



Imaging Core Sorting Guidelines
Flow Cytometry Facility at MGH-East
www2.massgeneral.org/aids/flow_cytometry.html

Things to remember for your sort!

1. Email COMPLETED sort request form with your reservation request to parcfacs@partners.org. Fill it out ACCURATELY with colors used, how many cells, and ALL populations that you wish to collect (up to 4 at a time). Please complete all fields, according to the instructions.
2. Resuspend sample to between 10 and 20 million per ml, or a min. volume of 200 ul.
3. FILTER your sample as a final step before bringing it to us. Either FACS tubes with cell strainer cap (Fisher 08-771-23, \$500 for case) or for volumes larger than 3 ml a cell strainer (Fisher 352350) works well. Filter caps should be replaced with a normal cap and ALL caps should be securely fastened for transport.
4. Remember to bring your single stained controls and an unstained control.
5. Bring collection tubes filled halfway with media. 1 million sorted cells will be in 1 to 2 ml of PBS, so 12x75mm FACS tubes hold 2 million cells, 15 ml conicals up to 7 million. Bring a few extra empty collection tubes for instrument setup and/or if some get contaminated due to a clog.
6. Use the smallest tube possible to collect your cells—using 15 ml conicals to collect less than 1 million cells will usually result in excessive cell loss.
7. Bring extra dilution media in case sample is too concentrated, and collection media in case more collection tubes are needed.
8. All tubes should be labeled with **ethanol resistant** markers (NOT Sharpie).
9. If you are running more than 30 minutes late, please notify us. We may not be able to adjust your ending time, so we may not be able to finish your sample.

I. TO SET UP A SORT, MAKE SURE YOU:

- A. Send an email to parcfacs@partners.org to reserve your sort time. It is usually a good idea to meet in person before the sort to discuss the details and make sure it will work!
 - a. Due to Lab Meeting and weekly maintenance, sorting on Mondays is limited to 2:30 to 4:30. On weeks with a Monday holiday, Tuesday morning will be reserved for these tasks and unavailable for sorting
 - b. Tuesday thru Friday, sorting may be booked between 10 AM and 4:30 PM. Sorting outside of this timeframe may be possible upon request.
 - c. Non-Ragon users cannot make a reservation more than 1 week in advance.
 - d. Tuesday and Thursday from 2:30 to 4:30 are reserved for members of the Ragon Institute. Any time not claimed will be released to all users on the day before the opening.
 - e. Available time slots can be viewed in the online Calendar, which can be found at www.schedulebook.com. Login with the username “flowguest” and the password “flow”. More detailed calendar instructions can be found linked from our “Core Documents” page on our website.
 - f. Times blocked as “Guest” are sort reservations for people that ONLY sort and thus do not have their own personal account—clicking on the “N” next to the name will display the name of the researcher.
- B. In your email requesting a reservation, for EVERY sort you request (do not just tell me “same as last time”), please include (as an attachment) a sort request form completed according to the instructions document including the fund number for billing and a phone number if we have to call you.
- C. A printout of an analyzed sample would be helpful for proper gating in some cases (especially if there is not a clearly defined + and – population).
- D. The sorter is inside a BL2 room (CNY 149, room 5555), which requires keycard access to enter the anteroom. If you would like access so that you can get to the sorter room without having to call the sorter room phone, please send me your employee ID number (on the back of your MGH ID card) or your Unit number (also known as your Blue Card number) and I will notify security to grant you access.

II. All sorting is done on a FACSAria IIu in a biosafety cabinet:

- A. All sorts are run by core personnel, there is no user run sorting.
- B. The Aria can detect 12 colors simultaneously, according to the chart below. We are able to change the filter setup, so if there is a reagent that you wish to use that is not in the list, let us know.
- C. It is possible to sort up to 4 populations at the same time into 12x75 mm tubes or eppendorf tubes, or 2 populations into 15 ml tubes.
- D. At the maximum flow rate (11), run time is around 7 minutes per 1 ml of sample (but note that this is a dilute sample, and a limit of 20k events/sec still applies—if you have 20 million/ml, cell rate will be over 20,000 per second at a flow rate of 3, which takes >20 minutes per ml).

- E. The Aria has an automatic sample line backflush mode, so manual cleaning (running bleach to flush the lines) between samples is not always necessary (carryover <0.1%), but can be performed if you prefer, and is recommended if you will be culturing the cells for an extended period of time. This will add 2 to 3 minutes per sample, so if you have multiple samples this can add up!
- F. If your cells are fragile, sorting can be done at slower speeds. If they are large (>30um), sorting can be done with a bigger nozzle (which also runs slower). Sample temperature can be set between 4 to 42 C (4C is the default) and collection tubes may be chilled upon request.
- G. LABEL TUBES WITH AN ALCOHOL-RESISTANT MARKER: due to aerosol generation, sample collection tubes and sample tubes must be wiped down with ethanol after a sort—if the marker wipes away, tubes have to be relabeled and risk of mixing them up is greater. Sharpies=Bad, Fisher or VWR marking pens=good.

The sorter configuration is:

	Fluorochrome	Detector	LP	BP
488 nm	PE-Cy7	Blue A	735	780/60
20 mW	PE-Cy5-5, PerCP-Cy5-5	Blue B	680	695/40
	PE-Cy5, PerCP, AO(RNA), 7AAD, mCherry	Blue C	635	675/20
	PE-Texas Red	Blue D	595	610/20
	PE, PI, DII, DsRed	Blue E	556	576/26
	FITC, GFP, AO (DNA), Alexa 488, YFP, Chameleon Yellow	Blue F	502	530/30
	SSC	Blue G		488/10

633 nm	APC-Cy7, APC-Alexa 750	Red A	735	780/60
25 mW	Alexa 700	Red B	685	720/40
	APC, Alexa 647, Sytox Red	Red C		660/20

405 nm	Pacific Orange, Alexa 430, Cascade Yellow	Violet A	535	560/40
	50 mW AmCyan, CFP	Violet B	480	500/20
	Pacific Blue, Alexa 405, Cascade Blue, Marina Blue, Cerulean	Violet C		450/40

III. For ALL sorts:

- A. The rate for sorting is \$100 per hour for all users.
 - i. Payment is usually done by fund transfer, so you must provide an MGH fund/grant number.
 - ii. Outside users can pay by check payable to “The General Hospital Corporation”
- B. If you are going to be more than half an hour late, please send me an email and let us know! We usually have a full schedule, so if you are late we

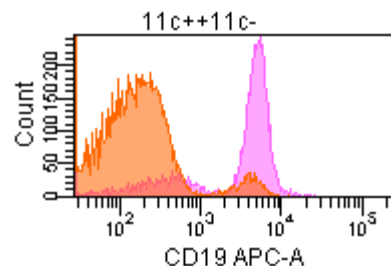
usually still have to stop at our scheduled finish time and may not be able to run the entire sample.

- C. Due to various factors (counting before staining, cell clumping, sample left over in tube that cannot be sorted) it has been found that sorters may count as few as half of the cells that the researcher reports. Keep this in mind. This factor will be reduced if you count your cells as a final step.
- D. Please do the math for your experiment! Calculate the number of cells you need to get back, the percentage of cells that fit your sorting requirements, and how many cells you will need to get that number. Then double that and that is how many cells you will need in order to get the cell number you want back. The maximum event rate is around 20,000 per second (or 60 million per hour) but keep in mind:
 - a. the frequency of your target cells affects how fast the sample can be run—if the population is low frequency (5% or less), then the sample cannot be run at full speed and total sorting time will be greater (twice as long or more).
 - b. the “event rate” also counts debris and dead cells, so if you have a “dirty” prep, then the actual event rate for your cells will be lower than maximum.
 - c. I can use “threshold” to block out some of the debris (the instrument basically ignores those events) but if they are ignored, they could end up in your sorted sample, which shouldn’t matter for cells put into culture, but may affect other assays. The debris is still there, so in extreme cases there may be enough to clog the instrument when running at high event rates.

For practical purposes, use 50 million per hour as the rate for calculating how long the sort will take (plus 15 minutes for setup/optimization).

- E. Cells should be provided preferably in PBS plus 2% FCS, but can also be run in media with NO MORE than 5% serum. No BSA should be in the sort sample, as it may cause high background fluorescence. You can use 12x75mm FACS tubes (polystyrene or polypropylene) or 15 ml conical tubes or 1 ml microtubes (Bio-Rad Laboratories, #223-9391, 1000 tubes per box) for your samples. Cells should be at a maximum of 20 million per ml, or a minimum volume of 200 ul.
- F. Proper set up of the instrument requires control samples. Included should be an unstained sample (should be GFP negative also, if applicable), and single stained controls for each color used (if experiment involves GFP, other control samples should be GFP-). Please make sure there are enough cells in the controls! Minimum 50k, but 100k (or more) better.
 - 1. You usually do not have to use your sample of interest for the controls if you have another source of the same cell type (lymphocytes from a different patient as controls for a patient of interest, for instance).
 - 2. Be sure to use a reagent that will give a BRIGHT, >5% positive population for the sample you are staining. It must be as bright or brighter than the reagent in your sample. A single stain control that only labels 0.1% of the cells, or is very dim, will **not** be useful in setting up the experiment! Antibody capture beads may be used instead.

- G. Bring COLLECTION TUBES to sort into. FACS tubes (12x75mm), eppendorf tubes, cryovials, 15 ml conical tubes, or almost any type of tissue culture plate may be used. It is a good idea to bring extras of your collection tubes so that I can use them to set up the instrument in case they are a different size than the tubes/plates I have in the lab, and also as backups in case a clog causes contamination of a collection tube in use. With plates, you may want to verify that they will fit on the instrument so check BEFORE the day of your sort.
- H. Each sorted cell is in a droplet of PBS. Depending on sorting mode, 1 million sorted events will have a volume of between 1 to 2 ml of mostly PBS (depends on if you sort 1 drop or 2 per event, 2 drops giving you better yield). If you are sorting more than 1 million events, a 15 ml conical with 8-10 ml of media is recommended. Smaller sorts can be into FACS tubes with 2.5 ml of media (use the numbers to calculate the volume for your sample). Some media types will form a precipitate when mixed with sufficient volumes of PBS, so the final mix of PBS to media after sorting should be less than 1:2. (the presence of serum will help dissipate charge, prevent cells sticking to the tube, and improve yield) or some other collection media (Trizol, PBS, etc). *Polypropylene* tubes may improve yield (fewer cells stick to walls).
- I. 96 well plate (or other plate) can also be used for cloning (or sorting a number of cells into a well so that no transfer is necessary). Please fill the wells at least halfway to improve viability of sorted cells (otherwise they may hit the bottom of the well and die). If sorted volume is a concern (will be mostly PBS), there are settings to reduce the drops but may also decrease yield.
- J. Bring extra collection media and dilution media (if other than PBS) in case a sort tube becomes contaminated or the sample needs to be diluted.
- K. Samples must be transported using a sealable secondary container. If you use filter cap tubes to filter your samples, be sure to replace the filter cap with a sealed cap to prevent spills in the event of an accident in transport. All caps should be securely fastened.
- L. For ALL sorts collecting multiple populations, make sure your target populations are exclusive, otherwise define the gating that will make them so. For example, if you are sorting 2 single positive populations, view them on the same plot to make sure there are no double positive events, or gate on the negatives of the first population to select the positives in the next. In the example below, events are displayed on a CD19 histogram and broken down into CD11c+ (pink) and CD11c- (orange)--you can see that the bulk of CD19+ events are CD11c+ also--your yield for your population depends on which you choose first.



- M. Groups are limited to 2 sorts and 6 cumulative hours per week. If it is necessary to cancel a sort, please do so as soon as possible. Any cancellations

within 1 hour of the scheduled start time will still incur a charge for setup time.

- N. No more than 4 occupants in the sorter room at any time, and all occupants must wear a disposable gown and gloves.
- O. Please leave all coats and bags either in the anteroom or near the analysis stations in 5233—bring only your transport container into the sorter room.

IV. PIBC approval required for all human samples

According to Boston and Cambridge city regulations, all work with human samples or human pathogens must be registered with an Institutional Biosafety Committee (IBC), which for MGH is the Partners Institutional Biosafety Committee, or PIBC (it was the Committee on Microbiological Safety, or COMS until Dec 2011) for approval of the work and the protocols to be performed. The PIBC website is http://resadmin.partners.org/RM_Home/Research_Support_Depts/Research_Oversight/PIBC/PartnersPIBC.aspx

--If you already have approval for cell sorting in your current application, your PIBC number will have to be provided on the sort request forms that you fill out to schedule a sort with the Ragon Institute's sorting facility. The number should have the following format: 2011B000001

--If you or your PI is already PIBC registered but sorting by flow cytometry was not covered in the original application, then an amendment will need to be submitted for approval to add this to your project

--If for some reason you are not registered then a new application is required.

Sorting of fixed cells or of non-human non-primate cells does not need to be registered. All unfixed human cells, including human cells transplanted into mice, would need to be registered.

All pathogens, animal or human, would also need to be registered. Please provide a description of the signs and symptoms of infection in the event of an accidental exposure.

To look up your new number:

Projects that have a COMS number MUST provide the updated PIBC numbers which look like: 2011B000001. To find out the new number, you must be the PI on the protocol or listed as the alternate, and go to the PIBC website:

http://resadmin.partners.org/RM_Home/Research_Support_Depts/Research_Oversight/PIBC/PartnersPIBC.aspx

click on "eIBC/Insight Research Portal" in the box on the right side of the page, and log in with your partners username and password. It will bring up the modules linked to your profile, click on "go to Biosafety" and it should list any protocols associated with your profile, and list the COMS and PIBC numbers.

If you are using human samples, you must include your PIBC number. For the Walker lab/Ragon Institute, this is 2011B000799 (formerly 09-175) or the other infectious protocol 96-111 is now 2011B000507. If you do not have or do not know your PIBC #,

please contact Anne Sallee, the MGH Biosafety Officer:

Ms. Anne Sallee
Environmental Health & Safety
West End House
16 Blossom Street
Boston, Massachusetts 02114
E-mail: ASallee@partners.org
Phone: 617-724-4579
Fax: 617-726-5126

BWH PI's are registered through Harvard's IBC, so Anne can also be contacted for questions from those users. Other places outside of the Harvard Affiliated Hospital/Institutions should have their own IBC.

V. Tips for Specific Sorts

--Sca+/Lin- samples: To properly set the gates for Lin-, it is preferable to have a sample of undepleted cells stained with the Lin cocktail and then with the proper fluorochrome.

--GFP samples: Dim + events may overlap with bright autofluorescent events in FL1, which can usually be resolved by looking at a bivariate plot of FL1 vs. FL2—GFP is often shifted higher on FL1 vs. autofluorescence, which will be seen on the 45 degree angle if initial setup is done properly.

--I have different modes available to sort with, which affect purity and recovery of your sorted sample. Speak with me if you have any specific requirements.

--Nozzle size is 70 um, optimum for around 20 um particles or smaller. If your cells are larger, I may have to use larger nozzle, which means slower sort rates.

--96 well sorting is possible, survival/colony formation dependent on cell type. Cell line clone formation usually around 50%.

--It is a good idea for plates to bring me a sample of the ones you use to make sure they will fit on our sorter. Some of them are too tall to fit.

--It has been observed with monocytes (FSC/SSC) and DC's (fluors) that the population will decline after running for a minute or two. Adding EDTA (1uM) will keep the cells from sticking together or to the tubing, and events will not decrease.

1/31/12