# A multi-omics approach to investigate the heterogeneity of memory T cells

## Immunology applications for the BD LSRFortessa™ X-20 cell analyzer

**Features**

- Design complex, high-resolution panels of up to 18 colors for a deeper understanding of the biology of immune cells
- Maximize panel resolution and minimize spillover by spreading fluorochromes across laser lines
- Standardize the performance of flow cytometry panels across BD platforms, analyzers and sorters
- Correlate gene expression with protein expression at the single-cell level
- Expand multicolor flow cytometry panels based on biological information obtained from genomic analysis

As researchers extend the boundaries of cellular knowledge and discovery, they need tools for deeper and detailed phenotypic and molecular analyses. For example, the post-thymic activation and development of T cells involves a heterogeneous population of cells at different stages of differentiation, including naïve ($T_N$), stem cell memory ($T_{SCM}$), central memory ($T_{CM}$), effector memory ($T_{EM}$) and terminal effector T cells ($T_{Eff}$). Studies have shown that these different T-cell subsets are themselves highly heterogeneous, and their identification and characterization requires not one or a few but, rather, a combination of several markers. This heterogeneity has heightened interest within the scientific community to find new markers that might provide insights into the T-cell differentiation and development pathway and potentially identify new cell populations. Such exploratory studies require development of multi-omics approaches that combine high-dimensional flow cytometry and single-cell RNA sequencing (scRNA-Seq) for phenotypic and genomic analyses at the single-cell level.

BD

Figure 1

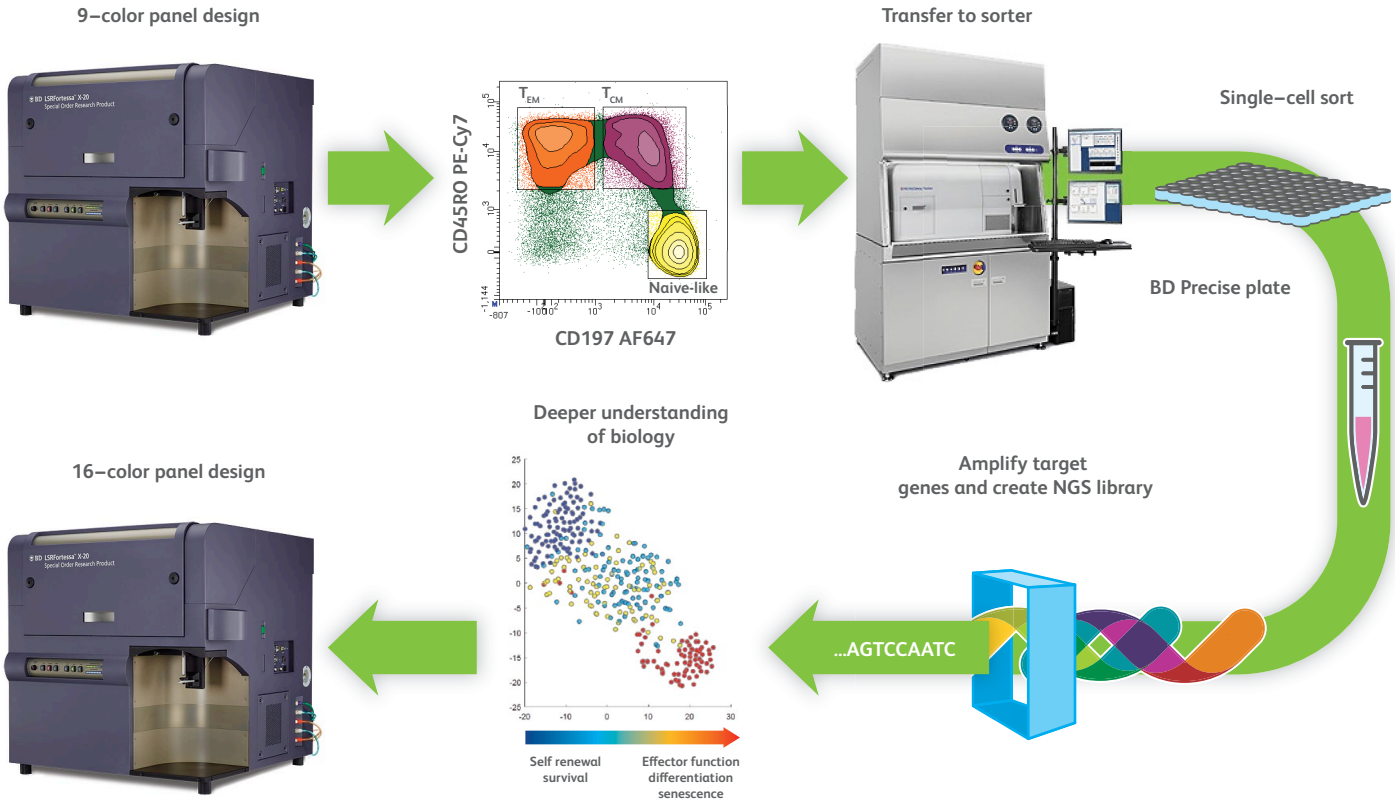


**Figure 1. Multi-omics workflow to identify markers for deeper characterization of immune cells**

A 9-color panel was optimized on a BD LSRFortessa X-20 cell analyzer for detection of $CD8^+$ $T_N$, $T_{SCM}$, $T_{CM}$ and $T_{EM}$ subsets, and then transferred to a BD FACSAria Fusion cell sorter for single-cell isolation of these subsets. Cells were deposited individually into a BD™ Precise plate, followed by target gene amplification, library preparation and scRNA-Seq. Differential gene-expression analysis was performed to identify genes encoding surface markers that might better identify the $CD8^+$ memory cell subsets. Finally, a 16-color panel was designed based on the RNA-Seq analysis, including potential markers to discriminate different $CD8^+$ subsets.

With up to 5 lasers and 18 fluorescence parameters, the BD LSRFortessa™ X-20 cell analyzer enables the development of integrated workflows to investigate these phenotype-genotype relationships. In this data sheet, we describe a complete workflow solution (Figure 1) in which a BD cell analyzer, a fluorescence-activated cell sorter and a sequencing-ready library preparation platform are used to identify, sort and characterize different $CD8^+$ T-cell subsets at the single-cell level.

We began by designing a 9-color panel that was optimized on the BD LSRFortessa X-20 analyzer for clearly resolving the $CD8^+$ T-cell subsets. This optimized panel was transferred to a BD FACSAria™ Fusion cell sorter to isolate single cells into BD™ Precise 96-well plates for downstream genomic analysis. We used scRNA-Seq to analyze gene expression of 200 T-cell specific genes, to identify markers that might further differentiate T cell subsets of interest. Taking advantage of the BD LSRFortessa X-20 system's multiple detectors, we designed an expanded 16-color flow cytometry panel to validate those markers' usefulness for further phenotypic analysis.

Table 1 shows the 9-color panel designed to identify $CD8^+$ $T_N$, $T_{SCM}$, $T_{CM}$ and $T_{EM}$ cell subsets on the BD LSRFortessa X-20.

Fluorochromes were chosen and assigned to each marker considering antigen co-expression and density as well as fluorochrome brightness and spillover. By distributing fluorochromes across the five lasers and accounting for cross-laser excitation and emission spectra, it is possible to design multicolor panels with minimal spectral overlap and maximum resolution.

**Table 1. Nine-color T-cell subset panel for cell analyzer and sorter**

BD LSRFortessa X-20 5-laser cell analyzer configuration: B-2, R-3, YG-3, V-6, UV-4
BD FACSAria Fusion 5-laser cell sorter configuration: B-2, R-3, YG-4, V-6, UV-3

| Marker | Clone | Laser | Fluorochrome/Dye |
|---|---|---|---|
| CD8 | RPA-T8 | Ultraviolet (355 nm) | BUV395 |
| CD27 | M-T271 | Ultraviolet (355 nm) | BUV737 |
| CD183 | 1C6/CXCR3 | Violet (405 nm) | BV421 |
| CD3 | UCHT1 | Violet (405 nm) | BV510 |
| CD45RA | HI100 | Blue (488 nm) | BB515 |
| CD19/CD14/CD4 | HIB19/MφP/SK3 | Blue (488 nm) | PerCP-Cy™5.5 |
| Live/Dead | — | Blue (488 nm) | 7-AAD |
| CD95 | DX2 | Yellow-Green (561 nm) | PE |
| CD45RO | UCHL1 | Yellow-Green (561 nm) | PE-Cy™7 |
| CD197 (CCR7) | 150503 | Red (640 nm) | Alexa Fluor® 647 |

Figure 2 shows the gating strategy used to identify $CD8^+$ T-cell subsets. After excluding lineage and dead cells, $CD3^+CD8^+$ cells were gated for analysis of CD45RO and CD197 expression. $T_{EM}$ and $T_{CM}$ cells were defined as $CD45RO^+CD197^-$ and $CD45RO^+CD197^+$ respectively. The $CD45RO^-CD197^+$ naïve-like population contains $T_N$ cells and a population of long-lived memory T cells endowed with enhanced self-renewal and multipotency. These cells, called stem cell memory T cells ($T_{SCM}$), can be distinguished from the $T_N$ cells based on high expression of CD183 (CXCR3) and CD95.[1] CD27 and CD45RA were also included in the panel to further illuminate the heterogeneity of $CD8^+$ cells as shown by the presence of noncytotoxic $CD45RA^-CD27^+$ memory cells and cytotoxic $CD45RA^+CD27^-$ effector cells.[2]

Figure 2A

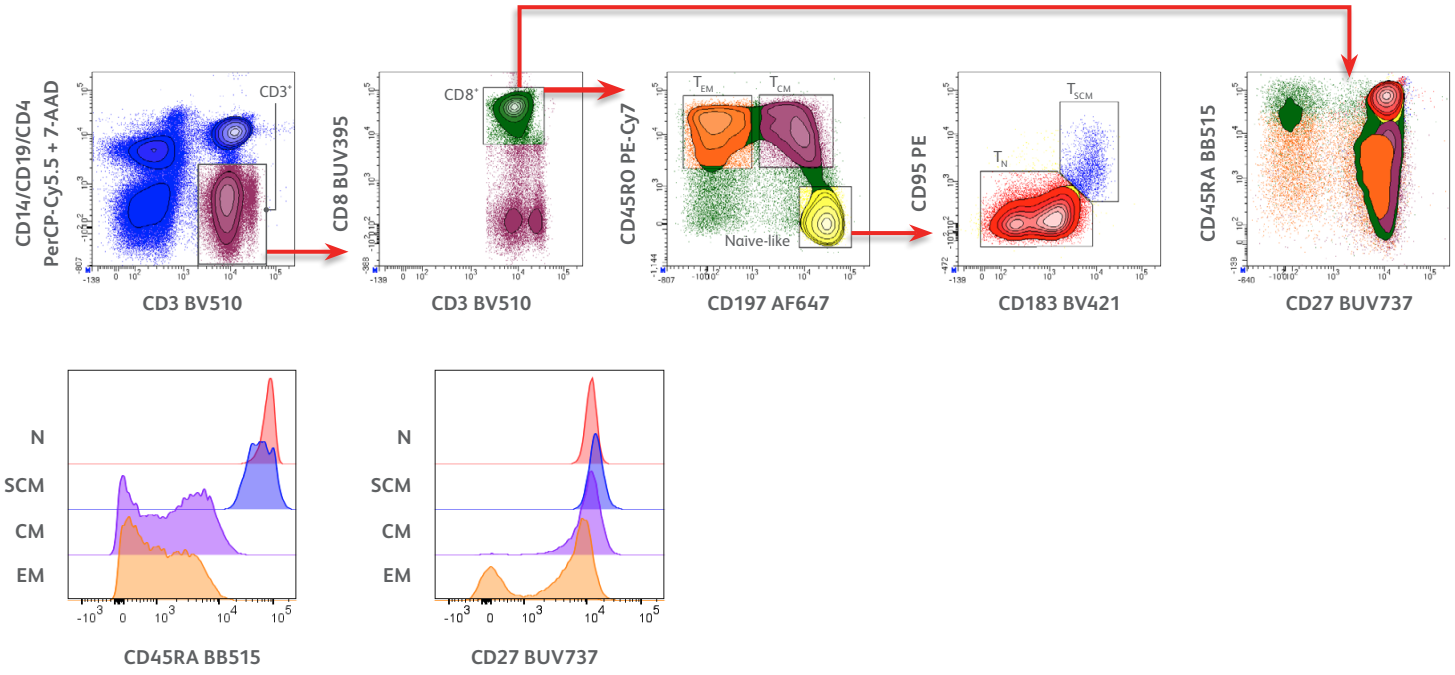


Figure 2B

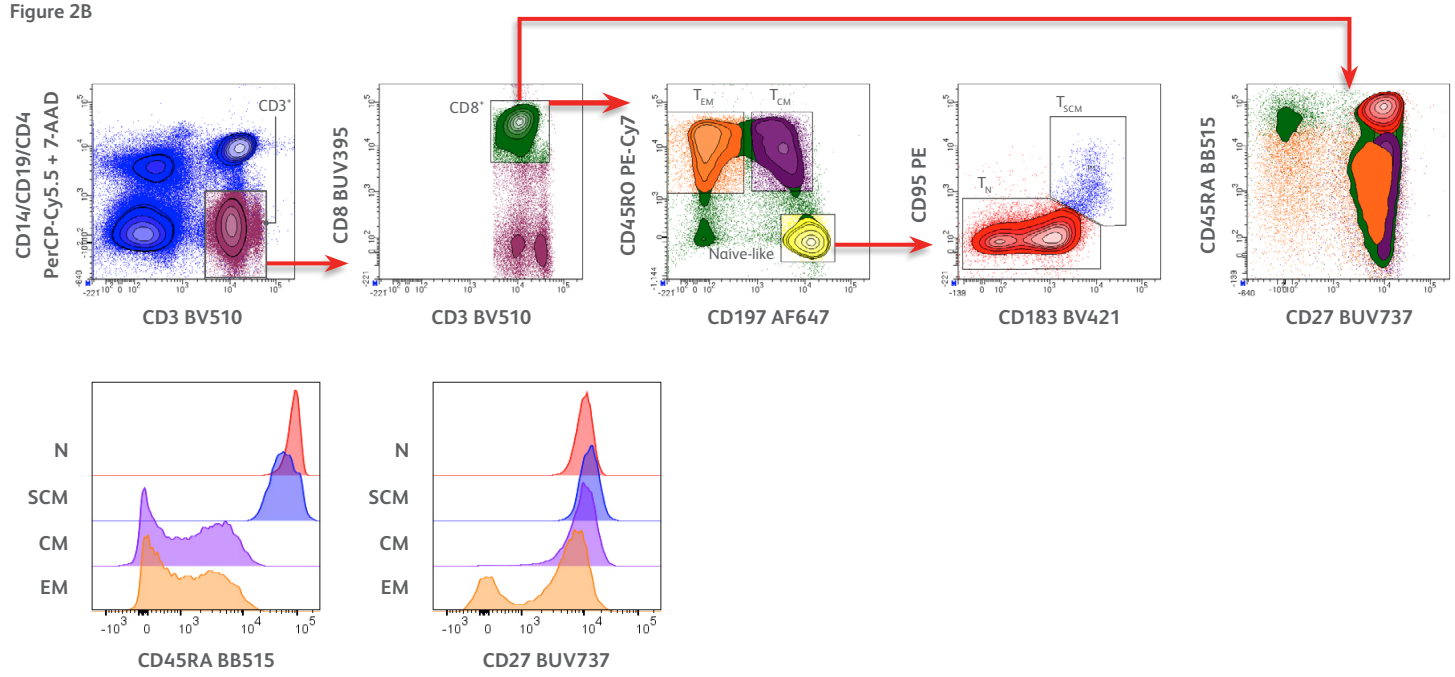


**Figure 2. Nine-color $CD8^+$ T-cell panel on the BD LSRFortessa X-20 and BD FACSAria Fusion systems**

**Method:** Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor. Cells were pre-stained with CD197 (CCR7) at 37°C. After 15 minutes, cells were stained with a premade cocktail of antibodies (Table 1) and BD Horizon™ Brilliant Stain Buffer at RT for 30 minutes. BD Pharmingen™ 7-AAD was added before acquisition to exclude dead cells from analysis. **Instruments:** Samples from the same staining were acquired on both **(A)** the BD LSRFortessa X-20 cell analyzer and **(B)** the BD FACSAria Fusion cell sorter with standardized mean fluorescence intensity (MFI) target values to maintain consistency between instruments. Data was analyzed using both BD FACSDiva™ and FlowJo® software. **Gating strategy:** Red arrows denote gating hierarchy. $CD8^+$ T cells were identified by excluding doublet cells and defined by lineage/7-$AAD^-$ populations and $CD3^+$ T cells. From the $CD8^+$ population, naïve-like ($CD45RO^-CCR7^+$), central memory ($T_{CM}$, $CD45RO^+CCR7^+$) and effector memory T cells ($T_{EM}$, $CD45RO^+CCR7^-$) were identified. Stem cell memory ($T_{SCM}$, $CD183^{high}CD95^+$) and naïve T cells ($T_N$, $CD183^{+/-}CD95^-$) were gated from the naïve-like T cells. Biexponental scaling for each instrument was set for optimal visualization. Overall, the target populations were similarly resolved upon cross-platform standardization.

With the subset identification panel optimized on the BD LSRFortessa X-20 cell analyzer, we transferred it to the BD FACSAria Fusion cell sorter to enable single-cell isolation of the cells for downstream genomic analysis. We standardized across platforms by staining human whole blood with the BD Horizon™ CD4 Fluorochrome Evaluation Kit and adjusting voltages on the BD FACSAria Fusion to match the MFI recorded on the BD LSRFortessa X-20. This approach resulted in overall similar resolution and clear detection of the target T-cell subsets without designing or optimizing a panel specifically for cell sorting (Figure 2B).

The cells were then sorted individually into a BD Precise 96-well plate for downstream genomic analysis. BD Precise kits contain a complete set of reagents for preparation of a sequencing-ready library. BD Precise kits also incorporate molecular indexing technology that enables highly accurate mRNA molecule quantification and eliminates noise that would otherwise result from PCR amplification bias. Finally, the technology allows samples from each well to be pooled into a single tube while maintaining the identity of each sample.

Following targeted scRNA-Seq using a panel of 200 T-cell-specific genes, we performed a comparative analysis of the gene expression profiles from the sorted $CD8^+$ cell subsets. We specifically focused on the analysis of genes coding for surface markers that could be prospectively used for flow cytometry analysis and sorting, upon validating the correlation between mRNA and protein expression. We identified four candidate genes that were differentially expressed in $T_N$, $T_{SCM}$, $T_{CM}$ and $T_{EM}$ $CD8^+$ subsets, which coded for the surface markers CD161, CD314, CD127 and CD184. For example, CD161 mRNA expression was exclusive to $T_{CM}$, and $T_{EM}$ cells, while CD184 and CD314 mRNA expression was higher in $T_{SCM}$ than in other subsets (data not shown).

Taking advantage of the many fluorescent parameters available on the BD LSRFortessa X-20, we expanded the original 9-color panel to a new 16-color panel (Table 2) to validate the differential protein expression of the gene markers identified from the scRNA-Seq study. The panel included two additional markers, CD58 and CD122, previously reported to improve the identification of $T_{SCM}$,[1,3] as well as CD4, to comprehensively and simultaneously characterize memory subsets of both $CD4^+$ and $CD8^+$ T cells. This approach significantly increases the amount of biological information that can be obtained from a single tube.

**Table 2. Sixteen-color T-cell subset panel with expanded marker set**

BD LSRFortessa X-20 5-laser cell analyzer configuration: B-2, R-3, YG-3, V-6, UV-4

| Marker | Clone | Laser | Fluorochrome/Dye |
|---|---|---|---|
| CD8 | RPA-T8 | Ultraviolet (355 nm) | BUV395 |
| CD127 | HIL-7R-M21 | Ultraviolet (355 nm) | BUV737 |
| CD183 | 1C6/CXCR3 | Violet (405 nm) | BV421 |
| CD161 | DX12 | Violet (405 nm) | BV510 |
| CD184 | 12G5 | Violet (405 nm) | BV605 |
| CD45RO | UCHL1 | Violet (405 nm) | BV650 |
| CD58 | 1C3 | Violet (405 nm) | BV711 |
| CD4 | SK3 | Violet (405 nm) | BV786 |
| CD122 | Mik-β3 | Blue (488 nm) | BB515 |
| CD19/CD14 | HIB19/MϕP | Blue (488 nm) | PerCP-Cy5.5 |
| Live/Dead | — | Blue (488 nm) | 7-AAD |
| CD95 | DX2 | Yellow-Green (561 nm) | PE |
| CD197 (CCR7) | 150503 | Yellow-Green (561 nm) | PE-CF594 |
| CD314 | 1D11 | Yellow-Green (561 nm) | PE-Cy7 |
| CD27 | M-T271 | Red (640 nm) | APC |
| CD45RA | HI100 | Red (640 nm) | APC-H7 |
| CD3 | UCHT1 | Red (640 nm) | Alexa Fluor® 700 |

The flow cytometry data (Figure 3) partially recapitulated and extended the results of the genomic analysis. For example, consistent with the scRNA-Seq data, CD161 was exclusively expressed in $T_{CM}$ and $T_{EM}$ cell subsets. These findings agree with previous characterizations of $CD161^+$ T cells, validating the multi-omics approach for identification of subset-specific markers that are differentially expressed at the molecular and protein level. We observed similar expression patterns for $CD4^+$ and $CD8^+$ subsets for all markers tested except CD314, which was expressed by all $CD8^+$ subsets but not by $CD4^+$ subsets.

The experiment demonstrates the usefulness of integrating flow-cytometry-based cell analysis and sorting with downstream genomic analysis to discover potential novel markers that can better identify and characterize cell populations of interest. scRNA-seq allows for the screening of a large number of genes. The list of prospective markers for phenotypic discrimination and cell sorting can be narrowed down after differential gene expression analysis, selection of genes coding for surface markers and validation by flow cytometry. The BD LSRFortessa X-20 lets you analyze more markers in a single tube by spreading key protein markers across multiple laser lines and detectors, allowing you to delve deeply into the biology of individual lineages and discover interrelationships between markers that would be lost across multiple tubes.

Figure 3A

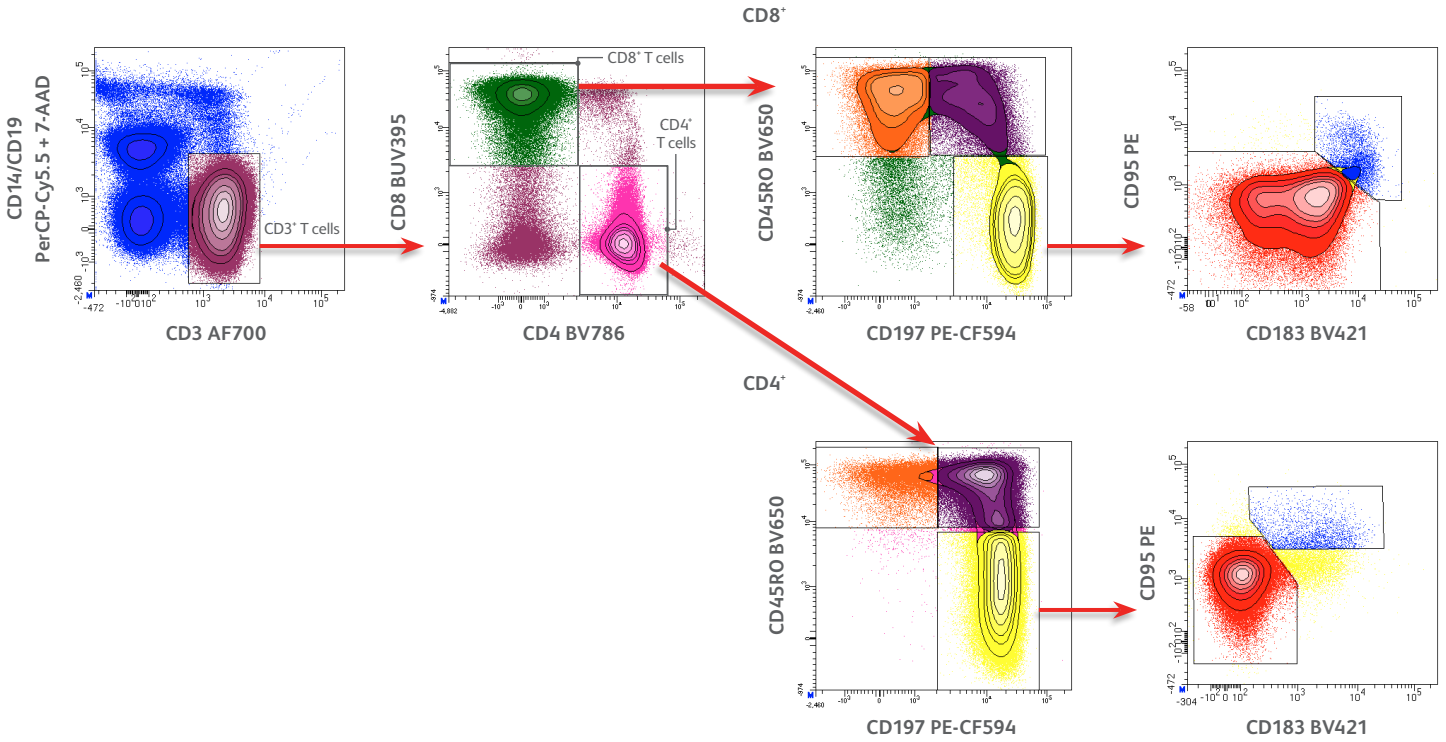


Figure 3B

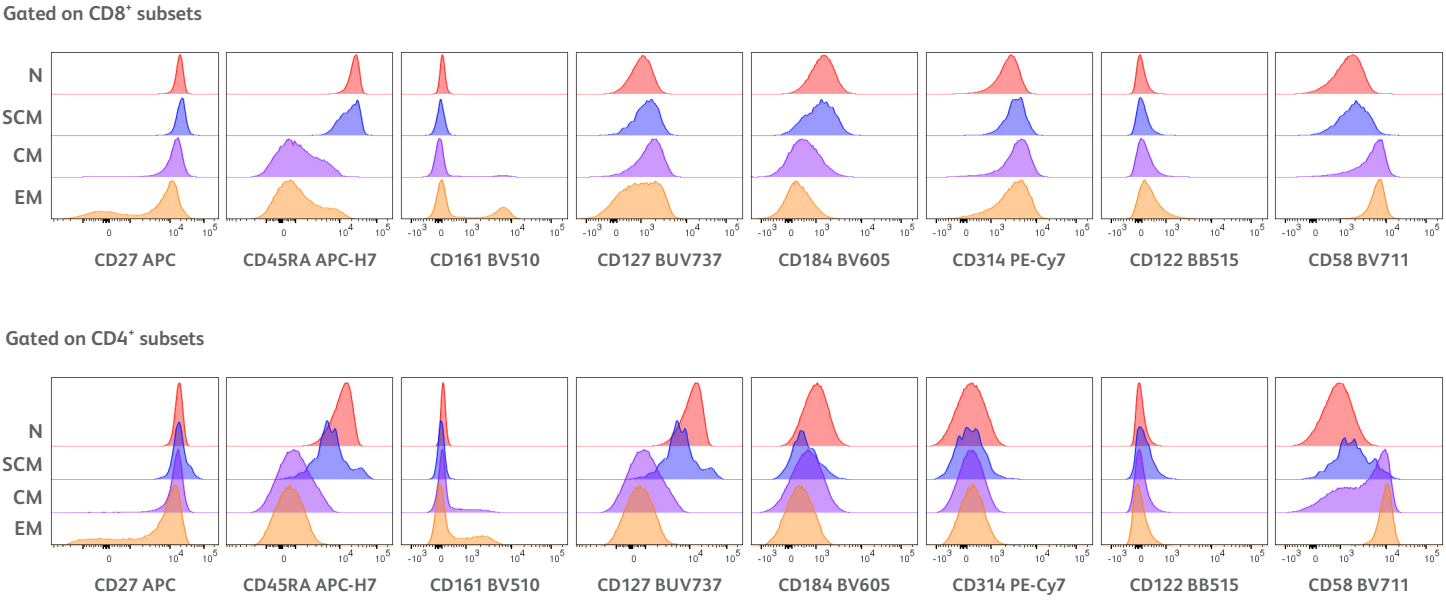


**Figure 3. Sixteen-color CD8⁺ and CD4⁺ T-cell panel on the BD LSRFortessa X-20 cell analyzer**

The 9-color CD8⁺ T-cell panel (Figure 1) was expanded to 16 colors on the BD LSRFortessa X-20 to analyze the expression level of markers reported in the literature and identified from the scRNA-Seq analysis. The staining protocol and gating strategy **(A)** were similar to Figure 2, adding additional markers and gates for CD4⁺ as well as CD8⁺ subsets. **Results (B):** Histogram overlays show expression levels of potentially differentiating markers within CD8⁺ and CD4⁺ subsets. The data provides a comprehensive phenotypic analysis of multiple T-cell subsets. For example, CD161 was exclusively expressed in $T_{CM}$ and $T_{EM}$ cells, in agreement with the gene expression analysis and previous publications. CD58 expression gradually increased as cells differentiated toward the $T_{EM}$ phenotype. CD314 was uniformly expressed by all CD8⁺ subsets but by no CD4⁺ subsets.


References

1. Gattinoni L, Lugli E, Ji Y, et al. A human memory T-cell subset with stem cell-like properties. *Nat Med*. 2011;17:1290–1297.
2. Hamann D, Baars PA, Rep MG, et al. Phenotypic and functional separation of memory and effector human CD8⁺ T cells. *J Exp Med*. 1997;186:1407-18.
3. Lugli E, Gattinoni L, Roberto A, et al. Identification, isolation and in vitro expansion of human and nonhuman primate T stem cell memory cells. *Nat Protoc*. 2013;8:33-42.

## Systems and software

| Description |
|---|
| BD LSRFortessa™ X-20 Cell Analyzer – Special Order |

## Ordering information: Reagents

| Description | Cat. No. |
|---|---|
| BD Pharmingen™ Alexa Fluor® 700 Mouse Anti-Human CD3 | 557943 |
| BD Horizon™ BV510 Mouse Anti-Human CD3 | 563109 |
| BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD4 | 566316 |
| BD Horizon™ BV786 Mouse Anti-Human CD4 | 563877 |
| BD Horizon™ BUV395 Mouse Anti-Human CD8 | 563795 |
| BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD14 | 562692 |
| BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD19 | 561295 |
| BD Horizon™ BUV737 Mouse Anti-Human CD27 | 564301 |
| BD Pharmingen™ APC Mouse Anti-Human CD27 | 561786 |
| BD Pharmingen™ APC-H7 Mouse Anti-Human CD45RA | 560674 |
| BD Horizon™ BB515 Mouse Anti-Human CD45RA | 564552 |
| BD Pharmingen™ PE-Cy™7 Mouse Anti-Human CD45RO | 560608 |
| BD Horizon™ BV650 Mouse Anti-Human CD45RO | 563750 |
| BD OptiBuild™ BV711 Mouse Anti-Human CD58 | 742729 |
| BD Pharmingen™ PE Mouse Anti-Human CD95 | 555674 |
| BD Horizon™ BB515 Mouse Anti-Human CD122 | 564688 |
| BD Horizon™ BUV737 Mouse Anti-Human CD127 | 564300 |
| BD Horizon™ BV510 Mouse Anti-Human CD161 | 563212 |
| BD Horizon™ BV421 Mouse Anti-Human CD183 (CXCR3) | 562558 |
| BD OptiBuild™ BV605 Mouse Anti-Human CD184 (CXCR4) | 740418 |
| BD Horizon™ PE-CF594 Mouse Anti-Human CD197 (CCR7) | 562381 |
| BD Pharmingen™ Alexa Fluor® 647 Mouse Anti-Human CD197 (CCR7) | 560816 |
| BD Pharmingen™ PE-Cy™7 Mouse Anti-Human CD314 (NKG2D) | 562365 |
| BD Pharmingen™ 7-AAD | 559925 |
| BD Horizon™ Human CD4 Fluorochrome Evaluation Kit | 566352 |
| BD Horizon™ Brilliant Stain Buffer | 563794 |

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