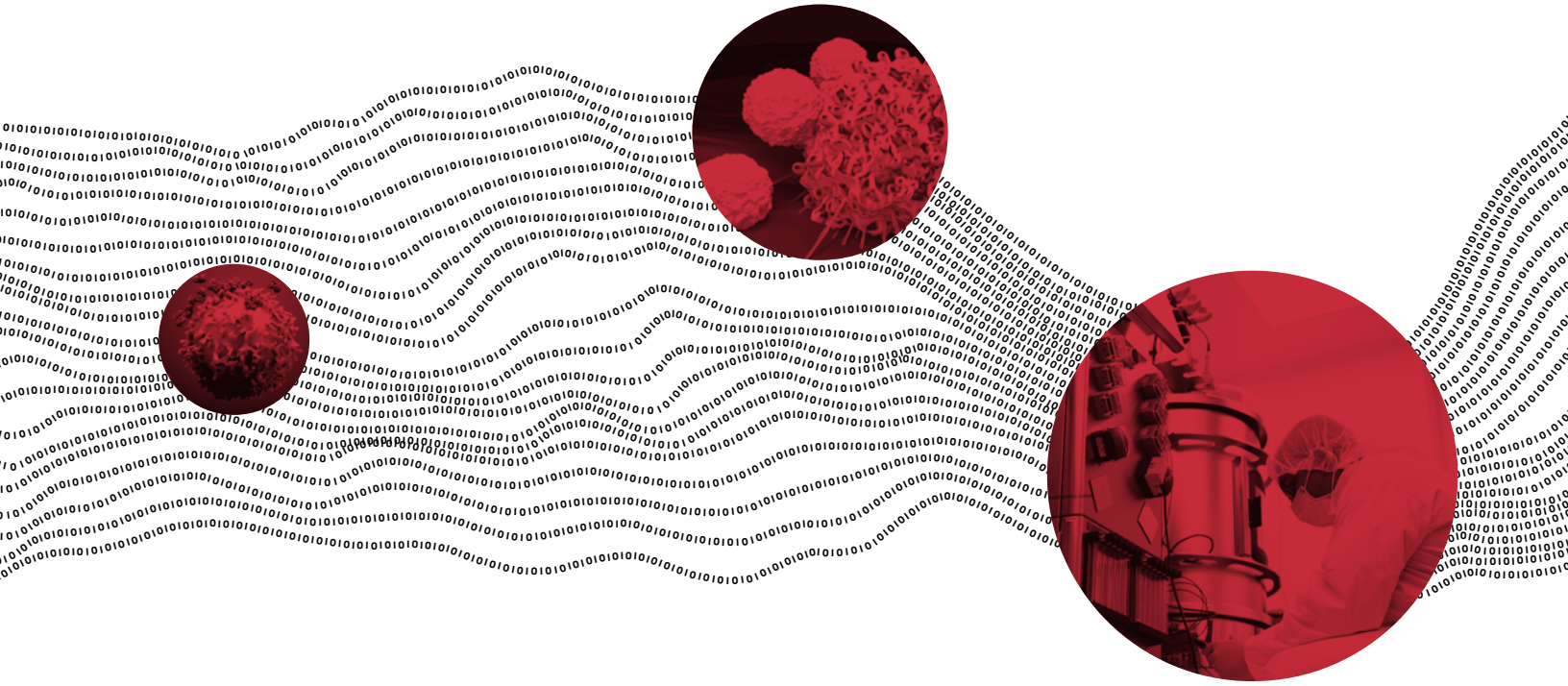
TECH NOTE

METHOD COMPARISON OF THE ACCELLIX PLATFORM VERSUS TRADITIONAL FLOW CYTOMETRY



Accellix

In this technical note, we demonstrate that the Accellix Automated Cell Phenotyping Platform provides comparable results to a traditional flow cytometer. Enabled by cartridge-based sample preparation and automated cell phenotyping and analysis, the Accellix workflow is five times faster and requires ten times fewer pipetting steps, which results in increased reproducibility and accuracy by reducing manual handling.

INTRODUCTION

Cell therapies, the use of human cells as medicines, have the potential to revolutionize treatment of a wide range of diseases, such as cancer, autoimmune, and neurodegenerative disorders.

By leveraging the intrinsic potential of cell-based immunological responses, these complex, multi-mechanism therapies surpass the single target nature of small molecule or antibody products.

Still, the promise of cell therapies brings many challenges, particularly in the areas of manufacturing reproducibility and consistent product administration to thousands of patients (Levine et al.). Methods that are sufficient for generating products on the scale of early clinical studies may not directly translate to commercial-scale yields and efficiencies. Thus, despite the success rates of current clinical trials, commercial-scale demands for cell therapies in common diseases could hinder the supply of these potentially life-saving therapies.

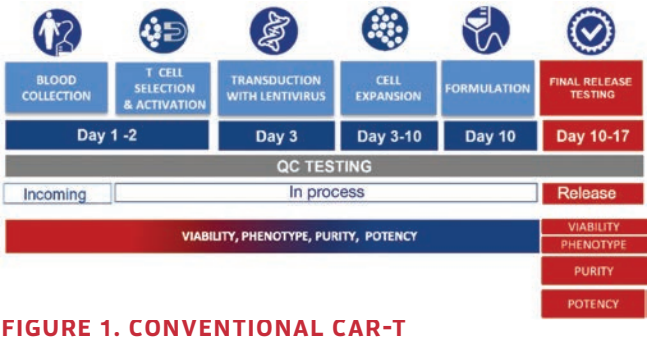


FIGURE 1. CONVENTIONAL CAR-T MANUFACTURING PROCESS

The production of cell therapies requires many carefully orchestrated steps, with Quality Control (QC) testing performed multiple times throughout the process (Figure 1). Using conventional CAR-T cell manufacturing as an example, the first step involves the use of leukapheresis to remove blood from the patient's body, isolate the leukocytes, and return the remainder of the blood to the circulation. The autologous leukapheresis product is enriched for T cells and the cells are then activated and transduced with a viral vector expressing the CAR transgene. Cells are expanded for a variable number of days, at which point they are harvested, washed, and prepared for infusion. At multiple points during this manufacturing process and prior to releasing the therapy for patient infusion, QC testing is performed, and several critical quality attributes are measured. These measurements serve to assess the quality of the incoming material, success of cell purification strategies, degree of genetic modification, cell phenotype and differentiation state, and finally, to define the dose of modified cells that the patient will receive.

Flow cytometry has become an invaluable and enabling tool in immunological research, cell biology, and cell therapy development. Using a single platform, cell therapy developers can measure a variety of parameters, including phenotype, function, cell health, and transgene expression. Despite its utility in cell therapy research and development, flow cytometry is not easily transferable into the GMP manufacturing environment due to complexity, variability, and manual steps associated with individual operator handling, reagents, instrument setup and maintenance, sample preparation, as well as data acquisition and analysis.

The Accellix Automated Cell Phenotyping Platform eliminates many of these limitations and automates the entire QC workflow from sample preparation to data analysis, generating rapid cell phenotyping results directly in the manufacturing suite. The platform consists of:

- Fully Automated Flow Cytometer: An entirely automated, compact, fan-less, easy-to-use benchtop flow cytometry system fully operatable within an hour of training.
- Cartridge-Based Assays: Includes a microfluidic cartridge and unitized, dried down, and room temperature stable reagents allowing for automated sample preparation, and increasing reproducibility and accuracy by reducing manual handling.
- User-Friendly Software: Fully automated algorithm-based data analysis (when enabled) and auto classification process computes a test result that is presented to the operator on the instrument screen at the end of the run.

In this technical note, we showcase the performance of the Accellix Platform in comparison to the CytoFLEX LX flow cytometer (Beckman Coulter), a traditional cytometer that operates in cell therapy QC labs. By measuring frequencies of common markers measured in a cell therapy development and manufacturing environment, we show statistically comparable frequencies detected between the two platforms. Along with comparable results, the Accellix workflow proves to be faster and easier, enhancing reproducibility and accuracy by reducing manual handling.

RESULTS

We sought to validate the accuracy of the Accellix Platform by comparing its performance to a predicate flow cytometer.

Matched healthy donor PBMCs were processed and analyzed on instruments at two lab facilities and the results were compared. For each donor, one vial of cells was shipped to a flow cytometry lab where manual sample processing and fluorescent antibody staining was performed, and the samples were acquired on the CytoFLEX. Matched vials of cells were shipped to a lab containing the Accellix Platform, where automated sample preparation and flow cytometry analysis were performed using two off-the-shelf Accellix kits: T Cell Non-Lyse (Catalog number: A1000-1NL), which is a T cell subset panel, and TBNK-16 Non-Lyse (Catalog Number: A1003-1NL), a lymphocyte subset panel that includes anti-CD16 antibodies. The TBMNK antibody panel used for the CytoFLEX experiments incorporated the same antibody clones as those used in the Accellix kits (Figure 2). Cell subset frequencies from each of the two methods were analyzed in FlowJo™, maintaining consistent gating strategies, and evaluated for comparability.

CYTOFLEX ANTIBODY PANEL

TBMNK
CD45-A700
CD3-PECy7
CD4-PerCP Cy5.5
CD8-BV650
CD14-FITC
CD19-PE
CD56-APC
CD16-APC
Viability

ACCELLIX ANTIBODY KITS

T CELL SUBSET ASSAY
CD45-AF488
CD3-PECy5
CD4-PE
CD8-PED594
Viability

LYMPHOCYTE SUBSET ASSAY
CD45-PECy7
CD3-PECy5
CD14-PED594
CD19-PE
CD16-AF488
CD56-AF488
Viability

FIGURE 2. ANTIBODY PANELS USED FOR CELL PHENOTYPING Table on left details fluorophore-conjugated antibodies used in the CytoFLEX experiments. Tables on right detail the fluorophore-conjugated antibodies included in the two Accellix off-the-shelf assays used in the Accellix experiments.

T CELL SUBSET PANEL

Sixteen PBMC samples were analyzed for expression of CD45, CD3, CD4, and CD8 and stained with a viability dye, using a manual sample prep workflow and flow cytometric analysis on the CytoFLEX, and an automated workflow on the Accellix Platform. A similar gating strategy was developed to compare the cell subset frequencies from both data sets (Figure 3). Given that the Accellix instrument does not measure side scatter, cells were differentiated from debris in the first gating step by selecting CD45+ cells with sufficiently high forward scatter (FSC). Single cells were then gated, with FSC-Peak serving a similar function as FSC-Height. Next, viable CD3+ cells were selected. CD4+ and CD8+ T cell populations were further gated.

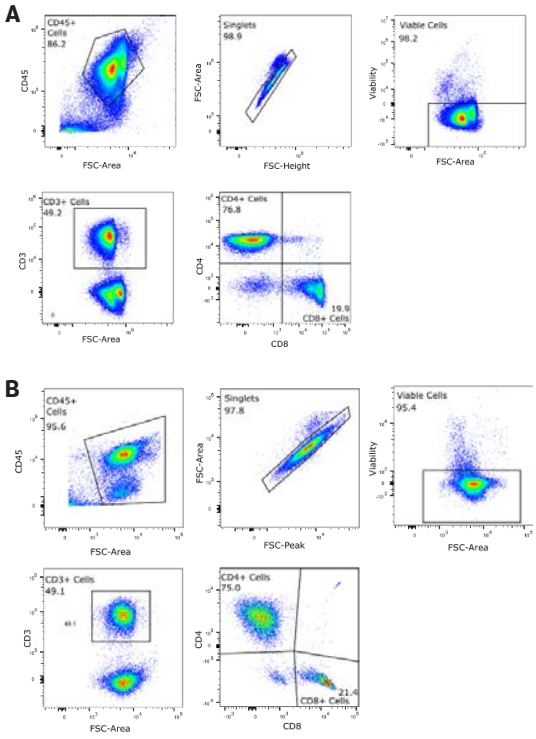


FIGURE 3. FLOW CYTOMETRIC ANALYSIS OF T CELL SUBSETS. A) Representative flow cytometry plots from the CytoFLEX platform, showing gating of CD45+ cells, followed by gating of singlets, viable cells, and CD3+ T cells. CD3+ cells are then subdivided into CD4+ and CD8+ cells. B) Representative flow cytometry plots from the Accellix Platform using the same gating scheme.

To quantify the relative concordance of measurements made on the two platforms, we first plotted the frequencies of CD3+, CD4+, and CD8+ cells as a proportion of viable CD45+ cells for each sample, measured on the Accellix instrument (x-axis) versus the CytoFLEX (y-axis). Linear regression analysis was performed and a strong correlation between the two datasets was identified, as measured by R^2 values for all populations greater than 0.9 and p-values less than 0.0001 (Figure 4a). Additionally, Bland Altman plots were generated to measure the bias values (differences between methods) across the range of cell frequencies (Figure 4b). For all three cell populations, bias values were less than 10% between the two methods, which meets the standard acceptance criteria for clinical validation of flow cytometers (Mfarrej et al.).

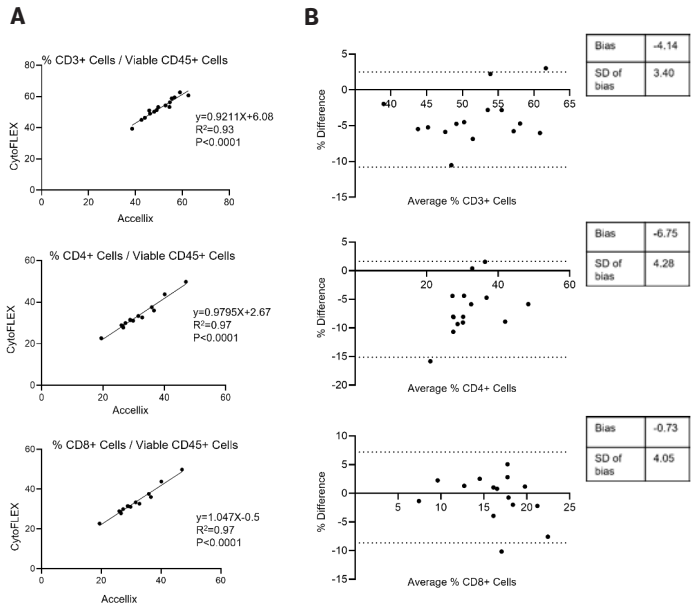


FIGURE 4. COMPARABILITY OF T CELL SUBSET ANALYSIS. A) Correlation plots show frequencies of CD3+, CD4+, and CD8+ cells as a proportion of viable CD45+ cells measured on the Accellix instrument (x-axis) versus the CytoFLEX instrument (y-axis). Linear regression analysis was performed and the resulting equation, along with the associated coefficient of determination (R^2) and p-value are reported. Each dot represents an individual donor, and a line of best fit is overlayed. B) Bland Altman plots represent the comparison data between the two instruments, in terms of measuring frequencies of T cell subpopulations. The x-axis represents the average frequency of each cell population measured across both instruments and the y-axis represents the bias (percent difference as calculated by (Accellix value – CytoFLEX value)/average). Dotted lines represent the 95% limits of agreement.

TBNK-16 LYMPHOCYTE SUBSET PANEL

In addition to the T cell subset analysis, we performed a similar comparative analysis on a general phenotyping panel for peripheral blood cells. Sixteen PBMC samples were analyzed for expression of CD45, CD3, CD14, CD19, CD56, and CD16 in the same fluorescent channel, and stained with a viability dye. A similar gating strategy was developed to compare the cell subset frequencies from both data sets (Figure 5). After selecting for viable CD45+ cells, the resulting data was analyzed to identify CD45+ cells that were CD14+ monocytes or CD14- lymphocytes. To ensure the purity of the monocyte population, we further gated on a population of CD14+CD3- cells. The CD14- population was divided into CD3+ T cells and CD3- cells. Finally, the CD3- population was subdivided into CD19+ B cells and CD56/16+ Natural Killer cells (NK cells).

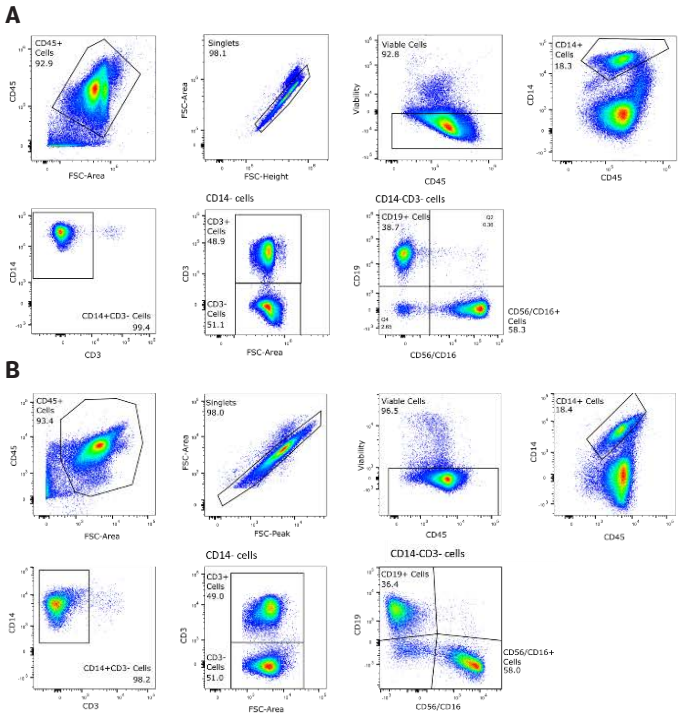


FIGURE 5. FLOW CYTOMETRIC ANALYSIS OF LYMPHOCYTE SUBSETS. A) Representative flow cytometry plots from CytoFLEX platform, showing gating of CD45+ cells, followed by gating of singlets, viable cells, and CD14+ cells. CD14+ cells were further gated to identify cells which are also CD3-. The CD14- population was subdivided into CD3+ T cells and CD3- cells. CD3- cells were further gated to identify CD19+ B cells and CD56+/ CD16+ NK cells. B) Representative flow cytometry plots from the Accellix Platform using the same gating scheme.

We plotted the frequency of CD3+, CD14+CD3-, CD19+, and CD56/CD16+ cells as a proportion of viable CD45+ cells for each donor, measured on the Accellix instrument (x-axis) versus the CytoFLEX (y-axis) (Figure 6a). Linear regression analysis was performed and, as in the T Cell Subset analysis, strong correlation between the two datasets was identified, as measured by R^2 values for all populations greater than 0.9 and p-values less than 0.0001. Bland Altman plots were also utilized to measure the bias values across the range of mean cell frequencies (Figure 6b). For all four cell populations, bias values were observed to be less than 10% between the two methods.

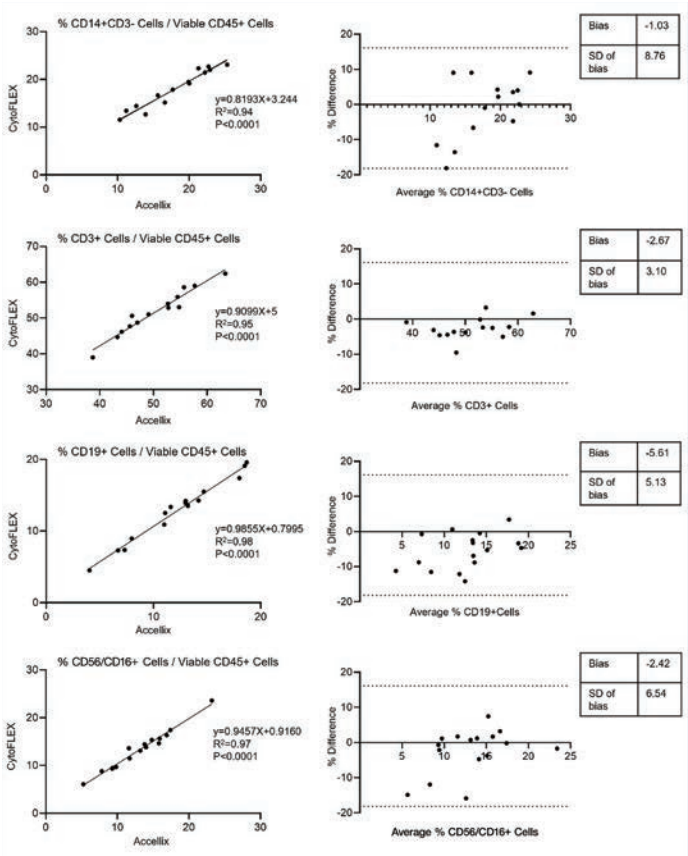


FIGURE 6. COMPARABILITY OF LYMPHOCYTE SUBSET ANALYSIS. A) Correlation plots show frequencies of CD14+CD3-, CD3+, CD19+, and CD56/CD16+ cells as a proportion of viable, CD45+ cells measured on the Accellix instrument (x-axis) versus the CytoFLEX instrument (y-axis). Linear regression analysis was performed and the resulting equation, along with the associated coefficient of determination (R^2) and p-value are reported. Each dot represents an individual donor, and a line of best fit is overlayed. B) Bland Altman plots represent the comparison data between the two instruments, in terms of measuring frequencies of lymphocyte subsets. The x-axis represents the average frequency of each cell population measured across both instruments and the y-axis represents the bias (percent difference as calculated by (Accellix value – CytoFLEX value)/average). Dotted lines represent the 95% limits of agreement.

WORKFLOW COMPARISON

After confirming that comparable data measuring cell population frequencies can be generated on the Accellix and CytoFLEX platforms, we compared the hands-on time associated with each workflow.

The traditional flow cytometry workflow is highly manual and requires roughly 13 steps: thawing, washing, cell counting, preparing of antibody cocktails, multi step blocking and cell staining, instrument setup, and acquisition. There are approximately 29 pipetting actions required to complete the process, with each pipetting step introducing the possibility of error. The complete workflow requires more than two hours before data is ready for analysis, with most of that time requiring active attention that cannot be diverted to another task (Figure 7A).

A

ASSAY STEP	# OF PIPETTE STEPS	TIME (MINUTES)
Thaw	1	2
Wash 2X	2	10
Count	2	5
Plate	2	5
Wash 2X	2	10
Make Antibody Cocktails	13	15
Surface Block	1	20
Surface Stain	1	25
Wash 2X	2	10
Fix	1	10
Wash and Concentrate	2	5
Cytometer Setup	-	30
Acquire Data	-	5
Total	29	152

B

ASSAY STEP	# OF PIPETTE STEPS	TIME (MINUTES)
Thaw	1	2
Load Sample into Reagent Tube	1	0.25
Vortex	0	2
Load Sample into Cartridge	1	0.25
Insert Cartridge into Instrument	0	0.25
Automated Sample Prep and Data Acquisition	-	30
Total	3	35

FIGURE 7. WORKFLOW COMPARISON. A) Table shows the manual steps required to analyze one PBMC sample on the CytoFLEX instrument, from the point of thawing cells to the point when data is available for analysis. For each step, the number of pipetting steps and time to execute is shown. B) Table shows a similar analysis of the manual steps required to analyze one PBMC sample on the Accellix instrument.

In comparison, automated sample preparation in a microfluidic cartridge using the Accellix Platform greatly reduces the number of steps and time required to generate similarly high-quality flow cytometry data. This workflow requires roughly six steps, from thawing cells, loading sample into the cartridge, and generating data, and entails only three pipetting actions. The workflow takes 35 minutes before data is ready to analyze, with more than 85% of that time being completely hands-off and allowing the operator to perform other tasks.

In addition to the time reduction associated with the Accellix workflow, fewer manual operations leave less opportunity for human error, reduce variability, and increase the reproducibility of the resulting data. This is especially critical in the cell therapy manufacturing environment, where many individual patient's therapies are being processed in parallel and small missteps in cell phenotyping can lead to under- or over-estimating potent cell doses for patient infusion. Given that staining reagents are unitized and pre-dried in the Accellix Assay Tube, there is no possibility for error in antibody-labeling, and the cartridge's QR code ensures that expired reagents are not utilized. Further, because cells can be loaded into the cartridge directly after thaw, with no washes required, the Accellix workflow reflects the true phenotype of the cells, without a bias for dead cells being preferentially lost during washes, or the biological sample changing while waiting to be analyzed.

CONCLUSION

While the future of cell therapies is bright and holds much promise, there are still challenges that must be overcome, especially in ensuring that commercial-scale demands can be met. The Accellix Platform brings automated sample preparation and flow cytometry to cell therapy manufacturing. The workflow saves time, reduces manual steps, and increases reproducibility, while maintaining comparable results to traditional flow cytometers. Implementation of the Accellix Platform streamlines quality control in cell therapy manufacturing , allowing cell therapy providers to confidently and quickly deliver their efficacious cell therapies to patients.

METHODS

ACCELLIX ASSAYS

Cryopreserved PBMCs from healthy donors (AllCells) were assayed using the standard Accellix T Cell Subset Reagent Tube Format (RTF) Assay kit with non-lyse buffer (T Cell RTF Assay (NL) and the Accellix Lymphocyte Subset Assay with non-lyse buffer (TBNK-16 RTF Assay (NL)). Each Accellix RTF Assay kit includes a microfluidic cartridge and a unitized room temperature-stable dried reagent tube containing antibodies, viability dye, and control beads. The antibodies included in the T Cell Subset Assay are CD45-AF488, CD3-PECy5, CD4-PE, CD8-PED594, and DIYO-3 viability dye. The antibodies included in the Lymphocyte Subset assay are CD45-PECy7, CD3-PECy5, CD14-PED594, CD16-AF488, CDI9-PE, CD56-AF488, and DIYO-3 viability dye. To summarize the workflow, 40 µl of cells were transferred into the tube and mixed on a pulsed vortex mixer for 2 minutes. After mixing, 20 µl of sample mixture were transferred into the loading port of the Accellix RTF cartridge. The cartridge was inserted into the Accellix Instrument and the assay run was initiated.

CYTOFLEX ASSAYS

Matching, cryopreserved PBMCs from healthy donors were analyzed in parallel on the CytoFLEX LX flow cytometer. Cells were thawed, counted, and washed. Cells were then resuspended in a blocking buffer containing I0% normal mouse serum and I00 µg/mL HulgG and incubated for I5 minutes at room temperature. Samples were incubated for an additional 20 minutes at room temperature with an antibody cocktail containing CD45-Alexa700, CD3-PECy7, CD4-PerCP Cy5.5, CD8-BV650, CD14-FITC, CD16-APC, CD19-PE, CD56-APC, and viability dye in triplicate. Finally, cells were resuspended in a 1% Paraformaldehyde solution prior to acquisition.

Data generated on both flow cytometry platforms were analyzed using FlowJo software (BD) and statistical analysis performed in Prism software (Graphpad).

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To learn more about the Accellix Platform, email us at **info@accellix.com**

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