Assay transfer of two 12-color panels for CAR detection and assessment of T-cell function using a BD FACSLyric™ Flow Cytometer

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Abstract

We compared assay transfer results between instruments for two 12-color immunophenotyping panels applied to identify CAR T-cells (anti-CD19 CAR/NFAT Jurkat cell line) and differentiate them from monocytes, B-, T-, and NK-cells along with assessing markers of T-cell function. Both panels share nine fluorescent conjugates: a set of seven backbone markers to identify the main cell subsets (CD45-BV786, CD3-BV510, CD4-BV711, CD8-APC-H7, CD19-R718, CD16/56-RB780, and CD14-BV605), a viability dye (7-AAD), and a CAR detection reagent (CD19Ag-PE). Panels differed on three markers applied to characterize Effector and Memory (E&M) T-cells (CD45RA-BB515, CCR7-APC, and CD95-BV421) or to characterize exhaustion states (TIM3-BB515, LAG3-AF647, and PD-1-BV421). Fluorochrome assignment was optimized to ensure clear resolution of target cell populations on a BD FACSLyric™ Flow Cytometer. Cells from healthy donor blood and CAR T cells were stained and processed with each panel, then mixed before sample acquisition. For the assessment of exhaustion markers, cells were stimulated in cultures for 72h prior to staining. Settings were optimized and samples acquired on a BD FACSLyric™ Flow Cytometer then successfully reacquired on two different BD FACSLyric™ Flow Cytometer, after transferring the settings using the BD FACSuite™ Application assay export and transfer feature. To compare across three instruments, CV% in MFI of T, B, NK, and CAR-T cells ranged 0.7%-6.2% for E&M panel, 1.7%-5.7% for T-exhaustion panel; while CV% in %Parent ranged 0.2%-3.0% for E&M panel, 0.2%-1.0% for T-exhaustion panel. Results of assay transfer for the two 12-color immunophenotyping panels were satisfactory.

Methods

Sample preparation:

- Study samples are composed of white blood cells (WBC) or Peripheral Blood Mononuclear Cells (PBMC) obtained from healthy volunteers spiked with CAR T cells [Anti-CD19 CAR / NFAT (Luciferase) Reporter Jurkat Cell Line (from BPS Biosciences)].
- For the assessment of T-Exhaustion markers, PBMC and CAR T cells were activated in cell culture for 72h prior to staining using Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (ThermoFisher Scientific).
- WBC were prepared from NH_4Cl lysing method of whole blood using BD Pharm Lyse™ Lysing Buffer. PBMC were isolated using gradient centrifugation with Ficoll-Paque™ PLUS (GE Healthcare).
- Staining Tube 1: contains normal donor WBC or PBMC to be stained with 10 antibody reagents in a panel (excluding CD19Ag-PE and 7AAD) plus the BD Horizon™ Brilliant Stain Buffer (BSB).
- Staining Tube 2: contains CAR-T cells to be stained with 8 antibody reagents in a panel (excluding B, NK, monocyte antibodies and 7-AAD) plus BSB.
- Stain cells in Tube 1 and Tube 2 for 30 min in the dark. WBC, PBMC and CAR T cells were washed twice with wash buffer (PBS with 0.5%BSA and 0.1%NaN_3).
- The stained/washed CAR-T cells were spiked into the stained/washed WBC or PBMC.
- 7-AAD was added to cells and incubated for 20 min before acquisition.

Table 1. Liquid antibody reagents used in this study

	Backbone markers							Viability	CAR T	T cell subpopulation markers		
Fluorochrome	BV510	BV711	APC-H7	R718	RB780	BV605	BV786	7AAD	PE	BB515	APC or AF647	BV421
Effector & Memory Panel	CD3	CD4	CD8	CD19	CD16+56	CD14	CD45	7AAD	CD19Ag	CD45RA	CD197 (CCR7)	CD95
Exhaustion Panel	CD3	CD4	CD8	CD19	CD16+56	CD14	CD45	7AAD	CD19Ag	CD366 (TIM-3)	CD223 (LAG-3)	CD279 (PD-1)

Sample acquisition criteria:

- Sample acquisition and analysis were accomplished using BD FACSuite Application software.
- CAR T-spiked WBC or PBMC samples were acquired on three FACSLyric instruments from the same tube.
- Stopping criteria was 30,000 CD3+ T cells.
- Data were analyzed to identify CAR T cells, T, B, NK, monocytes and T cell subsets.
- Cell percentages and MFI were generated and compared across three instruments.

Assay Transfer across three BD FACSLyric™ Flow Cytometers

- Performance QC (PQC) was performed on Instrument #1
- Tube-target values and Reference Settings were established using single-color controls for each panel in Experiment Mode
- On Instrument #1, user-defined Assays were created for each of the two 12-color panels (Effector & Memory and Exhaustion) and samples were acquired using a worklist.
- Both assays were exported from Instrument #1 and imported into the library of Instrument #2 and Instrument #3 after running PQC on both instruments.
- Assay/Tube Settings Setup was run on Instruments #2 and #3.
- Stained samples from the same lot were acquired on instruments #2 and #3 for both the Effector & Memory and the Exhaustion Panels

Consistent data across three BD FACSLyric™ Flow Cytometers after assay transfer using BD FACSuite™ Analysis Application

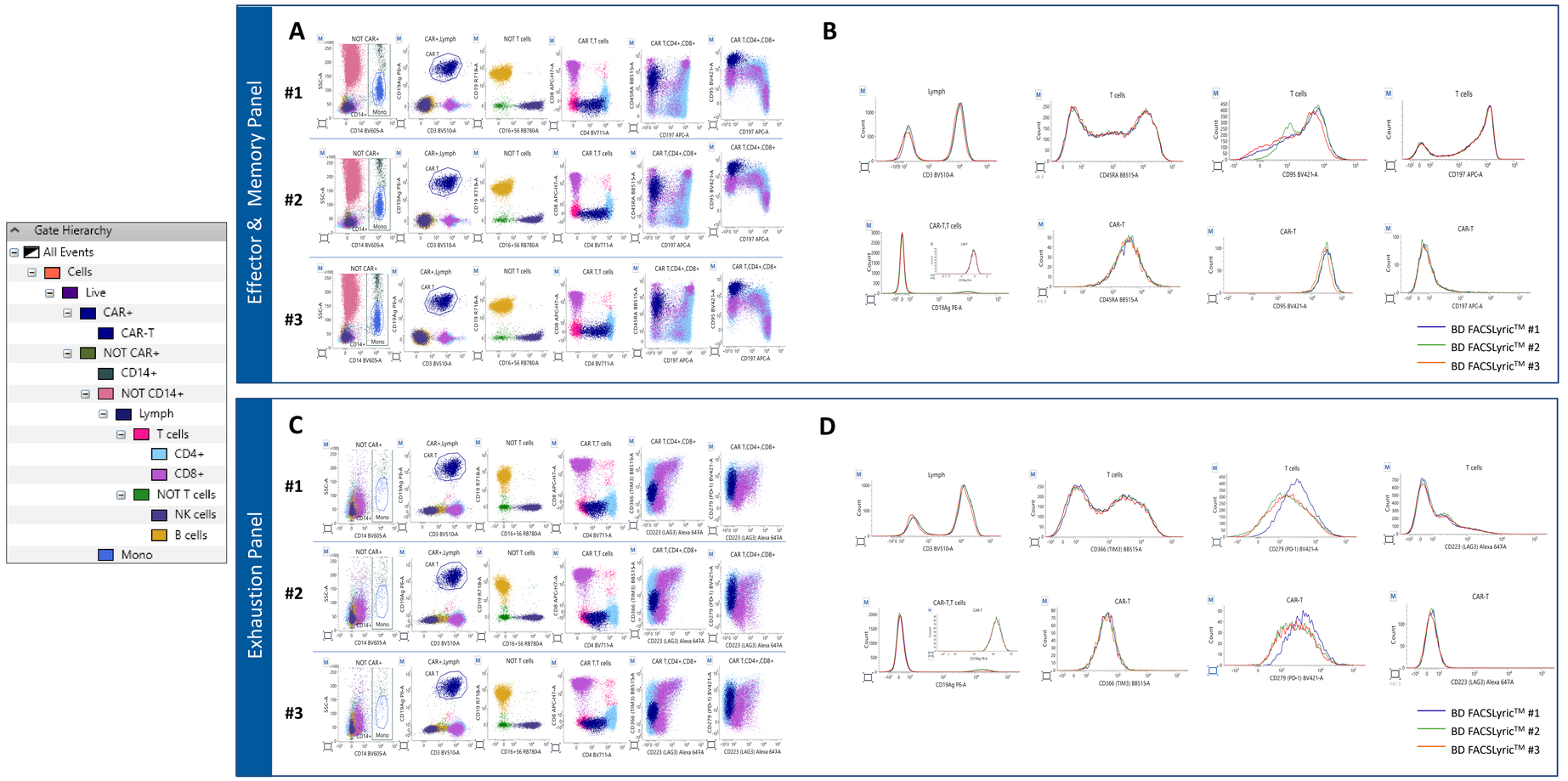
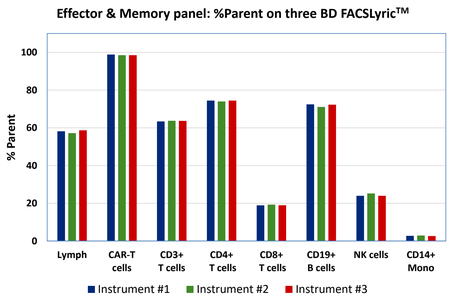


Figure 1: Consistent data across three BD FACSLyric™ Flow Cytometers after two assay transfers using BD FACSuite™ Analysis Application. 2D dot plots for the 12-color Effector & Memory (**A**) and Exhaustion (**C**) panels show similar distribution patterns and gating for different cell subsets, including spiked CAR T cells, across all three instruments (#1, #2, #3). The histogram overlay illustrates consistent MFI across all three laser lines and on all three instrument for lineage and functional makers for T cells as well as CAR T cells in both panels (**B** and **D**).

Reproducible population frequency and MFI is achieved with the assay transfer feature on the BD FACSLyric™ Flow Cytometer



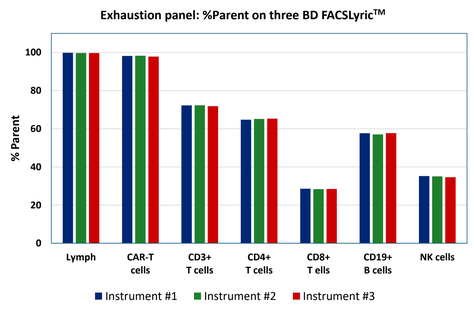
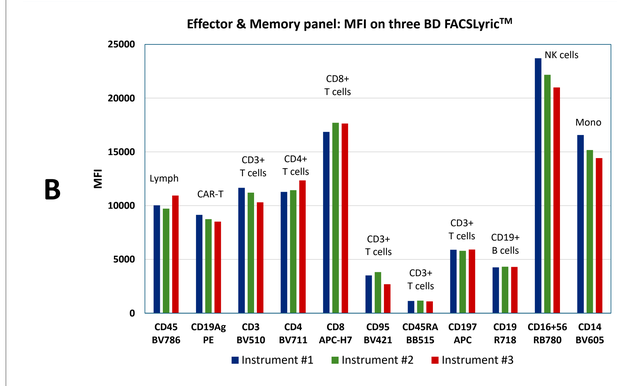


Table 2

	E&M panel (WBC and CAR T cells)			T-Exhaustion panel (activated PBMC and CAR T cells)		
Cell population	Average %Parent	SD	CV% of %Parent	Average %Parent	SD	CV% of %Parent
Lymphocytes	58.0	0.74	1.3	99.7	0.0	0.0
Monocytes	2.8	0.16	6.0	NA**	NA**	NA**
CAR-T cells	98.6	0.2	0.2	98.1	0.2	0.2
CD3+ T cells	63.6	0.2	0.3	72.1	0.2	0.3
CD4+ T cells	74.3	0.3	0.4	65.1	0.3	0.4
CD8+ T cells	19.1	0.2	1.1	28.5	0.1	0.3
CD19+ B cells	71.9	1.1	1.1	57.5	0.38	0.7
CD16+CD56+ NK cells	24.4	0.7	3.0	35.0	0.34	0.7



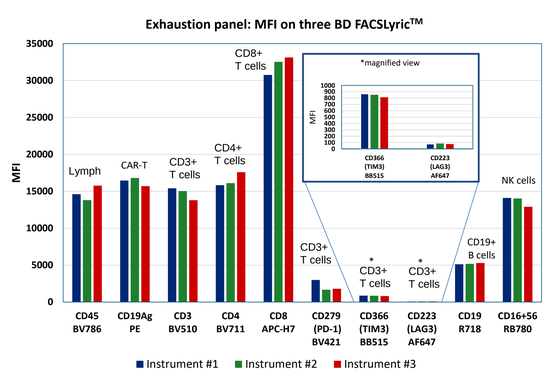


Table 3

		E&M Panel (WBC and CAR T cells)			T-Exhaustion Panel (activated PBMC and CAR T cells)		
Cell population	Channel	Average MFI	SD	MFI CV%	Average MFI	SD	MFI CV%
Lymphocytes	BV786	10233.3	631.3	6.2	14724.3	987.2	6.7
Monocytes	BV605	15134	1030.7	6.8	NA**	NA**	NA**
CAR-T cells	PE	8798.0	320.3	3.6	16322	565.1	3.5
CD3+ T cells	BV510	11058.0	690.3	6.2	14744	840.3	5.7
CD4+ T cells	BV711	11685.7	573.5	4.9	16504.7	946.3	5.7
CD8+ T cells	APC-H7	17402.7	469.4	2.7	32138	1235.8	3.8
CD19+ B cells	R718	4290.0	31.8	0.7	5194	86.1	1.7
CD16+CD56+ NK cells	RB780	22292.0	1363.2	6.1	13679.7	664.8	4.9

Figure 2: Consistent population frequencies and Median Fluorescence Intensity (MFI) across all three BD FACSLyric™ Flow Cytometers. Frequency of each population within the sample was reproduced across instruments (**A**), with CVs less than 3.0% for lymphocyte subsets in both panels and of 6.0% for the monocyte population in the Effector & Memory Panel (**Table 2**). MFI of each population was also consistent across instruments (**B**), with CVs below 6.8% for all cell populations on both Effector & Memory and Exhaustion panels (**Table 3**).

**The %CV of monocytes in the Exhaustion panel is not shown due to dramatic cell depletion after 72h-activation of PBMC using CD3/CD28 beads. Refer to Figure 1, CD14 BV605 vs SSC plots.

Conclusions

Using the assay transfer feature in the BD FACSuite™ Software, two user-defined assays were transferred across three BD FACSLyric™ Flow Cytometers. Target MFI from Instrument #1 were reproduced on Instruments #2 and #3 with %CV of 7.1 or less across all populations, indicating reproducible conditions across instruments. The frequency of each population within the sample was consistent across instruments, with CV < 3.0% for lymphocyte subsets in both panels and equal to 6.0% for monocytes in the Effector & Memory panel.
Altogether, these results indicate that the assay portability feature of the BD FACSLyric™ Flow Cytometer ensured reproducibility of conditions required for cell identification and characterization based on the phenotypic expression of markers used in two different 12-color panels and with two different sample types (WBC and PBMC, both spiked with Jurkat CAR T cells) across three different BD FACSLyric™ Flow Cytometers.