



3. Cell Sorting FAQ

Q1. When should I use fluorescence-activated cell sorting (FACS) over bulk separation methods like density gradient centrifugation, panning or magnetic bead separations?

- When very high purity (above 95%) of the target population is required.
- For separations on the basis of single/multi-color staining with fluorescent dyes or fluorophore-labeled antibodies.
- For separations on the basis of internal staining, e.g. of DNA or of internal antigens.
- For separation of populations that have a low density of receptors on their surface, to which fluorophore-labeled antibodies can be bound.
- For enrichment of populations on the basis of surface receptor density.

Q2. How large cells can I sort?

BD FACSAria Fusion is a sorter, equipped with a 70,85,100 and 130 μm nozzles. Generally, the diameter of the cells to be sorted should be at least 5 fold smaller than the nozzle size.

Q3. How many cells do I need for the sorting procedure?

In order to answer this, the following information is required:

1. How big is the population to be sorted (~percentage)?
2. How many cells do you require post sorting?
3. What is more important: high purity or total yield?
4. How fragile or how large are the cells?

The duration of the sorting procedure depends on all these parameters. Here is an example:

How many cells do I need to prepare to recover 1×10^6 of a population that comprises 10% of the cells?

$1 \times 10^6 = 0.1 [10\% \text{ target population}] \times 10 \times 10^6$ starting cell number. However, the actual recovery is usually around 80–90% of this theoretical value. Therefore, the starting cell number should be $10 \times 10^6 \times 100 / [80-90] \approx 11.0-12.5 \times 10^6$. However, the actual yield is significantly lower if the cells are of poor quality (low vitality, clumping, etc.) or if you demand high purity.

Also remember that we will consume some cells in setting up the sort. If this is the first time you are sorting this particular sample, we may need more cells initially than we will in subsequent sorts.

The actual percentages of cells that are recovered from a given sort depend on a multitude of factors:

- The quality of the cells to be sorted has the largest influence to the actual yield. In order to receive good sorting results, such as purity and yield, the cells must be clump-free and consist of a single cell suspension.
- Cell death that occurs pre- and post-sort and loss through adherence of cells to tube walls.
- Sort rate; the higher the sort rate the lower the recovery.
- Precision of sort set up.
- If it is an enrichment sort or a purity sort. Enrichment sorts have higher recovery than purity sorts.

Also, remember that there are often losses in the cell preparation.

When you arrive, and tell us you brought 1×10^7 cells this should be the number you determined by counting after all cell preparation including filtration to remove clumps or an estimate if we will do the filtering. The sorters are very good at counting the cells that go through the instrument. Therefore, if you tell us you brought 1×10^7 cells and the sorter sees only 0.6×10^7 , you did not deliver us 1×10^7 .

Remember that some sorted cells will be used to determine the effectiveness of the sort. In cases, where the yield



is extremely low we may waive this. This post-sort analysis is **essential data if you expect to publish** the results of your sort experiment!

Therefore, we recommend using at least 50% more cells than required by the theoretical value for the sorting procedure.

Q4. What % of the theoretical number of cells can be recovered in reality and what does it depend on? Generally, at least 50% of the theoretical number of cells can be expected to be recovered, most of the time the efficiency of sorting exceeds that percentage. The losses are caused by factors that have already been discussed in the answer to the question **Q3**.

Q5. Are there ways to improve sort recovery?

Yes:

- Use polypropylene tubes for cell processing and sorting.
- Use polypropylene collection tubes and fill them (~1/2 mL) with media (containing 30% serum, for eukaryotic cells).
- Count cells immediately prior to sorting (after all washes).
- Use “Enrich” sort mode to improve yield (nb. Purity will decrease!).
- Process collection tubes immediately after they are filled up or the sort is finished.

Q6. How many cells per second can be sorted on a BD FACSAria Fusion sorter?

Maximum event acquisition rate of the instrument is ~25,000 events per second. For certain cell preparations lower event acquisition rates such as 5,000 events per second or below can be advisable to obtain higher purity.

Q7. How long does the sorting procedure take?

Approximately 1 hour every working day are necessary for the operator to set up the instrument, stabilize the flow and perform the necessary QCs. Follow 15 minutes for setting sort regions for the cells, and about 15 minutes of post-sort analysis. Sorting time depends mainly on how many cells need to be analyzed and sorted. Theoretically BD FACSAria Fusion it can process up to 30 million events/hour.

The duration of the sorting procedure itself depends also on several parameters:

- On the quality of the cells.
- How big is the population to be sorted (~percentage)?
- How many cells do you require post sorting?
- What is more important: high purity or total yield?
- On the concentration of the cells. The time for sorting procedure depends on the dilution of the cells; the more diluted the cells are, the longer it takes. For diluted samples, the volume is also a factor that should be considered when estimating the time needed for sorting: usually, **1 mL of sample (30 million cells) can be processed in one hour.**

Q8. Will my cells be harmed by the sorting process?

We underline that Influence of the pre-sort cell staining toxicity on cell viability should be evaluated before the cell sorting experiments. Generally, cells might be have also mechanical sort stress during sorting process, but it could be minimized by maintaining cells continually at a temperature, pH and in media that is most suited to them. BD FACSAria Fusion is equipped with a 100 µm nozzle and can be operated with lower pressure than with smaller nozzles. This automatically reduces mechanical sort stress.

For temperature, the user has different options:

- **To defined temperature chamber**



- To define temperature of sorting collection tubes

Q9. How do I assure cell viability during the sort, and are there any ways of improving it?

Samples should be held in a rich medium that is most suitable to them. For eukaryotic cells, the addition of **serum** proteins is advisable, but should not exceed a concentration of 2–3% because the sort stream precision is detrimentally affected by high serum content in the sample to be sorted. For eukaryotic cell lines and adherent cells, the addition of 0.5 mM **EDTA** is recommended. The concentration of EDTA can be raised up to 5mM for extremely sticky cells. For sterile sorting, the addition of **antibiotics** is recommended. Moreover, the addition of 25mM **HEPES** pH 7.0 could be useful.

The collection tubes should contain a high serum concentration (30 %), as this concentration is diluted by sheath fluid from the sort droplets. Processing of the sorted cells as soon as possible after the completion of the sort helps to maintain cell viability.

Q10. What is the maximum purity of a population that can be achieved and what does it depend on?

Maximum purity is 99% to 100%. 95% to 100% purity can be expected for populations that are well resolved from the unwanted cells. It is dependent on sorter stability and the way sort gates are set. These gates should always be set with the investigator present to help make decisions. The purity of not well defined regions or for enrichment sorting might be less than 90 %.

Q11. How is the purity of the sorted population determined?

By re-analysis of the sorted population(s) on the cell sorter or another flow cytometer; for the second options, be sure that:

- the sensibility of the two instruments is the same.
- you acquire labeled cells for the sorting before the sorting on the other flow cytometry

Q12. How do the cells have to be resuspended for the sorting procedure?

Cell concentration. If you have too few cells, then the sort will take longer than necessary compromising viability. Too many cells can cause reduced purity and more chance of instrument blockages. An optimal cell preparation increases not only the vitality but also leads to a better yield. Here are some guidelines:

If you have fewer than 10×10^6 cells put them into a volume of 0.5 mL. Sample concentration for a BD FACSAria Fusion should be **30 million/mL** depending on the cell type. We can dilute the cells if they are too concentrated, but for practical reasons we will not concentrate them. If you are uncertain, we recommend bringing the cells in a concentration that is rather too high than too low since they can always be diluted. Count cells immediately prior to sorting (after all washes).

Cell resuspension buffer. In principle, any physiologic buffer may be used to resuspend the cells to be sorted. It is essential to have the cells well dissociated and to prevent clumping in order to achieve good recovery rates and to prevent clogging of the nozzle.

For prokaryotic cells, we recommend suspending them in PBS (phosphate buffered saline).

For eukaryotic cells, we recommend suspending them in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer, such as PBS (phosphate buffered saline) or HBSS (Hank's balanced salt solution) with low protein content ($\leq 2\%$ FBS or BSA). Note that standard eukaryotic cell culture media contain Ca^{2+} , Mg^{2+} and proteins from serum that favor cell clumping. In addition, many cells benefit from proteins in the buffer, for example 2% FBS or BSA. Please bear in mind that a serum concentration higher than 5% can lead to cell aggregation and clogging of the machine.

For eukaryotic cell lines and adherent cells, the addition of 0.5 mM EDTA is recommended. The concentration of EDTA can be raised up to 5mM for extremely sticky cells.

In cell suspensions with a high rate of dead cells the free solved DNA can cover the cells and lead to serious clumping of the cells. Here, an addition of DNase II (20–100 $\mu\text{g}/\text{mL}$; 10 U/mL) to the cell resuspension buffer may



help.

For pH-sensitive cells, the addition of HEPES (10–25 mM) is recommended, because it buffers the shifting of the pH value caused by the high pressure during the sorting procedure. Moreover, the addition of antibiotics is recommended for aseptic sorting

Sample volume. Recommended minimum sample volume is 0.4 mL/0.5 mL. Maximum 4 mL of sample can be processed in one run.

Q13. What needs to be observed when preparing the cells?

The cells must be clump-free and consist of a single cell suspension. Therefore, for example, large (eukaryotic) cells have to be filtered, ideally immediately before the sorting procedure, using a 20–50 µm cell strainers.

Q14. Which sample tubes do I need for sorting of the cells?

Sterile 5 mL FACS tubes are required: 5 mL Polypropylene Round-Bottom Tubes 12×75 mm style, sterile, with caps.

Other collection tubes can be prepared, if necessary.

Q15. How should I transport the cells?

We recommend transporting the cells on ice or at room temperature (on the basis of type cell) and protected from light.

Q16. What kind of tubes are used for the collection of the cells?

On BD FACSAria Fusion maximum four cell populations can be sorted in one pass (4 way sorting) in FACS tubes (5 mL Polypropylene Round-Bottom Tubes 12×75 mm style, sterile) or 15 mL Falcon conical tubes, or Eppendorf 1.5 mL. Please bring labeled collection tubes with approximately 1/2 mL of cell culture medium. For eukaryotic cells, FBS 100% may be added in collection tubes for a overnight coating to help keep the viability rates of sorted cells high. Sterile 5 mL FACS polypropylene tubes are required also for post-sort acquisition controls.

BD FACSAria Fusion has a support for collecting single cells in 96-(and 384-)well plates.

Q17. Which fluorochromes can be measured?

It has to be clarified if fluorescent proteins can be at all measured on the flow cytometer. Not all fluorochromes displayed by microscope can also be measured at the flow cytometer. This has to be checked beforehand.

So-called Spectrum Viewers are available on the web offered by different companies (e.g. <http://www.bdbiosciences.com/us/s/spectrumviewer>; <https://www.thermo-fisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectra-viewer.html>).

The configuration of the BD FACSAria Fusion:



	Excitation	Position	Fluorochromes	Filters	LP
laser blu	488 nm	B	FITC	530/30	502
		D	EMPTY		
		F	EMPTY		
		H	EMPTY		
		A	PERCP_Cy5.5/BRILLIANT BLU 700	685/40	655
		C	SSC	488/10	
		E	EMPTY		
laser giallo verde	561 nm	B	PE-Cy5.5	710/50	685
		D	PE	582/15	
		F	EMPTY		
		H	EMPTY		
		A	PE-Cy7	780/60	735
		C	PE-CF594	610/20	600
		E	EMPTY		
laser violetto	405 nm	B	BV711	710/50	690
		D	BV605	610/20	595
		F	BV421/PACIFIC BLU/V450	450/50	
		H	EMPTY		
		A	BV785	780/60	750
		C	BV650	660/20	630
		E	BV510/AMCYAN/V500	525/50	505
laser rosso	640 nm	A	APC-Cy7/APC-H7	780/60	755
		C	APC/ alexa 647	670/30	
		B	APC-Alexa 700/ R700/Alexa fluor 700	730/45	690
laser uv	640 nm	A	BUV 737	740/35	690
		C	BUV 395	379/28	
		B	BUV 496	515/30	450

Q18. Is it possible to sort single cells into microtiter plates?

Yes. The BD FACSAria Fusion cell sorter is capable of plate sorting. However, the sort set-up differs from bulk sorting, requiring extra time and advance notice.

Q19. What is index sorting?

Index sorting is a high content flow cytometric record of each individual cell that is sorted. This feature provides the phenotype information of every cell sorted into a multi-position sort device, such as a 96-well plate. This information includes light scatter and fluorescence channel MFI (median fluorescence intensity) values. The index sorting feature helps track the cell that is being sorted. It links the cell phenotype information with the cell gene expression profile, if analyzed post-sort.

Q20. How many parameters can be used simultaneously for sort decisions on the BDFACSAria Fusion? The maximum number of parameters currently available is 18: forward scatter and side scatter plus 16 fluorescent parameters.

Q22. What kind of controls should I bring?

The following controls are recommended for flow cytometry experiments:

1. Unstained cells as a negative control.
2. Unstimulated/untreated cells as a biological control.
3. Single-stained cells or beads as compensation controls.
4. Isotype or FMO control
5. If you are using a dead cell exclusion dye, you should also bring a separate viability control for correct gating, as dead cells cannot always be excluded by their light scatter characteristics. We recommend killing your cells and



mixing with live cells followed by staining with your viability dye (e.g. PI, 7-AAD, DAPI, TO-PRO-3).

Q22. Why should I remove dead cells in eukaryotic cell sortings?

Dead cells can be a significant problem with cell separation. Generally, if there are more than 10% dead cells in a given suspension, then steps should be taken to remove them. Below this level, their effect on subsequent separation is, while not ideal, usually acceptable. The reason for the need to remove dead cells from suspension is twofold: (a) dead cells release DNA that aggregates cells and (b) dead cells can bind non-specifically to antibodies leading to false positives. The first problem can be resolved by the addition of DNase I, reducing the potential for aggregation. In the second case, the problem of dead cells is exacerbated in the case of antibody-mediated cell separation because non-target dead cells will be labelled along with the target population leading to a reduction in the purity of the isolated cells. A more effective method is to remove the dead or dying cells completely with a pre-separation step.

Q23. Is the sorting procedure performed under sterile conditions?

The BD FACSARIA Fusion cell sorter is not encased in a class II biosafety cabinet. Due to the nature of sorting, no sort can be defined as “sterile”. Normally, every sort is accomplished aseptically regardless of what the user is doing with the cells. The sheath fluid passes through a 0.22 µm filter before it goes into the instrument. Moreover, the sorter is regularly (a working day before the sorting and after sorting experiments) decontaminated with FACSClean™ solution.

You need to ensure you prepare your sort samples in sterile conditions (a small aliquot of a stained pre-sort population is advisable to be kept as a sample pre-sort sterility control) and ensure that all material (FACS Sheath buffer/ collection media/ stains/ antibodies/ tubes/ pipette tips) you prepare are sterile! For the sorted eukaryotic cells, the addition of antibiotics to the medium, however, is recommended.

Q24. What other preparations are required?

Before the cells can be sorted, they must be characterized (by flow cytometry or by fluorescence-microscope). Please, bring a recently dated print out with you the first time you come for a personal meeting. The completed “Flow Cytometry Sort Request Form” has to be brought along as well. Please, come on time!

Q25. Data management of the CPT FACS-service

It is the responsibility of all users to copy their data to a secondary, non-FACS-service, medium at the time of collection and/or analysis, as the FACS-service is still not equipped with a server for data backups.

Q26. Billing policy for the CPT FACS-service

The cost of the experiment will be strictly dependent on complexity of the samples (charges are available on CPT website). For external users, standard research contracts will be stipulated with CPT.

Q27. Acknowledgements

To promote the FACS-service, please acknowledge it in all publications and grant applications where the BD FACSARIA Fusion cell sorter was used and expertise, provided by our staff, contributed to the final work product. Please, send us a pdf of your newly accepted papers if they contain data generated by the FACS-service. Also, please let us know if your grants that contain FACS-service-generated data are awarded.