Barcode Enabled Antigen Mapping (BEAM) Flow Cytometry Guidelines

Introduction

The Barcode Enabled Antigen Mapping (BEAM)-Ab and BEAM-T solutions provide a comprehensive, scalable BCR and TCR discovery solution, respectively. In this assay, user supplied antigens are tagged using 10x Genomics BEAM reagents. Tagged antigens are used to label B or T cells, followed by fluorescence-activated cell sorting for enrichment. This Technical Note provides general guidance on cell sorting, as well as specific tips and best practices for optimizing sample enrichment for the BEAM workflow.

Fluorescence-activated cell sorting is a flow cytometry technique that uses fluorescent markers to identify and sort specific cells. In this method, a cell suspension is loaded onto the cell sorter, where it is aspirated into a rapidly flowing stream of liquid. Vibrations within the machine cause the stream of liquid to break into individual droplets, which is calibrated so that the probability of a droplet containing multiple cells is very low. These droplets are passed through a laser, which excites any fluorophores associated with the cell in the droplet (Figure 1). Based on this signal, the sorter utilizes an electrical charge applied to the droplet to deflect the droplet into a downstream container, which now contains a purified subset of the initial sample.

Several parameters during flow sorting can be adjusted including the size of the nozzle, flow rate, the type of the sheath fluid, and the collection buffer. Additionally, the chosen flow sorter should have enough lasers to support the BEAM assay. The next several sections will provide recommendations on these parameters with respect to the BEAM assay.

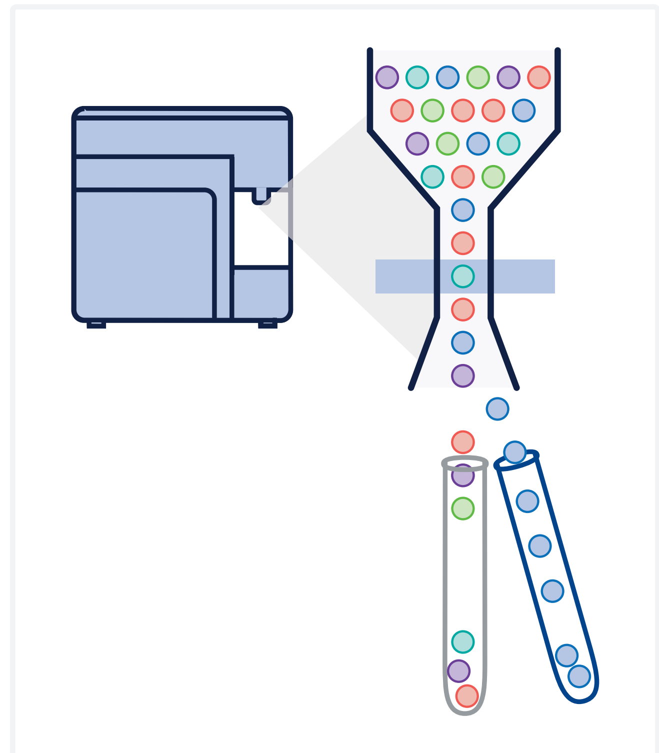

Figure 1. Flow sorting schematic. Cells are passed through a laser on the instrument and based on the excitation/emission of fluorophores on the cell surface, the sample is deflected into tubes for collection.

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Flow Sorter Parameters

Nozzle Selection

Nozzles with mild pressure help preserve cell viability and integrity, enabling optimum sample input for downstream single cell RNA sequencing (scRNA-seq) assays. The larger the nozzle size, the lower the pressure. Typical cell sorting guidance dictates that the nozzle should be at least three times larger than the average cell size; however, for the BEAM assay, larger nozzles (e.g. 100 µM) with gentler pressure are recommended.

Droplet Volume

Droplet volume may vary from sorter to sorter and should be determined prior to performing the BEAM assay. This may be done by sorting a known number of events into an empty tube, using the same sort mode and flow rate parameters as will be performed on the actual experiment. The resulting volume in the tube can be used to determine the per-droplet volume. The per-droplet volume can be used to predict the final volume that will be obtained after sorting a desired number of cells.

Flow Rate

Flow rate, defined as the volume of fluid per second flowing through the sorter, can be adjusted to influence the sample injection area width. For example, an increase in flow rate will widen the sample injection area and increase the number of droplets containing multiple cells, which will impact data quality.

Optimal flow rates will maintain sorting efficiency above 90%. If BEAM-T or BEAM-Ab positivity is less than 1%, a higher efficiency may be required. Table 1 demonstrates how the number of BEAM$^+$ events impacts the final sorted number of cells.

BEAM$^+$ Events	Target Sort Efficiency	Total Number of Cells	Sorted Number of Cells
0.1 %	>90%	10,000,000	9,000
10%	>90%	1,000,000	90,000

Table 1. Sorted number of cells given a desired percentage of BEAM$^+$ events.

Sheath Fluid

Sheath fluid is the liquid that flows through the flow sorter. Cells are injected into the sheath fluid, which carries it past the laser. Sheath fluids should not contain EDTA or excessive amounts of magnesium (less than 0.1 mM EDTA and less than 3 mM magensium).

Collection Buffer

5-20% FBS in PBS collection buffer is suitable for sorting up to 500,000 cells; however, if sorting will take longer than 30 minutes or if the cells are fragile, 100% FBS may be used. If more than 500,000 cells will be sorted, use 100% FBS.

If more than 50,000 cells will be sorted, they can be concentrated for counting and loading in a centrifuge chilled to **4°C**. Centrifugation time and speed should be optimized for each sample type. Centrifugation for **5 min** at **300 rcf** and **10 min** at **150 rcf** have both been tested by 10x Genomics. The sample can then be resuspended for loading in 5% FBS in PBS.

Lasers

The chosen cell sorter should include, a minimum, the following lasers:

- Blue (488 nm) - used to detect forward scatter, side scatter, and fluorophores such as FITC. This laser is also used to detect live/dead dyes such as 7-AAD.
- Yellow/Green (561 nm) - used to excite the the PE fluorophore on the BEAM reagent.
- Violet (405 nm) or red (647 nm) - additional lasers will ensure that other cell type specific markers can be gated for/against.

In addition to labeling cells with BEAM reagents, cells should be labeled with additional markers to optimize sorting. The next section will discuss recommended panel composition for BEAM samples.

Panel Design

Flow sorters are able to isolate specific populations of cells based on fluorescent markers. Tables 2-5 show example panels for both BEAM-Ab and BEAM-T. Marker panels for sample labeling should contain the following components:

Markers for Desired Cells: Antibodies specifically labeling T cells (BEAM-T) or B cells (BEAM-Ab) should be included.

Markers for Undesired Cell Lineages: Markers for cells that are not desired should be included so that these cell populations can be excluded during sorting.

Live Dead Marker: These markers selectively label dead cells, allowing the sorter to distinguish between live and dead cell populations. They function by entering dead cells (whose cell membranes are compromised) and binding to DNA or free amine groups. The BEAM assay was validated with 7-AAD.

Marker	Target Cell Type
CD3 (+)	T Cells
CD8 (+)	CD8 Cytotoxic T Cells
CD56 (-)	Natural Killer Cells
7-AAD	Dead Cells

Table 2. Example human BEAM-T panel. 7-AAD is added separately just before sorting.

Marker	Target Cell Type
CD3 (+)	T Cells
CD8 (+)	CD8 Cytotoxic T Cells
Ter119 (-)	Erythroid Cells
Ly6g (-)	Myeloid Cells
CD19 (-)	B Cells
CD14 (-)	Dendritic Cells
7-AAD	Dead Cells

Table 3. Example mouse BEAM-T panel. 7-AAD is added separately just before sorting.

Marker	Target Cell Type
CD19 (+)	B Cells
CD56 (-)	Natural Killer Cells
CD3 (-)	T Cells
CD14 (-)	Monocytes
7-AAD	Dead Cells

Table 4. Example human BEAM-Ab panel. 7-AAD is added separately just before sorting.

Marker	Target Cell Type
CD19 (+)	B Cells
Ter119 (-)	Erythroid Cells
Ly6g (-)	Myeloid Cells
CD3 (-)	T Cells
CD14 (-)	Dendritic Cells
7-AAD	Dead Cells

Table 5. Example mouse BEAM-Ab panel. 7-AAD is added separately just before sorting.

Panel Design Best Practices

When designing a labeling panel for the BEAM assay, consider the following:

Cell Sorter Laser: Determine the lasers and number of detectors available on the chosen cell sorter to determine possible fluorophores. At minimum, a yellow/green laser that can detect 561 nm (emission) is necessary for the PE marker on the BEAM reagent.

Compensation Controls: When using multiple fluorophores in one experiment, consider that the spectral overlap between fluorophores (sometimes called "spillover") will need to be corrected. To determine the true contribution of each fluorophore to the signal in a given detector, compensation must be performed.

- Use compensation beads (i.e. UltraComp eBeads Compensation Beads, 01-2222-41, Thermo Fisher Scientific) labeled separately with each antibody that will be used in the sort. If multiple antibodies labeled with the same fluorophore will be used in the sort, one antibody can be selected for preparing the compensation control for that fluorophore.
- For the PE compensation control, a PE-conjugated antibody, such as PE-anti biotin, may be used to label the compensation beads
- For a live/dead control, use a sample rich in dead cells labeled with the live/dead dye. A sample rich in dead cells can be generated by heating a sample of cells at 65°C for 5-10 min.
- Compensation controls should be made fresh on the day of the experiment and apply the compensation matrix to the sample of interest.

Fluorophore Brightness: If the number of proteins the chosen antibodies are targeting are abundant, choose a dimmer fluorophore. If the number of target proteins is not abundant, choose a bright fluorophore.

After the panel is designed, samples can be labeled according to the Reagent Assembly, Sample Labeling, & Flow Sorting for Barcode Enabled Antigen Mapping (BEAM) User Guide (CG000595) in preparation for the sort. The next sections provide guidelines for executing the sort.

Cell Sorting Best Practices

Before sorting BEAM samples, consider the following:

- If applicable, consult the flow cytometer operator about lead time for executing a sort.
- Pre-screen control samples labeled with the desired reagents on the same flow cytometer that will be used for the actual sort.
- Ensure that the proper controls are included:
  - Compensation
  - Live/dead
  - Fluorescence minus one (FMO). FMO controls are samples that are labeled with all fluorophores in the panel except for one. These controls are used to determine the maximum background signal for the missing fluorophore. For the BEAM assay, prepare an FMO control that contains all fluorophores except the PE fluorophore or the BEAM conjugate. This can be achieved by labeling cells with the BEAM-T or BEAM-Ab Antibody Panel, without the BEAM Assembly.

Sorting Modes: Cell sorters typically have a variety of sort precision modes available. Select a high purity mode. It is not necessary to use a "single cell" mode, as this will greatly reduce yield.

Sorting Efficiency: Maintaining a sorting efficiency of above 90% is critical for obtaining a pure target population and reducing contamination. The following recommendations will help improve efficiency:

- Flow rate should be low to medium to reduce the number of collinear events
- Improving sample preparation for debris-rich samples (filtering, buffer optimization, etc.) can minimize the amount of debris present during the sort.
- Ensure the gating strategy used, discussed in the next section, does not have any logical flaws.

Sorting Temperature: Keep cells on ice and in the dark prior to sorting. During sorting, the collection tube and sample should be chilled. Place the sorted cells on ice immediately after sorting.

Gating Strategy

Figures 2-5 show typical gating strategies when isolating samples of interest. Figures 6-7 show example sorting strategies for BEAM-Ab or BEAM-T samples.

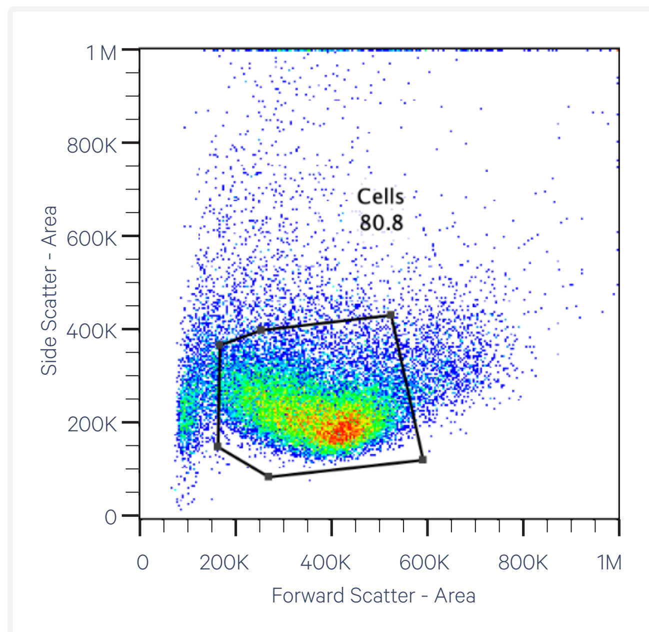

Figure 2. Debris exclusion. When forward scatter (FSC) and side scatter (SSC) are plotted against each other, draw a gate around the area with the highest number of events.

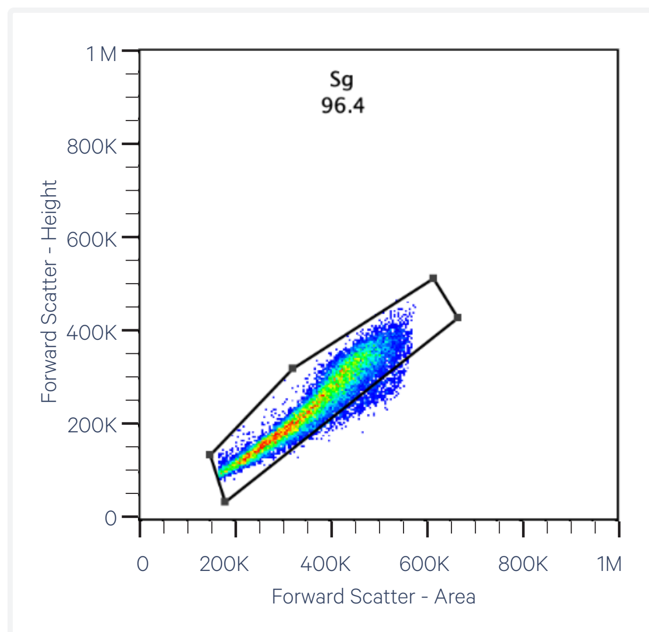

Figure 3. Doublet exclusion. Plotting height vs area for forward scatter allows for double discrimination - draw a gate around the events that show equivalent signal for both parameters.

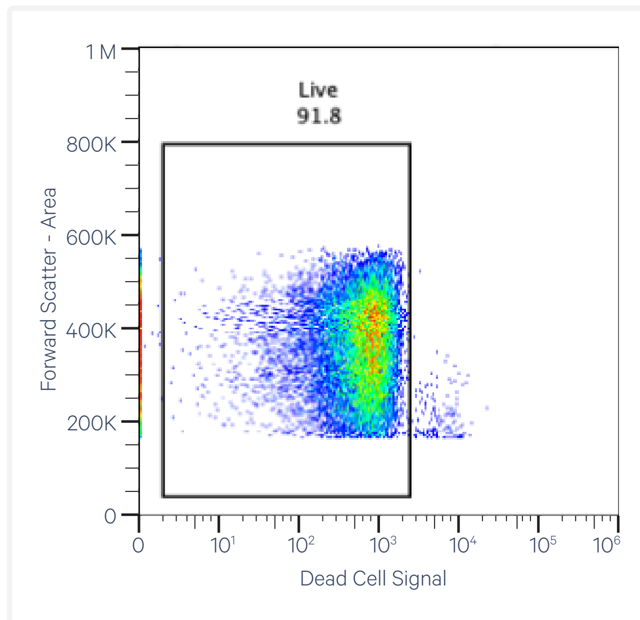

Figure 4. Dead cell exclusion. Plot forward scatter against the live/dead cell fluorophore allows for the exclusion of cells with high signal (dead cells).

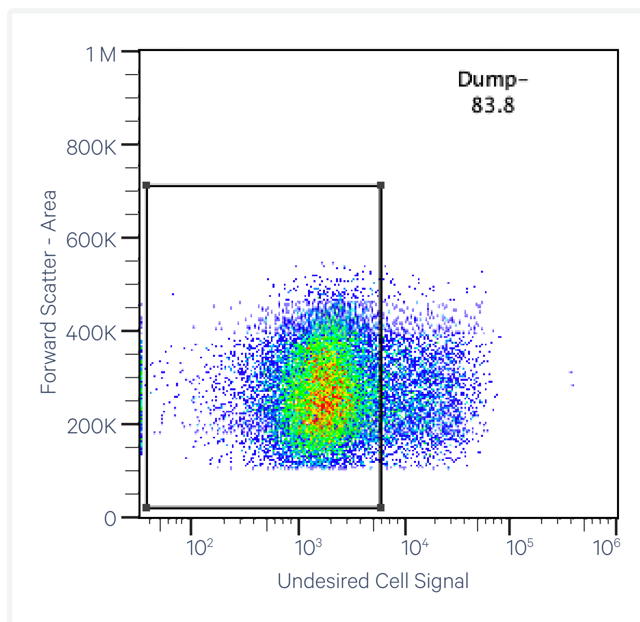

Figure 5. Undesired cell exclusion. Plot forward scatter against the fluorophore associated with markers for undesired cell populations. Gate the events that show a lower signal for the fluorophore in question, as shown above. Continue this process for all fluorophores associated with unwanted cell populations.

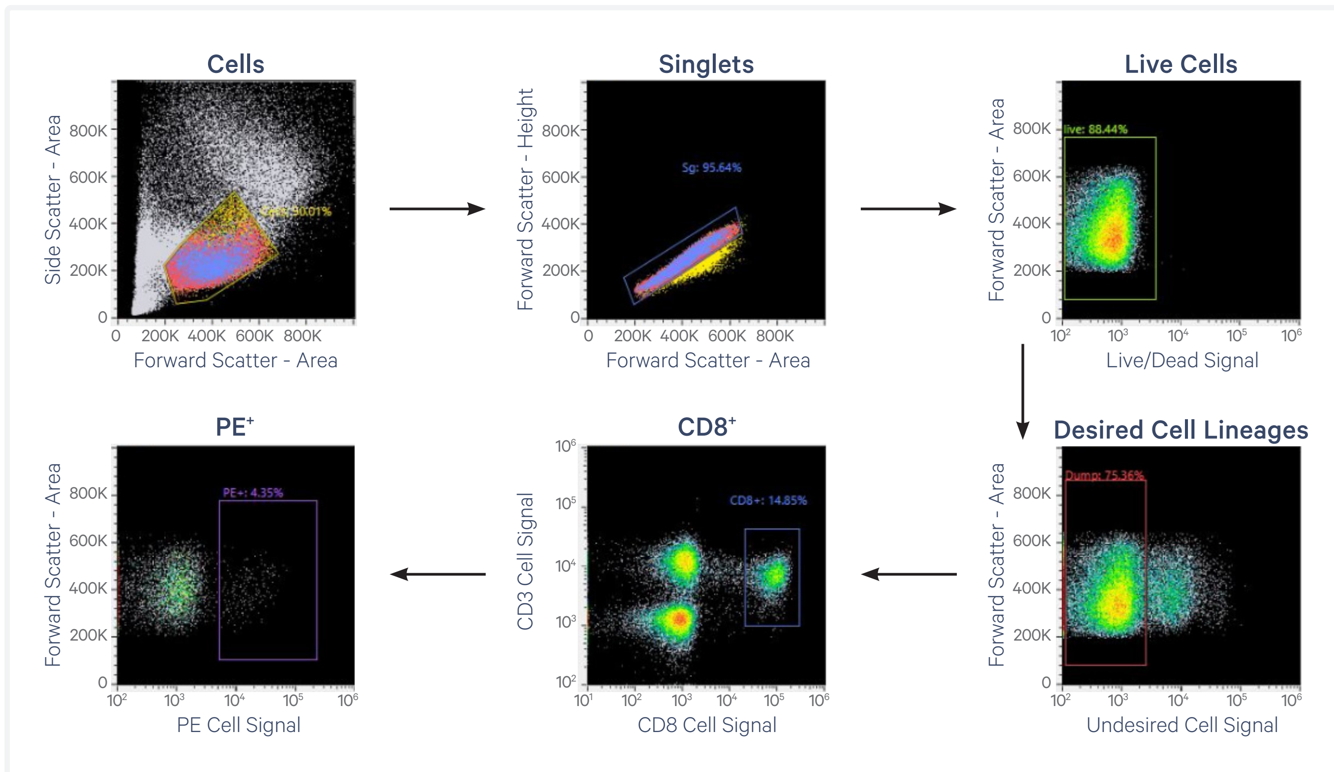

Figure 6. Example flow gating strategy for a BEAM-T sample. Events are gated for cells, singlets, live cells, desired cell lineages (negative gating), CD8⁺, and PE⁺ cells.

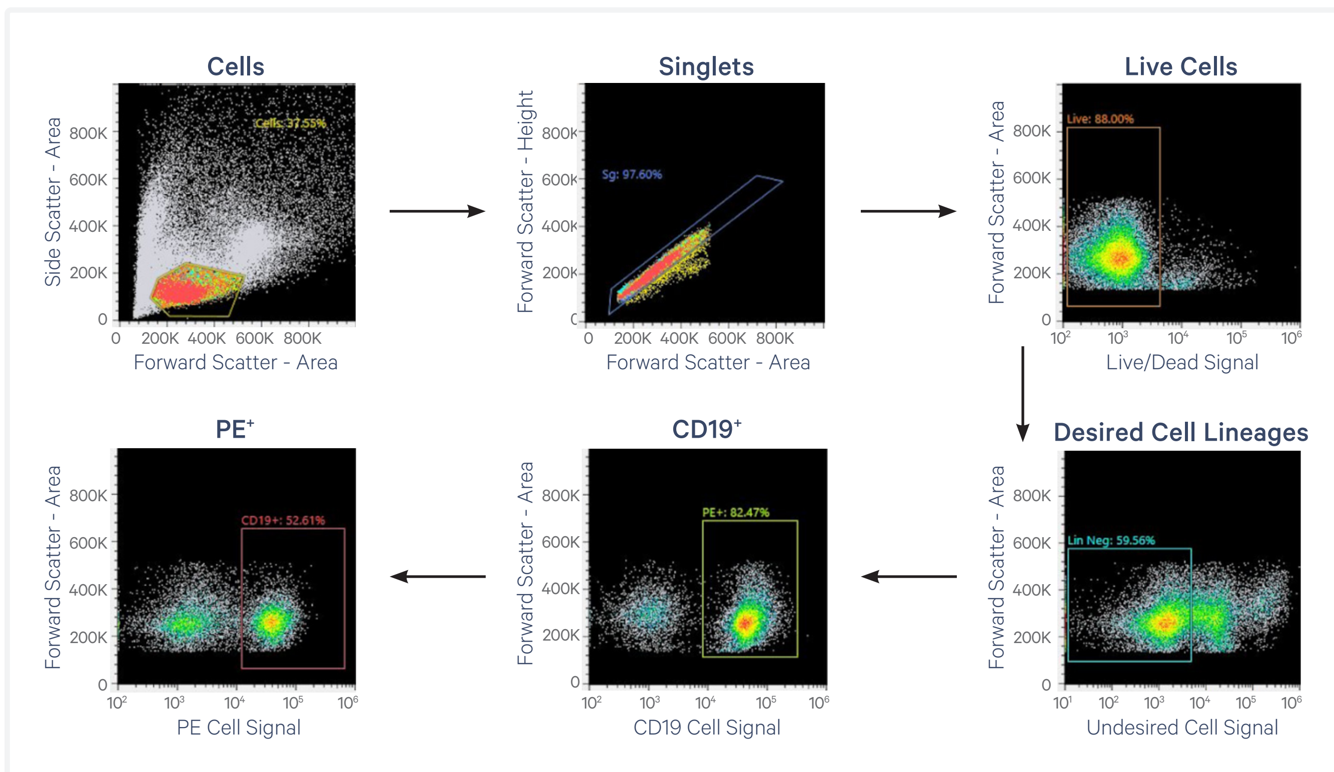

Figure 7. Example flow gating strategy for a BEAM-Ab sample. Events are gated for cells, singlets, live cells, desired cell lineages, CD19⁺, and PE⁺ cells.

Table 6 below demonstrates how gating during flow sorting results in a smaller number of PE⁺ events as compared to the initial number of events.

Sorting Step	Number of Events	Percent of Preceding Population	Percent of Total Events
All Events	100,000	N/A	100%
Cells	37,548	37.55%	37.55%
Single Cells	36,645	97.60%	36.65%
Live Cells	32,247	88.00%	32.25%
Desired Cell Lineages	19,206	59.56%	19.21%
$CD19^+$	15,840	82.47%	15.84%
PE^+	8,334	52.61%	8.33%

Table 6. Example results demonstrating how gating results in an increasingly smaller number of events as compared to the initial number of events. 100,000 events represents recorded events, which is a subset of the total number of events for this experiment. ~65% of sorted cells loaded onto the 10x Genomics Chip will be captured for downstream processing. Results may vary depending on sample type, sample quality, and flow sorting settings.

References

1. Reagent Assembly, Sample Labeling, & Flow Sorting for Barcode Enabled Antigen Mapping (BEAM) User Guide (Document CG000595).

Document Revision Summary

Document Number	CG000598
Title	Barcode Enabled Antigen Mapping (BEAM) Flow Cytometry Guidelines
Revision	Rev B
Revision Date	February 2023
General Changes	Updated for general minor consistency of language and terms throughout
Specific Changes	• Added additional tested centrifugation condition to page 2. • Corrected Ter19(+) to Ter19(-) in Table 5. • Clarified that both the sample and the tube should be kept cold while sorting on page 4.

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