAMPLE AND SORTING CONDITIONS:

#### ***A successful sort is dependent on appropriate sample and sorting conditions!***

The presorted cells should be in a buffer that maintains live, clump-free cells in suspension for the duration of the sort. In addition, it is vital to select the correct instrument settings for the cell type being sorted.

#### Cell Sorting Buffer:

Culture media is not an ideal sort buffer as high pressure during sorting increases the partial pressure of CO<sub>2</sub> which in turn leads to a drop in pH. Therefore, a buffer with strong pH buffering capacity like 25mM HEPES is recommended to avoid cell death after sorting. Also, media containing phenol red dye can result in increased fluorescence background. High protein concentration in the sample buffer is known to adversely affect the scatter properties of cells. Therefore, cells should be resuspended in a low protein buffer, such as Ca<sup>++</sup>/Mg<sup>++</sup> free PBS (or phenol red-free HBSS) to help reduce clogging and increase efficiency.

Cell concentration in Basic Cell Sorting Buffer should be in the range of 10 to 20 x 10<sup>6</sup> cells/mL and if you have fewer than 5 x 10<sup>6</sup> cells, then a volume of 300uL should be used. Not all cell types can handle the recommended concentrations. Final cell concentrations may need to be

adjusted (diluted) by the sorter operator to avoid aggregation of the sample so please bring extra sorting buffer to your appointment. The following is a suitable buffered medium, however, modifications may be necessary for your particular sample. This buffer can be filtered and stored at 4°C and antibiotics may be added to prevent microbial growth.

**Basic Cell Sorting Buffer:**

- **1x PBS (Ca/Mg<sup>++</sup> free) or HBSS (preferred)**
- **0.5-2% BSA or up to 2% heat-inactivated FBS [dialyzed against Ca/Mg free PBS]**
- **25mM HEPES pH 7.0**

These are the supplements that are highly recommended to reduce cell aggregation:

- **1mM EDTA (chelates Ca/Mg ions that promote cell aggregation)** A maximum of 5 mM EDTA could be used to prevent macrophages/monocytes from sticking to tubing; Some activated cells become clumpy and the chelators (EDTA) help reduce cation-dependent cell to cell adhesion.

**OR**

- **10U/ml DNase- (to remove DNA-induced cellular aggregation)** 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 lead to severe clumping. The addition of 10U/mL DNAase to the buffer recipe will help reduce DNA associated clumpiness.

**Note:** DNase should not be used in combination with EDTA, as EDTA chelates the ions required for DNase function.

Filtering Your Cells:

It is important that you filter your cells immediately prior to the sort. Even if you filtered your cells during the sample prep, they should be filtered immediately before sorting using a 35-µm cell strainer tube (Falcon 352235).

Selecting Sorting Conditions:

The type of sorter, sheath pressure and nozzle size can greatly impact cellular function and experimental outcome for downstream applications. Selecting the correct parameters is largely dependent on cell size and morphology in the sorted sample. While the sorter operator will determine the appropriate settings, the client should make sure that they communicate all necessary experimental details for this to occur.

## 5. SAMPLE COLLECTION CONDITIONS:

***A successful sort is dependent on optimal collection conditions!***

### Optimal Collection Media:

The collection media is the post-sort solution that receives the droplets containing target cells in sheath from the sorter. If the sheath is PBS, the calcium chloride in most culture media is not compatible with the phosphate component of the PBS leading to precipitation of calcium phosphate crystals when a large number of cells are sorted. The optimal collection media will depend on the downstream experiments planned for the sorted cells, but below are some suggestions.

Fetal Bovine Serum 100% to 50% in PBS

OR

Your own culture media with antibiotics

OR

PBS if collecting cells for RNA or DNA

OR

Lysis buffer from RNA isolation kit (e.g. buffer RLT from Qiagen kit)

Lysis buffer dilution could be a problem depending on number of cells collected and nozzle size. For tips on sorting for RNA analysis, visit the [Vermont Cancer Center website](#).

### Collection options:

All collection tubes should be filled 1/3 full with the appropriate collection media. Below is a table indicating the types of collection vessels that can be used on our instruments and how many populations can be collected for each vessel type.

Tube Type	Number of Sorted Populations
<i>1.5 ml or 2 ml microfuge</i>	<i>Up to 4</i>
<i>12x75 mm tube</i>	<i>Up to 4</i>
<i>15 ml conical</i>	<i>Up to 2</i>
<i>Multiwell plates or slides</i>	<i>One population at a time</i>

**Note:** 15ml Tubes are preferred when collecting more than a million cells using 100µm or 130µm nozzle.

### Sterility:

All tubes, plates, slides and buffers that are brought for collection should be sterile irrespective of the sterility requirement of your downstream process (even if it is for RNA/DNA/Protein). We require this to maintain an aseptic environment in the instruments and to avoid contamination of the actual sterile sorts. RNase free tubes should be considered if collecting the cells directly into RNA lysis buffer.

### Tube pre-coating:

If recovery of live cells is the sorting goal, pre-coat the tubes with 1% BSA to avoid sorted cells sticking to the sides of the tubes. Fill the tubes with 1% BSA in PBS, cap and invert for at least

half an hour prior to the sort. However, if performing Western blot for downstream analysis, tubes should not be treated with external protein.

Post sort analysis:

A small sample of the sorted cells can be analyzed to determine the effectiveness of the sort. Post sort analysis may be required if the result of the sort is to be published.

**Post this handy checklist when preparing for a sort!**

**WHAT TO KNOW WHEN SCHEDULING A SORT:**

- CELL TYPE** – Communicate all information about the type of sample you are bringing including cell size, species, source, biosafety information (any infectious agents?).
- MARKERS/FLUORS** – Be clear about what exact fluors and markers will be detected. Remember to include fluorescent proteins (ie. GFP), viability dyes, functional dyes etc.
- SAMPLE/CELL NUMBER** – Know the exact number of samples and number of total cells per sample to determine the length of the sort. We cannot always accommodate last minute add-ons.
- POPULATIONS COLLECTED** – Knowing the expression patterns and frequency of your populations will help determine the length of the sort and will aid in the setup process.

**WHAT TO BRING TO YOUR SORT APPOINTMENT:**

- SAMPLES** – Bring your samples in clearly labeled tubes. It is highly recommended to filter samples immediately before the sort.
- CONTROLS** – Bring unstained and single stained controls in clearly labeled tubes to **every sort**.
- SAMPLE TRANSPORT** – Bring samples on ice and with a lid. For samples requiring handling at BSL2 or BSL2+, the tubes must be transported in the following manner:
  1. Sealed primary tube (snap caps or screw caps)
  2. Sealed secondary container
  3. Absorbent between primary and secondary containers to absorb the entire contents
    - o This can be accomplished by placing your samples in a tube rack into a sealable plastic container with paper towels or other absorbent. These containers are inexpensive and can be purchased in local retail stores. **Remember to place a biohazard sticker on the outside.**
    - o If you need to place a large number of specimens on ice, this can be done by placing the secondary container in an oblong ice pan or Nalgene pan. Or you can place your entire ice bucket with sealed primary tubes and absorbent into a biohazard bag and seal the bag sufficiently so that no liquid can escape.
- BUFFER** – Bring extra cell sorting buffer to dilute your sample, if necessary.
- COLLECTION TUBES** – Bring plenty of clearly labeled collection tubes at least 1/3 full of appropriate collection media for your cells.
- PAPERWORK** – Bring necessary paperwork, including billing sheet, to every sort appointment and biohazard information for Biosafety Level 2+ sorts.

**AFTER THE SORT:**

- COMMUNICATE** – Give us feedback on the endpoint of your sort. This allows us to determine best practices for different cell types.