Cell sorting

Bigfoot Cell Sorter: factors affecting efficiency, purity, and recovery

Introduction

Cell sorting continues to be an essential tool for a host of scientific endeavors, but despite more than 50 years of development and improvements, cell sorting continues to require compromise between high purity and recovery. Here we discuss aspects of cell sorting that affect efficiency, purity, and recovery, along with new and improved sorting features afforded by the Invitrogen™ Bigfoot™ Cell Sorter.

The following definitions are used:

- **Efficiency**—The reported number of cells sorted by the cell sorter as a percentage of the number of target cells present in the sample. Sasquatch software provides continuous monitoring of live sort efficiency.
- **Purity**—The target cells found as a percentage of the total cell number in the sorted sample. Determination of post-sort purity requires flow cytometric analysis of the collected sample, which may not be possible if the number of collected cells is low.
- **Recovery**—The number of actual target cells found in the output collection tubes or plates compared to the number deposited as reported by the instrument. This can be difficult to measure when few cells are collected, or when collected cells adhere to the collection tube or microtiter well surface.

When presented with the choice between excellent target cell purity or collection of the maximum number of target cells, researchers frequently respond that both purity and recovery are essential for a successful experiment. Most present-day sorters provide excellent purity if the desired cell population is clearly defined by the fluorescent probes and gating strategy. However, efficiency and recovery are affected by many additional factors, including nozzle tip size, event rate, target population abundance, cell aggregation, gating strategy, sort mode, and sort envelope. The reported sort efficiency along with the post-sort measurement of cell purity are regarded by many to be the criteria that determine the success of a sort experiment. However, recovery rather than efficiency dictates the number of sorted cells a researcher takes back to the laboratory. Efficiency is the percentage of target cells sorted, and recovery is the actual deposition counts into the catch vessel. Recovery, while hard to verify, can be improved by ensuring accurate sort stream deposition into the collection vessel and accurate drop delay timing. The Bigfoot Cell Sorter includes several innovative features to help guide the sort streams into the collection medium in the center of the selected tube or plate well, to ensure optimal cell deposition and to accurately calculate the timing for the drop delay.

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Modeling expected cell sorter purity and efficiency

Jet-in-air cell sorters process a stream of saline solution, composed of particles of interest, into a series of droplets that can then be charged, deflected, and sorted. A timing calculation known as the drop delay is responsible for identifying the position of the last attached droplet in the stream. When a desired particle reaches the breakoff point, which is the last attached droplet, a charge is applied to the stream. The charged droplet is then detached from the stream and deflected toward an oppositely charged deflection plate. The Bigfoot Cell Sorter can simultaneously sort up to six different populations by use of two charged deflection plates and three levels of charge for each side of the center waste stream. It is important to remember that a cell sorter sorts droplets and any particles contained in those droplets. The cell sorter must maintain precise stream pressure and alignment, droplet generation, and charging consistency for optimal sorting performance.

It is helpful to predict the expected efficiency and purity of a sort to select the appropriate instrument settings for an experiment. In 1990, Lindmo et al. discussed the relationship between accurate drop delay settings and efficiency [1]. Models of cell sorter performance based on Poisson-distributed target-particle arrival times were also examined in a 2000 book chapter by Durack, which explored the effects of drop delay error, event rate, and target percentages in the then newly developed high-speed sorting instruments [2]. The Lindmo equation is shown below:

$$\text{Sort efficiency} = e^{(-m(1-a)nT)} \times 100\%$$

Where:

m = average analysis rate (events/sec)

$a = x_{in}/x_{tot}$ (ratio of positive to total events)

x_{tot} = total number of events that pass through the laser(s)

x_{in} = number of events that should satisfy the sort criteria and pass through the laser(s)

n = number of droplets analyzed for each sorting event

T = period of droplet generator (1/frequency)

Using this equation, the effects of sample analysis rate, ratio of positive to total events, sorting criteria, and drop drive frequency are factored into the estimation for purity-mode sorting. It is important to note the ratio of target cells is calculated using the total events detected, not the gated events. The number of events above the threshold, and therefore detected by the cell sorter, greatly affects sort efficiency, because debris and noise are classified as unwanted particles and their presence leads to aborts. Sort gates used to eliminate debris and doublets maintain desired sort purity but do not change the sorting efficiency.

It is also important to understand how the number of droplets analyzed affects efficiency. To maintain very high purity, the Bigfoot sorter checks for contaminants in each drop being analyzed and the nearest quarter of each adjacent drop. This means that a total of 1.5 drops are analyzed and must be clear of contaminants for the sort to be allowed.

As an example, consider a purity sort with a detected event rate of 15,000 events per second, with 5.0% positive cells of the total detected population, at 90,000 Hz droplet formation frequency, on a Bigfoot Cell Sorter analyzing 1.5 drops. The equation using these values is shown below.

$$e^{(-15{,}000(1-0.05)\times 1.5(1/90{,}000))} \times 100\% = 78.9\%$$

For this example, the expected efficiency of a well-mixed sample is 78.9%.

To evaluate the accuracy of the formula above, a mixture of fluorescent and nonfluorescent microspheres was sorted at varying event rates. The test sorts accurately mirrored the predicted efficiencies at the various event rates (Figure 1).

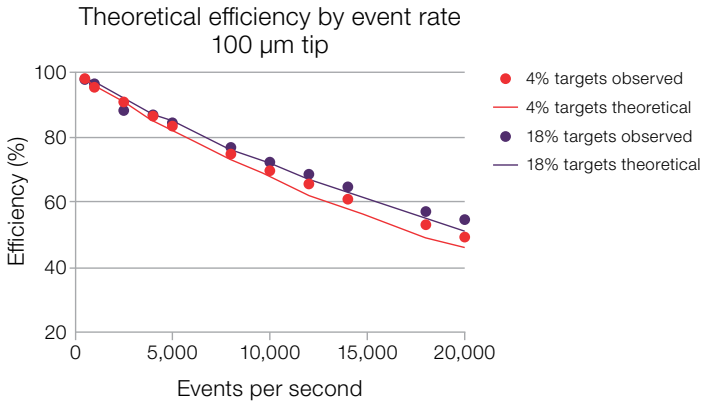
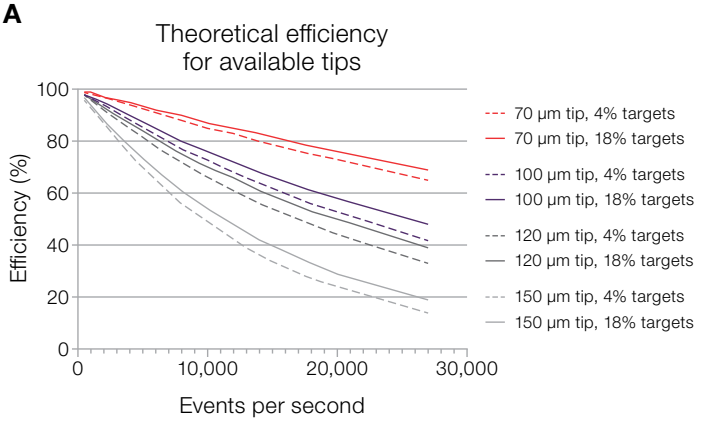


Figure 1. Verification of the efficiency model equation. A mixture of 4 µm blank microspheres (Spherotech, Cat. No. PP-40-10) and 3 µm multi-level fluorescent beads (Spherotech, Cat. No. RCP-30-5 6) suspended in 0.01% NP-40 in water were sorted on the Bigfoot Cell Sorter with a 100 µm tip and sheath pressure of 30 psi. Two different target percentages (4% and 18%) were sorted at various event rates to verify the predicted efficiencies. Post-sort purities of all data points were ≥99%.

Efficiency variations by tip size

The Bigfoot Cell Sorter has four interchangeable nozzle tip sizes: 70 µm, 100 µm, 120 µm, and 150 µm. The optimal system pressure and frequency ranges for each tip size is shown in Figure 2B. These values are related to the physics of fluidic droplet generation, with preferential values of drop drive frequency, amplitude, and sheath pressure for each tip size. The number of generated droplets per second is determined by the drop drive frequency, which, when paired with the smallest (70 µm) tip, generates 70,000–90,000 droplets per second. When paired with the largest (150 µm) tip, drop drive frequency generates 10,000–20,000 droplets per second. Smaller nozzle tip orifices can generate more droplets per second, and thus allow more cells to be processed and sorted in a given time frame. Ideally, ≤25% of droplets should contain a particle of interest, but this is dependent on the abundance of target events. This queue spacing reduces the number of aborted sorts caused by the presence of unwanted particles in, or droplets next to, the target droplet. The 70 µm tip allows an optimal event rate of 27,000 particles per second, and the 150 µm tip can optimally sort at 6,000 particles per second. A sort efficiency of 75% can be expected when sorting a 4% target population (Figure 2B).

A



B

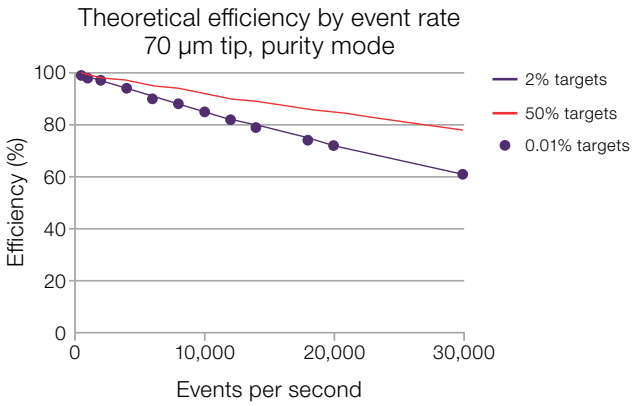
Nozzle tip size	Sheath pressure	Drop drive frequency range	Events per second for 75% efficiency with a 4% target
70 µm	50–60 psi	75–90 kHz	18,000
100 µm	20–30 psi	25–45 kHz	9,000
120 µm	15–20 psi	25–35 kHz	7,000
150 µm	9–12 psi	10–20 kHz	4,000

Figure 2. Nozzle tip size and sheath pressure determine the cell sorter drop drive frequency, and thus optimal sort event rates are dependent on target population percentages. (A) Predicted efficiencies by tip size in relation to increasing event rates for 4% and 18% target populations. **(B)** Nozzle tip sizes with standard system pressure ranges, drop drive frequency ranges, and event rates for predicted efficiencies of 75% when sorting a target population of 4%.

Effect of event rate and target cell frequency on efficiency

As the event rate increases and progressively more particles enter the droplet queue, adjacent events increase, which results in more aborts and reduced efficiency (Figure 3A). Conversely, as target cell numbers increase as a percentage of the total events, efficiency increases because fewer nontarget cells are present, and therefore fewer events are aborted (Figure 3B).

A



B

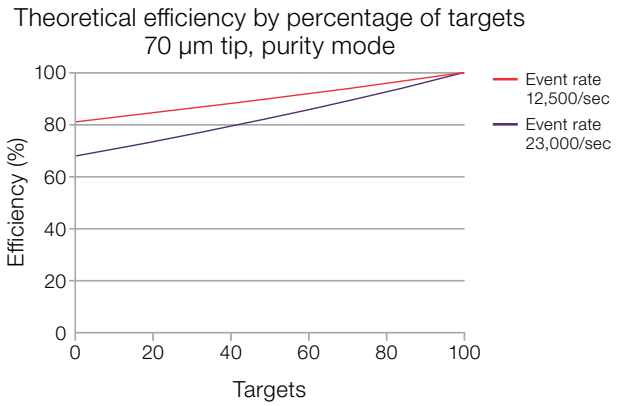


Figure 3. Models of expected efficiency by event rates and target percentages using purity sort mode. (A) Efficiency declines as the event rate increases. As the percentage of targets reaches less than 2%, the difference in efficiency is minimal. **(B)** Sort efficiencies are greater when more target particles are present in the sample.

Drop mode selection and efficiency

The Bigfoot Cell Sorter offers specific sort modes and sort envelopes that are related to the target particle position and the presence or absence of other events in the selected droplet. The available drop modes are illustrated in Figure 4.

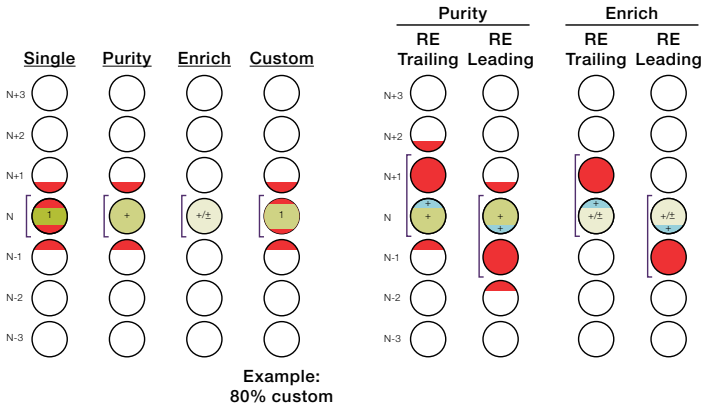


Figure 4. Single, purity, enrich, and custom sort modes, along with a more specialized recovery enhance (RE) option for purity or enrich modes. The number of droplets included in the mode assessment is set by the sort envelope.

Single

The sort is executed if exactly one event is in the target portion, depicted in dark green, and there are no particles of any kind in the adjacent areas, depicted in red. This is the most restrictive drop mode and efficiency will be reduced by approximately 50% of the purity mode value because of the strict position requirement. Single mode provides the best flight trajectory of single cells into small vessels, such as 96- or 384-well plates.

Purity

The sort is performed if one or multiple positive particles are in the target portion, depicted in medium green, and there are no negative particles in the adjacent areas, depicted in red. Positive particles can exist in the red areas and will be included in the sorted fraction. Purity mode provides higher efficiency than single mode and should be used for experiments where both efficiency and purity are important.

Enrich

The sort is performed if at least one positive particle is in the target portion, depicted in light green, regardless of the presence of other particles, either negative or positive. Purity is reduced in enrich mode, while efficiency is expected to be 100%.

Custom

Custom mode is identical to either single mode or purity mode, which is selected by the user, and allows the user to set the droplet centering mask percentage. The value entered in the custom sort mask field is the percentage of the droplet that will be used as the centering mask. For example, in custom single mode, a value of 80% excludes the outer 10% of each droplet.

Recovery enhance

For purity and enrich mode sorts, the recovery enhance (RE) option can be enabled. If the positive particle is in the outer quarter of the droplet, depicted in blue, and there are no negative particles in the adjacent area, depicted in red, an additional droplet will be sorted to increase the recovery of the positive particles. Note that for purity mode with recovery enhance enabled, the adjacent area extends quarter droplet farther to ensure that the purity is not compromised by adjacent negative particles. However, in enrich mode with recovery enhance enabled, a smaller adjacent area is used to maximize recovery.

The modified Lindmo equation for each primary drop mode is shown below:

Sort efficiency (%) in **single** mode = $e^{(-m(1-a)nT)}/2 \times 100\%$

Sort efficiency (%) in **purity** mode = $e^{(-m(1-a)nT)} \times 100\%$

Sort efficiency (%) in **enrich** mode = 100%

The expected sort purity for both single and purity modes is greater than or equal to 98%, but efficiency is expected to be less than 100% because of the exclusion of nontarget particles (Table 1). Conversely, basic enrich mode is expected to have an efficiency of 100% with a lower purity than the single and purity modes because the instrument ignores coincident events, and thus includes them in the sorted output. Similarly, the RE option in purity mode has an expected purity of >99%, but the efficiency is even lower than purity mode because of the exclusion of more adjacent droplets. Enrich mode with recovery enhance enabled maximizes recovery but results in lower purity than enrich mode with recovery enhance disabled.

Table 1. Maximum theoretical and observed efficiencies and purities of the three basic sort modes. A mixture of 4 µm blank and fluorescent 8-peak microspheres were sorted at 10,000 events/sec using a 100 µm tip at 30 psi. The target sort region included a selected population of the 8-peak beads representing 5% of the total beads. Predicted efficiency was calculated using the modified Lindmo equation for purity mode, with the expected efficiency for single mode being one half of that amount based on sorting the middle 50% of the target droplet and expected efficiency for enrich mode being 100% by definition.

Mode	Predicted efficiency (%)	Observed efficiency (%)	Expected purity (%)	Actual purity (%)
Single	44	38	98	98
Purity	88	77	98	98
Enrich	100	100	<98	86

Stable and accurate drop delay is essential for optimal efficiency and recovery

Figure 5 demonstrates a direct relationship between ideal drop delay timing and deviation from the ideal drop delay resulting in lower bead recovery. If less than expected sort recovery is encountered, the cell sort sample or beads of an equivalent size can be used to manually verify and adjust the drop delay setting if needed.

Figure 5. A representative screenshot of the automated drop delay plot. The Bigfoot Cell Sorter uses fluorescent beads and a miniature cytometer to advance through increasing and then decreasing increments of drop delay settings. The initial starting point for these scans is based on historical reference settings for the nozzle size and instrument serial number. The mean drop delay of the maximum signals detected in each direction is selected as the optimized drop delay setting.

The difficulties associated with measuring sort recovery using cellular material have led many researchers to conclude that high sort purity is a proper indication of sort quality. However, sort purity is often the easiest goal to achieve in cell sorting because multiple factors adversely affect cell recovery more quickly and dramatically than purity. Factors that reduce sort purity include drop delay timing errors, cell aggregation, viability, gating schemes, threshold settings, and resolution of cell populations.

If sort purity is slightly lower than expected, the operator may attribute the problem to other factors while overlooking a drop delay timing error. High sort purity is not a reliable indicator of the accuracy of the drop delay setting, because errors in drop delay may not cause a significant loss of purity until the error is quite large. Likewise, the live efficiency values reported by any cell sorter will not reflect errors in the drop delay setting. If the drop delay is incorrect, the live efficiency reading will be based on this incorrect value, and the droplets sorted frequently may not contain the desired particles. The Bigfoot Cell Sorter's mini cytometer with a laser and detector system for determining incremental drop delay settings is one of the most accurate technologies of its kind.

Minor drop delay errors of a fraction of a droplet dramatically reduce sort recovery, while sort purity is not as greatly affected unless sorting is done at extremely high event rates or of rare populations. Specifically, sort purity can remain above 95%, while the sort recovery drops to 80% because of a drop delay timing error of 20% of one droplet. As the amount of error approaches one entire droplet, the sort purity drops to the percentage of target cells in the input sample.

Options on the Bigfoot Cell Sorter that affect efficiency and recovery

Charge plate polarity flip option during a sort

The buildup of droplets on the inner walls of the collection vessel is common in cell sorting. Droplets can sometimes be observed to "bounce" out of vessels with wide openings. This is caused by a buildup of charge in the collected volume that repels the like charge of sorted incoming droplets. To mitigate this phenomenon, the Bigfoot Cell Sorter features a polarity flip (PF) option that reverses the polarity assignments of the droplet charges and charge plates to minimize the charge buildup in the collection vessel. The PF process requires approximately one second during which sorting is paused, but the stream flow continues. Depending on the percentage of target events present in the sample, the PF can also affect sort efficiency (Figure 8A). The frequency of switching is based on the accumulated volume in the collection vessel and is therefore driven by the volume of the most abundant targets. Although the reduction in efficiency is generally minimal, when a region of a very abundant target is sorted, PF will occur frequently and will negatively impact the efficiency of less abundant targets. Table 2 lists the default PF volume values in the software that can be optimized for each experiment. Evaluate individual experiments during sort setup to ensure volume values are appropriate. PF is most useful when the expected sort volume is high, to prevent sorted droplet repulsion. PF may reduce the reported efficiency, but overall, sort recovery is enhanced. This is due to reduced cell loss on the collection tube walls where cells adhere and dry, and less cell expulsion from the sort vessel altogether.

Table 2. Default values for PF are based on the sorted droplet volume added to each vessel type. PF can be adjusted or disabled as needed.

Nozzle tip size	Collection vessel	Polarity flip volume
100 µm	1.5 mL tubes	0.01 mL
	5 mL tubes	0.13 mL
	15 mL tubes	0.25 mL
	50 mL tubes	0.36 mL
70 µm	1.5 mL tubes	0.01 mL
	5 mL tubes	0.02 mL
	15 mL tubes	0.03 mL
	50 mL tubes	0.05 mL

Stream optimization and droplet spacing

Droplet spacing is a technique that can be used to reduce stream fanning, especially for droplets in the outermost streams, R3 and L3. If the outer sort streams are assigned to more abundant target populations, charged droplets that are close together in the droplet queue may interact with each other through air turbulence and cause deflection variations and stream fanning. As a result of this fanning, droplets may be deposited on the side walls of the output collection vessel or may entirely miss the collection tube or well. To reduce this fanning, the Bigfoot sorter aborts sort events located within the assigned droplet spacing limit. Keep in mind droplet spacing can have a significant deleterious effect on sort efficiency (Figures 7–9 and Table 3). The software includes default droplet spacing for each stream that assigns greater spacing to the outer streams (Table 3). We recommend careful investigation and adjustment of droplet spacing for each stream when new sorting assays are developed.

Table 3. Default droplet spacing for stream optimization. N/A indicates directions that are not available for the tube size.

Tube size	Tip size	Stream position		
		L3/R3	L2/R2	L1/R1
1.5 mL	70 µm	16	8	4
	100 µm	8	4	2
5 mL	70 µm	16	8	4
	100 µm	8	4	2
15 mL	70 µm	N/A	8	4
	100 µm	N/A	4	2
50 mL	70 µm	N/A	N/A	4
	100 µm	N/A	N/A	2

Droplet spacing can be changed or disabled if needed. For optimal results, the more abundant targets should be assigned to the inner streams, and the scarcer targets should be assigned to the outer streams. Larger sort vessels may be necessary to provide a wider opening to accommodate a fanning stream, and may not require droplet spacing. A larger nozzle tip or a slower event rate will also increase the spacing of target droplets. Aggregated cells in the sort sample will result in clusters of target cells in the sort queue, and thus further reduce efficiency. It is important to remember that while efficiency appears to be reduced, recovery and viability are enhanced by the reduction of stream fanning.

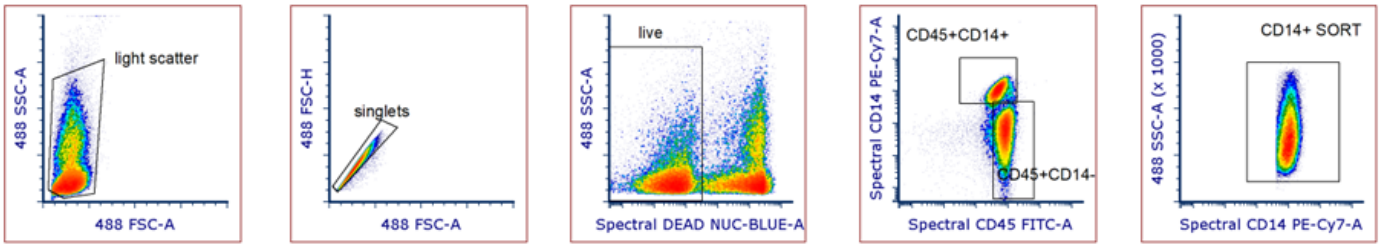
We used a ten-color spectral panel to evaluate the effects of drop mode, droplet spacing, and polarity flip on efficiency (Figure 6). Five phenotypes were sorted using purity and enrich mode, droplet spacing on and off, and polarity flip on and off. Efficiency was normalized as the ratio of the observed efficiency to the predicted efficiency. Purity mode with droplet spacing off and polarity flip off achieved greater efficiency than purity mode with droplet spacing and/or polarity flip on. As expected, efficiency was 100% in enrich mode when droplet spacing and polarity flip were off, but efficiency was reduced when droplet spacing and/or polarity flip were on. The use of enrich mode with droplet spacing sends conflicting instructions to the software—enrich mode requires all target cells to be sorted, while droplet spacing instructs the software to not sort some positive targets. We found this reduction of positive cell sorts varied greatly as the percentages of the phenotypes changed between samples. Purity remained high for all combinations in purity mode (≥97%) but was reduced in enrich mode (70–90%), as expected.

Cumulative effects of droplet spacing and polarity flip on efficiency, purity, and recovery

Droplet spacing (DS) and polarity flip (PF) show cumulative effects on the efficiency of cell sorting with the Bigfoot Cell Sorter (Figures 7 and 8). Efficiency is highest when both DS and PF are not used, but avoiding these features may not always be the best option. When maximum recovery is the main priority, it may be advantageous to use DS and PF and allow for slightly lower efficiency. DS reduces stream fanning, and PF reduces droplet repulsion caused by charge buildup. Efficiency is at its lowest when both DS and PF are used.

While DS appears to have a significant effect (Figure 7), it is highly dependent on the percentages of sorted target cells in the sample. While DS and PF reduce efficiency, purity is not affected. DS appears to reduce efficiency more than PF, but both can vary with changes in the percentages of the cell populations. DS and PF are intended to increase cell recovery, which is the percentage of cells sorted and captured in the collection vessel. This apparent decrease in efficiency may result in greater recovery due to the contributions of PF and DS.

Verification requires an accurate method for measuring recovery. We measured recovery by weighing the empty collection tube before adding medium and after sorting to determine the total volume contained in the tube. The sorted sample was analyzed and gated in a similar manner as the Bigfoot Cell Sorter on the Invitrogen™ Attune™ volumetric flow cytometer to determine the absolute count of target cells per mL. The total volume in the collection tube and the cells per mL were used to calculate the number of cells collected. The recovery percentage was determined from number of cells collected divided by the number of cells sorted as reported by the Bigfoot Cell Sorter.



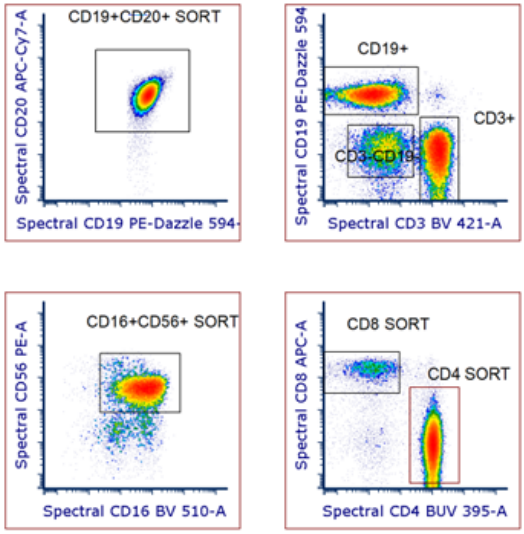


Figure 6. Sorting layout used for efficiency evaluation. 10-color spectral data of a fresh lysed human blood sample were sorted. Five sort regions were gated as follows:

Light scatter - singlets - live - $CD45_{pos}$ $CD14_{pos}$ - $CD14_{pos}$ Sort

Light scatter - singlets - live - $CD45_{pos}$ $CD14_{neg}$ - $CD3_{neg}$ $CD19_{neg}$ - $CD16_{pos}$ $CD56_{pos}$ Sort

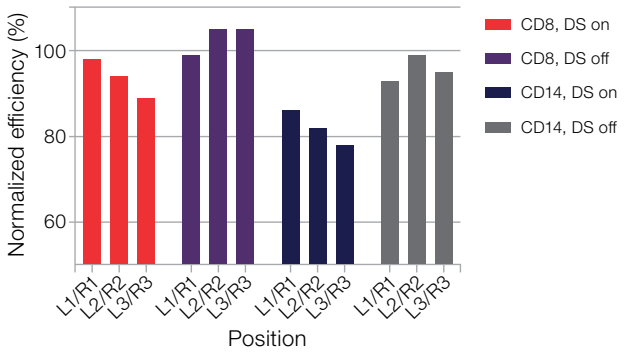
Light scatter - singlets - live - $CD45_{pos}$ $CD14_{neg}$ - $CD3_{pos}$ $CD19_{neg}$ - $CD4_{pos}$ $CD8_{neg}$ Sort

Light scatter - singlets - live - $CD45_{pos}$ $CD14_{neg}$ - $CD3_{pos}$ $CD19_{neg}$ - $CD4_{neg}$ $CD8_{pos}$ Sort

Light scatter - singlets - live - $CD45_{pos}$ $CD14_{neg}$ - $CD3_{neg}$ $CD19_{pos}$ - $CD19_{pos}$ $CD20_{pos}$ Sort

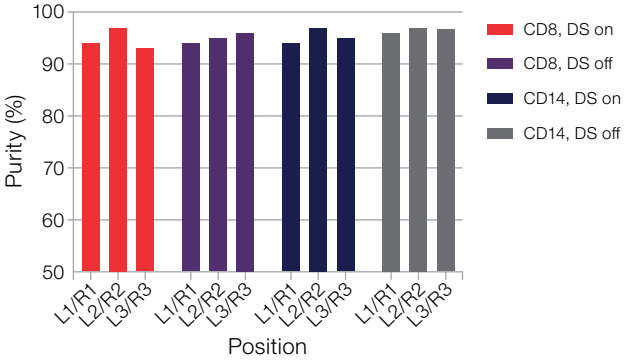
These data were spectrally unmixed and sorted at 28,000 events per second using a 70 µm nozzle tip at 60 psi sheath pressure. 50,000 events are shown on the plots, but many more were actually sorted.

A



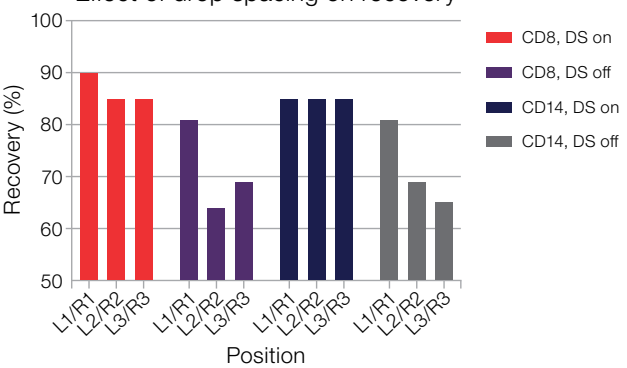
	CD8, DS on	CD8, DS off	CD14, DS on	CD14, DS off
L1/R1	96	99	86	93
L2/R2	94	105	82	99
L3/R3	89	105	78	95

B



	CD8, DS on	CD8, DS off	CD14, DS on	CD14, DS off
L1/R1	94	94	94	96
L2/R2	97	95	97	97
L3/R3	93	96	95	97

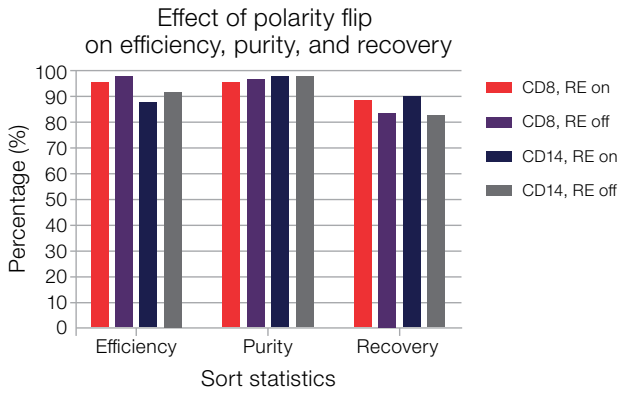
C



	CD8, DS on	CD8, DS off	CD14, DS on	CD14, DS off
L1/R1	90	81	85	81
L2/R2	85	64	85	69
L3/R3	85	69	85	65

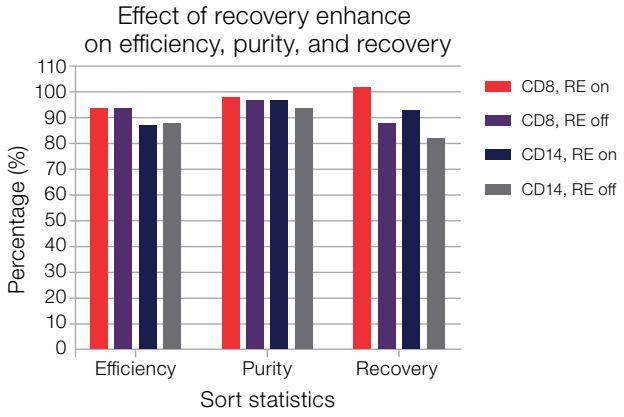
Figure 7. Effects of drop spacing (DS) using the 70 µm tip on (A) efficiency, (B) purity, and (C) recovery. R1/L1 have default values of 4, R2/L2 have default values of 8, and R3/L3 have default values of 16 on the 70 µm tip. Normalized efficiencies were calculated as the ratio of observed efficiency to predicted theoretical efficiency. Designations of tube positions were determined based on the most abundant cells and the highest priority to sort. All populations were completed at the default settings based on the tube location.

A



	CD8, RE on	CD8, RE off	CD14, RE on	CD14, RE off
Efficiency (%)	96	98	88	92
Purity (%)	96	97	98	98
Recovery (%)	89	84	90	83

B



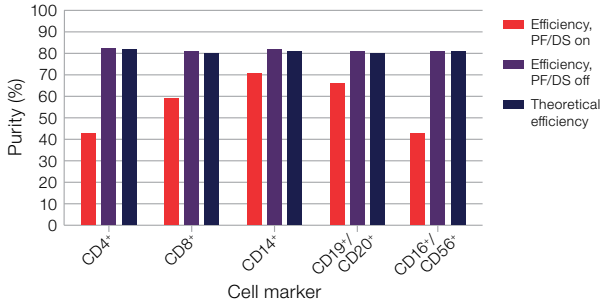
	CD8, RE on	CD8, RE off	CD14, RE on	CD14, RE off
Efficiency (%)	94	94	87	88
Purity (%)	98	97	97	94
Recovery (%)	102	88	93	82

Figure 8. Cumulative effects of the recovery enhance (RE) feature on the 70 µm tip. Effects of **(A)** polarity flip and **(B)** recovery enhance on efficiency, purity, and recovery. Polarity flip and drop spacing were defaulted to normal values.

Mode selection and droplet spacing and polarity flip

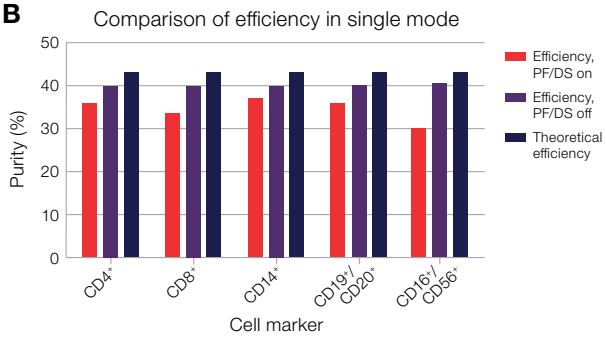
Specific sort modes and sort envelopes are related to the target particle position and the presence or absence of other events in the selected droplet (Figure 9).

A



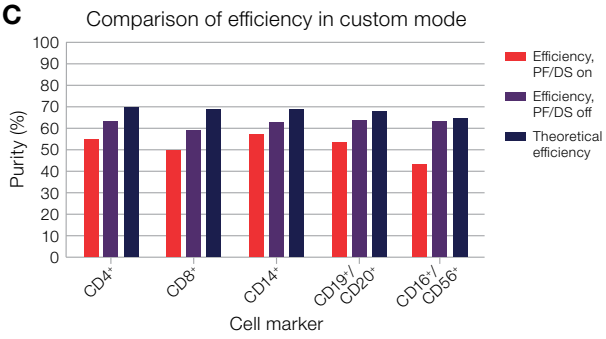
	Efficiency (%)			ΔEfficiency$_{theoretical–actual}$ (%)	
	PF and DS on	PF and DS off	Theoretical	PF and DS on	PF and DS off
CD4+	43	83	82	39	–1
CD8+	59	81	80	21	–1
CD14+	71	82	81	10	–1
CD19+/CD20+	66	81	80	14	–1
CD16+/CD56+	43	81	81	38	0

B



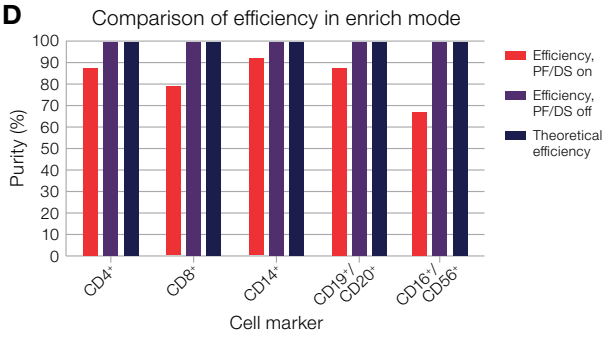
	Efficiency (%)			ΔEfficiency$_{theoretical–actual}$ (%)	
	PF and DS on	PF and DS off	Theoretical	PF and DS on	PF and DS off
CD4+	36	40	43	3	7
CD8+	34	40	43	3	9
CD14+	37	40	43	3	6
CD19+/CD20+	36	40	43	3	7
CD16+/CD56+	30	41	43	3	13

C



	Efficiency (%)			ΔEfficiency$_{theoretical–actual}$ (%)	
	PF and DS on	PF and DS off	Theoretical	PF and DS on	PF and DS off
CD4+	55	64	70	6	15
CD8+	50	59	69	10	19
CD14+	57	63	69	6	12
CD19+/CD20+	54	64	68	4	14
CD16+/CD56+	43	64	65	1	22

D



	Efficiency (%)			ΔEfficiency$_{theoretical–actual}$ (%)	
	PF and DS on	PF and DS off	Theoretical	PF and DS on	PF and DS off
CD4+	88	100	100	0	12
CD8+	79	100	100	0	21
CD14+	92	100	100	0	8
CD19+/CD20+	88	100	100	0	12
CD16+/CD56+	67	100	100	0	33

Figure 9. Examples of droplet spacing and polarity flip effects on various sorting modes. (A) When sorting in purity mode, the measured efficiency was 4% lower than the predicted theoretical maximum. **(B)** When sorting in single mode, the predicted efficiency is reduced by half when compared to purity mode, as expected. **(C)** Custom single mode increases the expected efficiency as compared to single mode. **(D)** As expected, efficiency was 100% in enrich mode when DS and PF were off, but efficiency was reduced when DS and/or PF were on. The use of enrich mode with DS sends conflicting instructions to the software—enrich mode requires all target cells to be sorted, but DS instructs the software to not sort some positive targets. We found that this reduction in positive sorted cells varied as the percentages of the phenotypes varied between samples. Purity remained high for all combinations in purity mode, but was reduced in enrich mode, as expected.

Sample considerations

Sort samples frequently contain cells of varying sizes along with cell aggregates caused by adherent and/or dead cells. We used lysed whole human blood cells to investigate these additional parameters that can affect efficiency and purity in cell sorts.

Cell aggregates

Cell aggregates adversely affect sort efficiency, purity, and recovery. Despite the use of singlet gates, cell aggregates still reduce the overall sort purity and efficiency. If a negative cell is adhered to a positive cell, and the negative target cell lacks any distinctive marker that can discriminate it from the true positive cell target, the negative cell will be sorted along with the desired positive cell, leading to a reduction in purity. While doublet gating plots are useful to overcome this scenario, inevitably some cell aggregates will not be detected using these plots.

Furthermore, loosely aggregated cells can be disassociated by the sorting process, which disrupts the random order of the cell queue and leads to clusters of cells in the queue. These cells are aborted because of their proximity to other cells or their location within a droplet spacing assignment. Cryopreserved cells, tissue digests, or cell samples with low viability form aggregates in the presence of free DNA that has been released from dead cells. It is helpful to add DNase to cell staining and sort buffers to reduce cell adhesion for these types of samples.

Debris

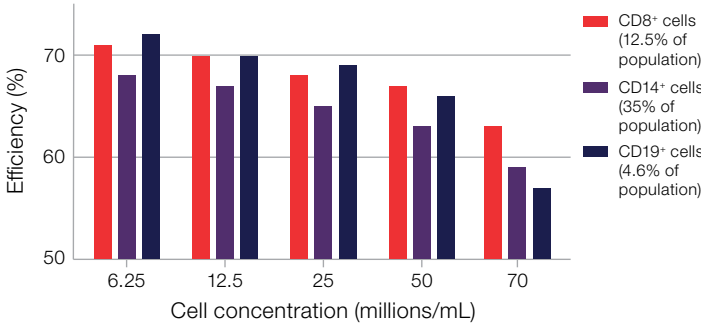
A large amount of sample debris reduces sort efficiency because the debris particles are classified as nontarget events. While much of the smaller debris can be excluded by the threshold trigger setting, some of the debris close to the threshold boundary will inevitably be seen in the sorted sample. The instrument is essentially blind to debris that falls below the threshold and that may be included in the sorted material. Careful evaluation regarding the effect of this undetected debris on downstream experiments should be considered.

Cell size

It is known that cell size and shape can affect the stream breakoff point. Large or oddly shaped cells may require an adjustment to drop delay, which may be done in Sasquatch software using the integrated “Confirm drop delay with cells” feature [3]. Large cells may also cause stream fanning if the nozzle tip size is too small. In general, the nozzle tip should be 4–6 times greater than the diameter of the cells. When sorting a mixed-size population of cells, the tip size should be determined by the diameter of the largest cells in the sample. Stream fanning can be reduced by changing to a larger tip size.

Cell concentration

While high-speed sorting most frequently relies on highly concentrated cell samples, efficiency on the Bigfoot Cell Sorter may be optimized if concentrated samples are diluted. A flow rate of 100–180%, in addition to diluting the samples, often results in better efficiency compared to running at a lower flow rate and a higher cell concentration (Figure 10). In doing so, efficiency will improve, and recovery will increase without an increase in total sort time. When the flow rate is increased, the core stream widens, which can result in a loss of data plot resolution. Therefore, flow data plots, especially the linear light scatter parameters, should be carefully monitored.



Concentration (millions/mL)	Efficiency (%)		
	CD8+ cells	CD14+ cells	CD19+ cells
6.25	71	68	72
12.5	70	67	70
25	68	65	69
50	67	63	66
70	63	59	57

Figure 10. Efficiency is improved by diluting cells and increasing the flow rate. Cryopreserved cells (7 x 10^7 cells/mL) were stained with CD8 APC, CD19 FITC, and CD14 PE. Cells were washed and resuspended in DNase buffer, diluted, filtered, and sorted using a 100 µm tip at 30 psi. Cells were sorted with DS off and PF off at an event rate of 10,000 cells per second for all samples.

Optimizing sort recovery

When sort efficiency does not perform as expected, explore the following circumstances, and adjust the instrument or assay accordingly.

Issues that affect efficiency	Suggestions for optimal performance
Aggregates	• Use a pipette to gently agitate the cell suspension. • Filter the sample. • Add DNase, RNase, collagenase, or EDTA to the cell staining buffer. • Use commercial cell disassociation buffers. • Dilute the sample and increase the flow rate.
Droplet spacing	• Confirm that the droplet spacing is optimized for the experiment.
Polarity flip	• Verify that polarity flip is needed, and if not, turn it off. • Increase the volumes used for polarity flip to optimize for your experiment and sort output collection. • When possible, add a sort limit to stop sorting for more abundant targets that cause the polarity flip frequency. • Use polypropylene collection tubes instead of polystyrene, as polystyrene does not dissipate charge as efficiently as polypropylene. • Use a larger volume of collection media and increase polarity flip volume or turn it off.
Cell concentration	• Dilute the sample. • Maintain the event rate by increasing the flow rate.
Debris	• Increase the threshold setting, if possible, to eliminate debris from detection. • As with all sorters, debris below the threshold may be included in the sort output. • Perform a pre-enrichment sort or use extra washes to remove excess debris.

Summary—optimizing cell sorting on the Bigfoot Cell Sorter

Efficiency, purity, recovery, and viability are some of the factors used to measure the success of cell sorts. Efficiency and purity are easily measured during and/or after the sort process, and can be predicted by models such as the Lindmo equation. Using these predictions, a researcher can know that efficiency will be sacrificed for purity, or that purity will be reduced for higher efficiency.

Efficiency is reported by the cell sorter software in real time as the sort progresses. However, efficiency, which represents the portion of target cells processed for sorting as reported by the cell sorter, does not confirm the cells are in the desired capture location.

Purity and viability are measured by analyzing a portion of the collected cells, stained post-sort with a live/dead cell indicator, using the cell sorter or a flow cytometry analyzer. However, the quantification of the percentage of truly dead cells in the post-sort cell collection does not guarantee that the remaining cells are unharmed and viable for downstream applications.

Recovery is a measure of the number of cells that were collected in the desired sort output vessel, and is a priority for cell sorting experiments. Recovery is not as easily measured as efficiency and purity.

The Bigfoot Cell Sorter includes two new features, droplet spacing and polarity flip, which are meant to enhance cell recovery and viability. While the data presented in this paper show an increase in efficiency with the droplet spacing and polarity flip off, this selection can reduce recovery because of stream fanning and charge repulsion. Droplet spacing can also improve viability because the sorted droplets are more accurately deposited in the desired locations since the sorted streams experience less fanning.

There are many post-sort applications, such as PCR, experimental treatments on sorted cells, isolation and growth of desired pure colonies, and clone selection, that depend on the purity of the sorted sample. It can also be essential for researchers to recover as many cells as possible, especially for studies of rare populations. Therefore, the droplet spacing and polarity flip features on the Bigfoot Cell Sorter provide excellent value for investigators who conduct diverse biological research. Efficiency, purity, recovery, and viability are all important aspects to balance when using a cell sorter. The polarity flip and drop spacing features provide investigators the flexibility to modify settings based on various cell types and experiments to get the most out of each cell sorting experiment.

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