Resolving human circulating innate lymphoid cell heterogeneity using advanced single cell multiomic analysis

Application of BD Rhapsody™ Single-Cell Analysis System and BD FACSymphony™ high parameter cell analyzer for cell signature discovery and deep immunophenotyping

Features

- Simultaneous analysis of protein and gene expression at the single cell level
- Identification of signatures defining distinct subsets of human circulating innate lymphoid cells (ILCs)
- Single cell multiomic screening provides biological information critical for the development of high parameter flow cytometry panels

Innate lymphoid cells (ILCs) constitute a recently identified family of lymphoid cells that do not express rearranged antigen receptors and have key functions in inflammation, infection, cancer, metabolic disorders and allergies. They represent a heterogeneous population including classically defined natural killer (NK) cells as well as more recently described non-cytotoxic ILCs. Three major groups of non-cytotoxic ILCs have been characterized in mucosal and non-mucosal tissues based on distinct patterns of cytokine production and transcription factor expression. However, recent publications using single cell RNA sequencing (scRNA-Seq) or high-parameter mass cytometry showed the existence of more than just three groups of ILCs; hence better understanding of ILC



heterogeneity is needed to determine their roles in disease pathogenesis and to determine whether targeting of ILCs will help to prevent or treat these diseases. The emergence of single cell multiomic (scM) technologies provides an opportunity to further resolve ILC heterogeneity by enabling integration of phenotypic and transcriptional data from the same cell.

This data sheet provides an example of a complete workflow solution for deep characterization of ILCs (Figure 1). ILCs were enriched by sorting on a fluorescence-activated cell sorter prior to single cell multiomic analysis. Computational data analysis was used to identify proteins and genes that are differentially expressed by distinct subsets of ILCs. Information about relative antigen density and co-expression was then used to quide the design of a high-parameter flow cytometry panel to assess the distribution of ILC1 subsets across different donors.



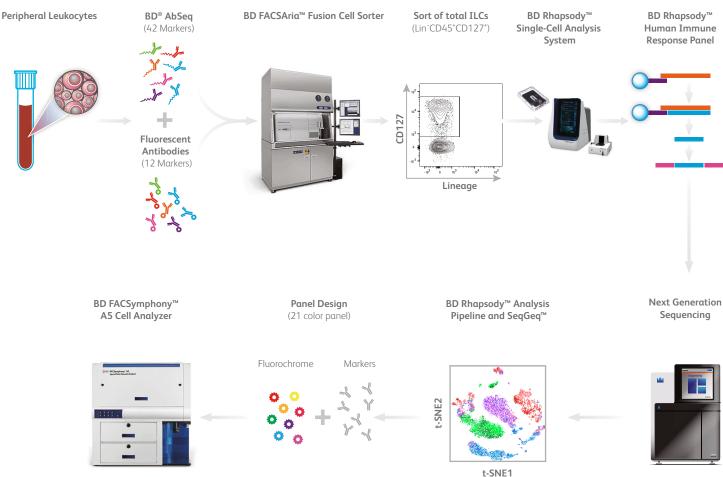


Figure 1. A complete workflow for high-parameter single cell ILC characterization using the BD Rhapsody Single-Cell Analysis System and the BD FACSymphony A5 Cell Analyzer

Peripheral blood leukocytes were isolated using HetaSep (STEMCELL Technologies) and labeled with a panel of 12 fluorochrome-conjugated antibodies for cell sorting (Table 1). Cells were also simultaneously stained with 42 oligonucleotide-conjugated AbSeq antibodies for downstream protein detection via next-generation sequencing. A BV510-labeled cocktail of CD19, CD14, TCRγδ, TCRαβ, CD123, CD1a, BDCA2 and FCεR1α antibodies was used as a dump channel to exclude lineage cells. To ensure clear resolution and exclusion of T and NK cells, CD3 and CD56 antibodies were additionally included in the sorting panel. Total ILCs were defined as Lineage-CD45*CD127* cells and were sorted using a BD FACSAria Fusion cell sorter and loaded into the BD Rhapsody Single-Cell Analysis System for single cell capture. After sample retrieval, single cell BD® AbSeq and mRNA (BD Rhapsody Human Immune Response Panel) libraries were prepared for sequencing. Data was analyzed using BD Rhapsody Analysis Pipeline and SeqGeq™ software. Biological information obtained from the sequencing screening was used to guide the design of high parameter flow cytometry panels. The information derived from multiomic analysis was validated by analyzing peripheral blood ILCs from 4 different healthy donors using a 21-color flow cytometry panel on the BD FACSymphony A5 cell analyzer.

Table 1. List of fluorochrome-conjugated antibodies used for sorting of Lineage CD45 CD3 CD56 CD127 total ILCs

Marker	Clone	Fluorochrome	
CD19	SJ25-C1		
CD14	МФР9		
ΤϹRγδ	11F2		
ΤCRαβ	IP26	BV510	
CD1a	HI149		
CD123	9F5		
BDCA2	V24-785		
FCεR1α	AER-37		
CD45	2D1	FITC	
CD3	SK7	APC-H7	
CD56	B159	PE-Cy7	
CD127	HIL-7R-M21	BV421	

Table 2. List of AbSeq oligonucleotide-conjugated antibodies used for single cell multiomic analysis of sorted total ILCs

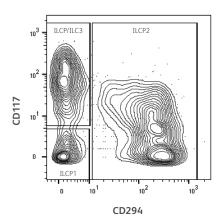
Cells were co-stained with the markers CD3, CD56, CD14 and CD19 in fluorochrome- and oligonucleotide-conjugated formats in order to further exclude contaminant lineage cells during downstream multiomic analysis. The use of two distinct clones for CD56 was necessary in order to achieve resolution of both fluorochrome- and oligo-conjugated antibodies.

BD® AbSeq Panel							
Marker	Clone	Marker	Clone				
CD2	RPA-2.10	CD90	5E10				
CD3	SK7	CD94	HP-3D9				
CD4	SK3	CD98	UM7F8				
CD5	UCHT2	CD103	BER-ACT8				
CD7	M-T701	CD117	YB5.B8				
CD8	RPA-T8	CD152 (CTLA-4)	BNI3				
CD11b	M1/70	CD161	DX12				
CD11c	B-LY6	CD183 (CXCR3)	1C6/CXCR3				
CD14	МфР9	CD184 (CXCR4)	12G5				
CD16	3G8	CD196 (CCR6)	11A9				
CD19	SJ25C1	CD223 (LAG-3)	T47-530				
CD25	2A3	CD226 (DNAM-1)	DX11				
CD27	M-T271	CD274 (PD-L1)	MIH1				
CD28	CD28.2	CD275 (B7-H2)	2D3/B7				
CD34	581	CD278 (ICOS)	DX29				
CD45RA	HI100	CD279 (PD-1)	EH12.1				
CD49a	SR84	CD294 (CRTH2)	BM16				
CD49d	9F10	CD314 (NKG2D)	1D11				
CD56	NCAM16.2	CD335 (NKp46)	9E2/NKP46				
CD62L	DREG-56	CD336 (NKp44)	p44-8				
CD69	FN50	CD366 (TIM-3)	7D3				

Data analysis was initially performed by manually gating the three major groups of circulating ILCs conventionally defined as CD117⁻CD294⁻ ILC1, CD117^{+/-}CD294⁺ ILC2 and CD117⁺CD294⁻ ILC progenitors/ILC3 (ILCP/ILC3) (Figure 2A). We then performed a differential expression analysis between the three ILC groups using single cell AbSeq and mRNA data and identified unique protein and mRNA signatures for each group (Fig 2B). We also observed expected expression patterns for key transcription factors defining ILC groups. For example, the gene encoding T-bet (TBX21) was exclusively expressed in a subset of ILC1 cells, whereas GATA3 was expressed at highest levels in ILC2 cells. As recently reported, RORC, encoding $RORy\delta$, was partially expressed by circulating ILC progenitors displaying an ILC3 phenotype ($CD117^+CD294^-$ ILCP/ ILC 3 cells).

The single cell heatmap also revealed heterogeneity within each ILC group as most of the represented markers were not uniformly expressed by all the cells. These data therefore suggest that additional subsets exist within each group of ILCs. For example, CD4 was exclusively expressed by the majority, but not all of ILC1 cells. This observation is in agreement with previous reports and was further confirmed by bi-variate plot analysis clearly showing discrete subsets of CD4⁺ and CD8⁺ cells within ILC1 but not ILC2 or ILCP/ILC3 cells (Figure 3A). To further investigate the heterogeneity of ILC1 cells, we then performed differential gene and protein expression analysis between the three subsets of CD4⁺CD8⁻, CD4⁻CD8⁺ and CD4⁻CD8⁻ ILC1 cells. Figure 3B shows a single cell heatmap displaying representative protein and gene signatures associated with each subset and illustrated the high heterogeneity and complexity of the ILC1 subset. These data demonstrate the power of a single cell multiomic approach to screen a high number of proteins and genes and discover critical markers required to define and discriminate unique cell subsets within highly heterogeneous samples.

Figure 2A Figure 2B



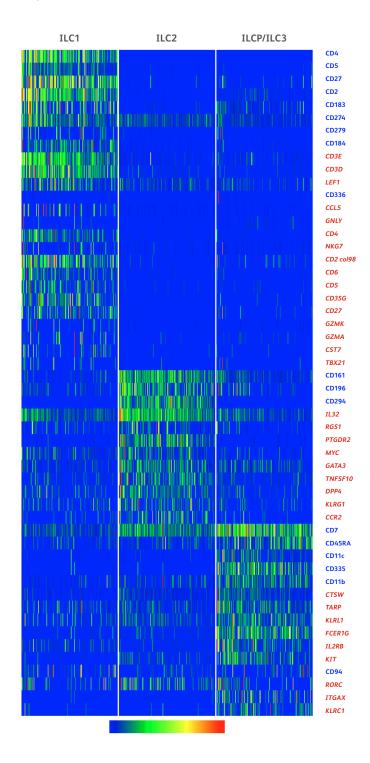
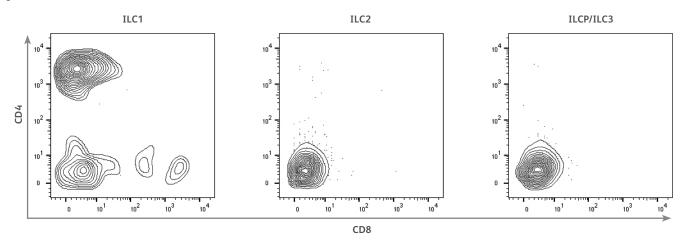


Figure 2. Single cell multiomic analysis reveals heterogeneity of total ILCs

A. Gating strategy for analysis of ILC groups. Cells were gated based on conventional phenotypes, ILC1 (CD117⁻CD294⁻), ILC2 (CD117⁺CD294⁻), ILC progenitor cells ILCP/ILC3 (CD117⁺CD294⁻). B. Single cell heat map displaying representative proteins (blue) and genes (red) differentially expressed between the three main ILC groups.



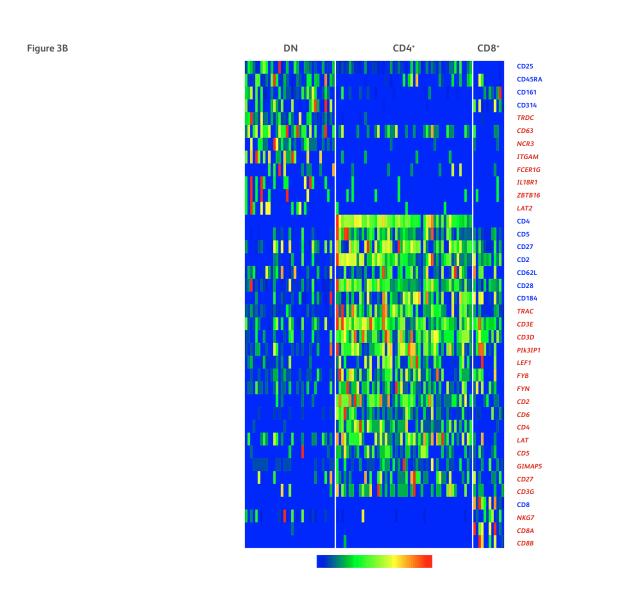


Figure 3. Single cell multiomic analysis reveals heterogeneity of ILC1 cells

A. Contour plots showing CD4 and CD8 expression for three groups of ILCs, demonstrating CD4 and CD8 expression exclusively in ILC1 cells. B. Single cell heat map displaying representative proteins (blue) and genes (red) differentially expressed between double negative (DN), CD4*CD8* (CD4*) and CD4*CD8* (CD8*) ILC1 subsets.

Next, we demonstrate how the information obtained by single cell multiomic screening can be leveraged to design high parameter flow cytometry panels for routine use, for example, to deeply characterize ILCs across a large number of donors or tissues. Of the 42 AbSeq markers tested, our analysis identified 13 surface markers differentially expressed between the three subsets of ILC1. We also identified one differentially expressed gene (*CD63*) coding for the surface protein CD63 (Fig. 3B). We therefore designed a 21-color flow cytometry panel that included lineage markers, markers required for the gating of the three main ILC groups, the 13 differentially expressed markers identified by AbSeq screening, and the surface marker CD63 based on the differential expression of its corresponding gene. The design of this panel was guided and facilitated by the information on relative antigen density and co-expression patterns that were obtained from the single cell multiomic screening (Table 3).

Table 3. List of fluorochrome-conjugated antibodies used for deep characterization of ILC1 cells via high-parameter flow cytometry

Clone	Fluorochrome or Dye	ILC1 subsets		
		DN	CD4⁺	CD8*
N/A	7-AAD	-	-	-
SJ25-C1				
МфР9				
11F2				
IP26	BB700	-	-	-
HI149				
9F5				
V24-785				
AER-37				
UCHT1	BUV661	-	-	-
NCAM16.2	BUV563	-	-	-
HP-3D9	BV786	-	-	-
YB5.B8	BV711	-	-	-
BM16	PE-CF594	-	-	-
HIL-7R-M21	BV421	+	+	+
SK3	APC-H7	-	+	-
RPA-T8	BUV496	-	-	+
UCHT2	BUV805	+/-	++	+
M-T271	APC	+	+	+
	BV605	-	++	++
	. –	+/-	+	+/-
		+	+	+
		+		+
		+	+/-	-
		+/-	++	+
		+	-	+
	,	+	+	-
			+/-	+/-
	N/A SJ25-C1 MфP9 11F2 IP26 HI149 9F5 V24-785 AER-37 UCHT1 NCAM16.2 HP-3D9 YB5.B8 BM16 HIL-7R-M21 SK3 RPA-T8 UCHT2	N/A 7-AAD SJ25-C1 MфP9 11F2 IP26 BB700 HI149 9F5 V24-785 AER-37 UCHT1 BUV661 NCAM16.2 BUV563 HP-3D9 BV786 YB5.B8 BV711 BM16 PE-CF594 HIL-7R-M21 SK3 APC-H7 RPA-T8 BUV496 UCHT2 BUV805 M-T271 APC RPA-2.10 BV605 12G5 PE SK11 BB515 HI100 BU395 2A3 BV480 CD28.2 BV750 DX12 BUV737 H5C6 PE-Cy7 11A9 APC-R700	N/A 7-AAD - SJ25-C1 M\$\text{M\$\text{PP}\$} 11F2 IP26 BB700 - HI149 9F5 V24-785 AER-37 UCHT1 BUV661 - NCAM16.2 BUV563 - HP-3D9 BV786 - YB5.B8 BV711 - BM16 PE-CF594 - HIL-7R-M21 BV421 + SK3 APC-H7 - RPA-T8 BUV496 - UCHT2 BUV805 H-/- M-T271 APC + RPA-2.10 BV605 - 12G5 PE +/- SK11 BB515 + H1100 BUV395 + H1100 BUV395 + CD28.2 BV750 +/- DX12 BUV737 + H5C6 PE-Cy7 + IA9 CPADD - SINTO BUV737 + H5C6 PE-Cy7 + IA9 APC-R700 H INDEX BUV737 + IND	N/A 7-AAD

To confirm equivalent protein measurement via AbSeq and flow cytometry methods, cells from the same donor as used for the single cell multiomic study were used for ILC1 characterization by flow cytometry. After exclusion of lineage and dead cells (dump channel), CD127⁺CD117⁻CD294⁻ ILC1 subset was identified and divided into three groups based on CD4 and CD8 expression (Figure 4A). The expression of each target marker was then assessed within each ILC1 subset (Figure 4B). The protein expression patterns across these three ILC1 subsets were overall consistent with the AbSeq results (Figure 4C) confirming the correlation between AbSeq and flow cytometry. For example, using either method, expression of CD314 (NKG2D) was specifically observed in subsets of DN cells, in the majority of CD8⁺ cells, and not in the CD4⁺ cells.

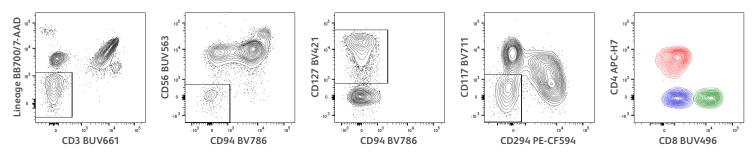
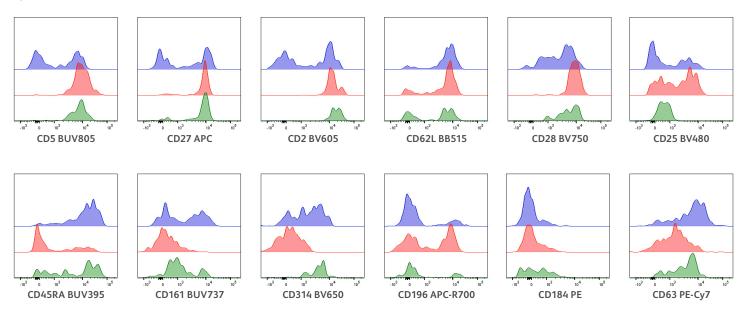


Figure 4B





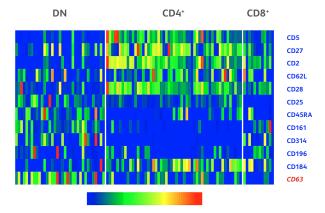


Figure 4. Assessment of markers on the surface of ILC1 cells using a 21-color flow cytometry panel

Fresh PBMCs from the same healthy donor used for the single cell multiomic study were stained with the fluorochrome-conjugated antibodies listed in Table 3. Samples were acquired on the BD FACSymphony A5 Cell Analyzer. A. The gating strategy to define ILC1 cells is shown. Lineage CD3 CD56 CD127 CD117 CD294 ILC1 cells were further gated into three subsets: CD4 CD8 (blue), CD4 CD8 (green). B. Protein expression patterns across the three ILC1 subsets. C. Summary of the AbSeq and scRNA-Seq data described in Figure 3 showing overall consistent protein (blue) expression patterns, as compared to high-parameter flow cytometry analysis. Relatively similar patter expression for the gene CD63 (red) was also observed.

Having demonstrated the quality and validity of the high parameter panel, this assay was then used to investigate the distribution of ILC1 subsets across 4 healthy donors. We used unsupervised computational analysis to eliminate bias introduced by manual gating and to facilitate the analysis of high dimensional data. First, we gated on ILC1 cells and performed dimensionality reduction resulting in the generation of a t-Distributed Stochastic Neighbor (t-SNE) plot for all 4 concatenated donors. Next, we used PhenoGraph algorithm for the identification of distinct ILC1 sub-clusters. To facilitate visualization, the 13 clusters identified by PhenoGraph were overlayed onto the t-SNE map (Figure 5A). Subsequently, the same t-SNE map was generated for each individual donor to visualize differences in cluster distribution. Cluster 3 (orange) is evident only in donor 2 while cluster 4 (green) is present mainly in donors 2 and 4. Figure 5B shows the quantitation of the frequency of cells belonging to each cluster across the 4 donors.

Figure 5A

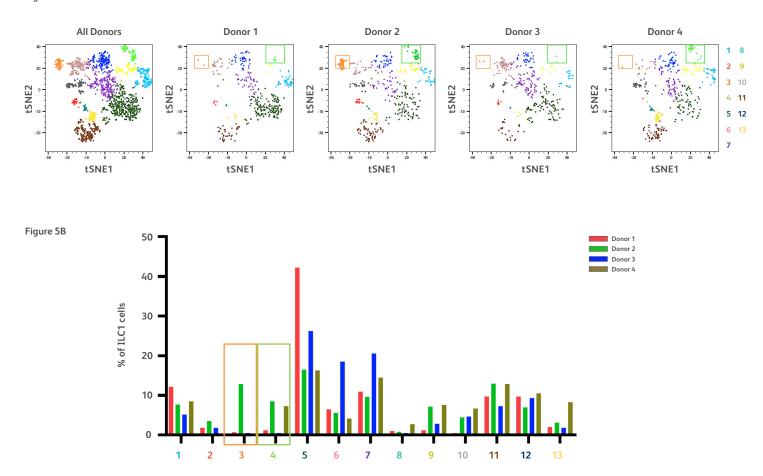


Figure 5. High-dimensional analysis of subpopulations of ILC1 cells across different donors

Fresh leukocytes from four healthy donors were stained with the fluorochrome-conjugated antibodies listed in Table 3. A. Data from all four donors were combined and analyzed by FlowJo PhenoGraph plugin, then shown for each individual donor. B. The distribution of each cluster within the ILC1 subset was quantified across all donors.

We then used the Cluster Explorer FlowJo plug in to characterize the immunophenotype of the cells in each cluster. This tool provides information on the levels of expression of selected markers in cells belonging to the chosen cluster. For example, the cells in cluster 3 were defined by high expression of CD4, CD62L and CD25, and the lack of expression of CD45RA and CD27. A manual gating strategy was developed accordingly to validate the signature and confirm the presence of this CD4⁺CD62L⁺CD45RA⁻CD25⁺CD27⁻ ILC1 subset only in donor 2 (Figure6).

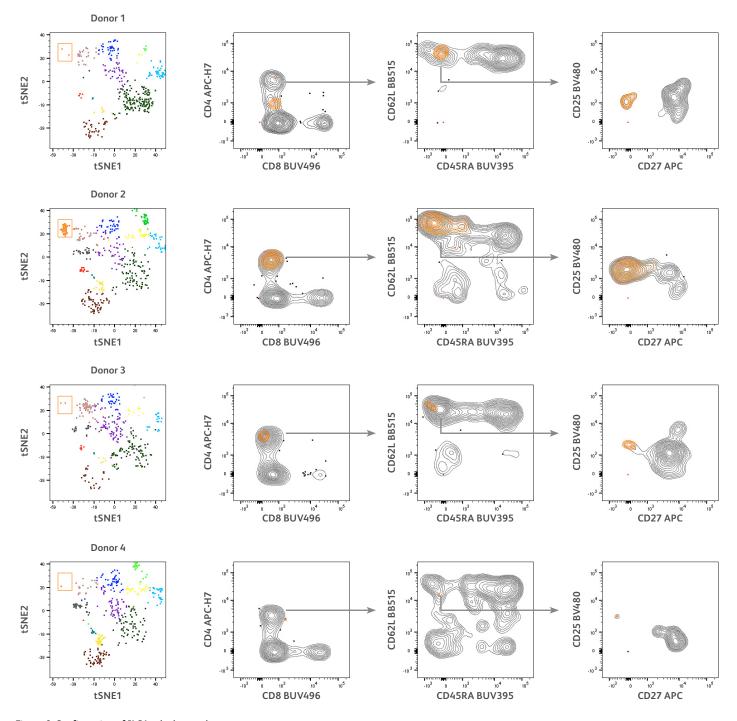


Figure 6. Confirmation of ILC1 sub-cluster phenotype
The phenotype of cluster 3 was validated using conventional manual gating strategy.

In conclusion, we have demonstrated a complete workflow for deep characterization of circulating human ILCs. Cell sorting enabled purification of total ILCs for simultaneous downstream analyses of the expression of 42 proteins and 399 genes. Single cell multiomic analysis revealed complex immunophenotypic and molecular signatures refining the identity of the three main groups of ILCs. The analysis also revealed heterogeneity within ILC1 cells leading to the discovery of markers defining distinct ILC1 cell populations. The biological information provided by the single cell multiomic screening was instrumental for the design of a high parameter flow cytometry panel that could be used to deeply characterize ILC1 cells from multiple donors. Unsupervised computational analysis, mediated by plugins available in FlowJo software, led to the observation of differential distribution of ILC1 cell populations across different donors. It also enabled the identification of a specific cluster that was present in only one of the donors tested. The advent of high parameter cell sorters will enable purification of cells and facilitate the downstream workflows demonstrated here to ultimately define the identity and function of putative new subsets of ILCs.

Product information

Systems and software

Description

 $BD\ Rhapsody^{\scriptscriptstyle{\mathsf{TM}}}\ Single\text{-}Cell\ Analysis\ System$

BD FACSAria™ Fusion Cell Sorter

BD FACSymphony™ A5 Cell Analyzer

FlowJo™

SeqGeq™

Reagents

Description

BD Rhapsody $^{\scriptscriptstyle{\text{M}}}$ Human Immune Response Panel

BD® AbSeq Antibody-oligonucleotide Conjugates

BD Fluorescent Antibodies

BD Rhapsody $^{\!\scriptscriptstyle{\mathrm{M}}}$ Targeted mRNA and AbSeq Reagent Kit

Class 1 Laser Product.

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23-22288-00

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