CELL & GENE THERAPY INSIGHTS

DOWNSTREAM BIOPROCESSING

SPOTLIGHT

EXPERT INSIGHT

Responding to the challenges of flow cytometry in GMP product testing, a technical evaluation of the Accellix platform

Kevin W Johnson, Conor O'Malley, Steve Kunitake, Amanda Trent, Aaron Gassman, Jyostna Ivatury, Anna Brown and Geoff Hodge

Flow cytometry is a powerful tool used in the research and development of cell and gene therapy products. With this tool the researcher can gain valuable insight into the phenotype and function of populations of individual cells and how those cells respond to perturbations in their respective environments. In the development of cellular medicine, flow cytometry is used for assessment of culture health, phenotypic characterization of in process culture and final product, as well as functional characterization to quantify the effect of potential process changes as well as indicate the labs' capability of making a safe and effective product on the lab bench.

Cell & Gene Therapy Insights 2019; 5(9), 1195–1205

DOI: 10.18609/cgti.2019.125

Issues can and do arise with this valuable technology; yet, when development efforts are successful, the product proceeds onto clinical development and the need to translate research assays into a regulated environment is imperative. Cross-site reproducibility and the relative scarcity of a labor pool trained in this technology within the GMP testing environment are examples of the challenges facing the implementation of flow cytometry-based assays broadly to inform cell product characterization and release. Manufacturers of flow cytometry instrumentation must rise to these challenges in order to make their technology broadly applicable to the rigors required for this type of testing. To this end, Accellix Inc. has developed the Accellix Platform, a measurement and analysis system composed of a compact benchtop flow cytometer instrument and a single-use cartridge. Herein we report the results of a technical evaluation of the Accellix platform's automated sample staining and data acquisition functionalities. We will assess its performance in key assay metrics of linearity and precision by repeatability, in order to assess its capabilities for its proposed implementation in GMP processes, e.g., cell phenotype enumeration, cell population characterization, process quality control (QC), etc., that are critical in the manufacture of a T-cell immunotherapy product.

BACKGROUND & ACCELLIX OVERVIEW

Personalized, cell-based therapy is emerging as a paradigm-shifting strategy in healthcare. To fully harness the potential of cutting-edge cellular engineering, stringent process controls must be put into place that monitor and measure critical parameters of the cell product at specific points in the manufacturing process, from patient sample acquisition to drug product release.

In order to increase accessibility to a wide range of cell and gene therapy products, streamlining and simplification of process and analytical procedures will be necessary as to reduce timelines and costs associated with the manufacture of these promising therapies. Of the analytical procedures employed in cell therapy manufacturing, flow cytometry has proven to be one of the most intractable methods to be streamlined and simplified. Traditional flow cytometry is the heretofore "gold standard" for cell phenotyping, enumeration and characterization. Yet, the challenge of incorporating the method into a commercially viable cell therapy manufacturing workflow remains, due to the complexity and time-intensive nature of the operation and maintenance of traditional flow cytometers. Standardization and maintenance of instrumentation, requirement for fluorescence compensation, reagent variability, and subjective expert data analysis are all features of traditional flow cytometry that constitute significant challenges when considering its candidacy as a platform for robust analytical methods which can pass the stringent validation procedures of a regulated manufacturing environment.

The Accellix System is designed to offer a streamlined workflow solution by migrating traditional flow cytometry assays onto a relatively small footprint instrument. The system combines automated sample preparation within a single-use microfluidic cartridge, customized ambient-stable reagents, high sensitivity fluorescent event detection, and assay-specific auto analysis (which is out of the scope of this particular study). Sample processing, including fluorescent staining and fixing of the cells, RBC lysis (if needed), and sample dilution are executed wholly within a closed microfluidic cartridge. To initiate the assay, a lab user pipettes patient sample directly into a customized, dried reagent formulation; this fluorescent staining of the cells potentially allows for significant gains in intermediate precision over traditional flow cytometry assays and should be tested in future studies.

EXPERIMENT RATIONALE & OVERVIEW

The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) has set guidelines for determining if an assay is validated for its intended use. The criteria to consider for complete validation of a quantitative assay include assessment of Specificity, Linearity, Range, Accuracy, Precision, Robustness and Stability [1]. We evaluated the linearity and precision of the Accellix System to establish proof of concept and to determine if this technology warrants further development for our analytical procedures. We used the off-the-shelf T-cell cartridge and a custom cartridge designed to identify our construct, an antibody-coupled T-cell receptor (ACTR) composed of the extracellular domain of CD16 linked to a CD3ζ signaling domain and to a costimulatory domain. ACTR-expressing T cells are universal and can be flexibly paired with desired therapeutic antibodies to target tumor antigens. The performance of the Accellix assays were benchmarked against an in-house conventional polychromatic flow cytometry procedure. For testing material, we first evaluated Beckman Coulter's Cyto-trol™ reagent, a QC reagent of fixed, lyophilized cells with stable cell populations which can be compared across instruments as well as to the manufacturer's certificate of analysis (COA). We then proceeded to test previously frozen, ficoll-purified PBMC from healthy donor apheresis and our post-harvest ACTR T cell product to represent the beginning and end of our manufacturing process, respectively. In our manufacturing process, a donor subject's leukocytes are collected via leukapheresis and purified to PMBCs via density gradient centrifugation. T cells within these PBMC's are activated and transduced with the ACTR construct *ex vivo* and then expanded by continued culture. These samples are thawed, cryoprotectant is washed out with fresh media and cells and split for analysis on each platform. For manual flow cytometry, the media is washed out and cells are resuspended in a staining buffer consisting of Dulbecco's Phosphate Buffered Saline and Bovine Serum Albumin before being stained with a panel to identify T cells and high-level subsets. (see Table 1 for an overview of the panels used in each instrument). For samples run on the Accellix instrument, thawed/washed

DOI: 10.18609/cgti.2019.125
Cell & Gene Therapy Insights - ISSN: 2059-7800

TABLE 1.

Overview of experiments used to compare Accellix to traditional cytometry.

Cells Tested	Samples (n)	Replicates	Traditional Cytometry Antibody Panel (Clone)	Accellix Cartridge Panel (Clone)	Endpoints
Coulter Cyto-trol™	1	2-3	CD45 (HIT3) CD3 (UCHT1) CD4 (RPA-T4) CD8a (RPA-T8)	CD45 (HIT3) CD3 (UCHT1) CD4 (RPA-T4) CD8a (RPA-T8)	CD3 (% of CD45) CD4 (% of CD3) CD8 (% of CD3)
PBMC and ACTR T-cells	5 of each	3	Viability (7AAD)[1] CD45 (HIT3) CD3 (UCHT1) CD4 (RPA-T4) CD8a (RPA-T8)	Viability (DiYO-3™) CD45 (HIT3) CD3 (UCHT1) CD4 (RPA-T4) CD8a (RPA-T8)	
ACTR T-Cells/ MOCK controlled mix dilution series	1 series of 6 dilutions	2	Viability (7AAD) CD45 (HIT3) CD3 (UCHT1) CD16 (B73.1)[1]	Viability (DiYO-3™) CD45 (HIT3) CD3 (UCHT1) CD16 (B73.1)[2]	ACTR T-cells (CD3+CD16+)[2]

Four types of samples were used to assess performance of the Accellix. Coulter CytoTrol™ cells, PBMC, ACTR T-cells and MOCK transduced T-cells were used to test reproducibility and linearity. Panels with identical antibody clones, but different fluorochromes optimized for each instrument, were tested with endpoints of CD3, CD4, CD8 and ACTR T-cell relative frequency in each sample.
7-Aminoactinomycin D
The extracellular domain of ACTR is characterized by the corresponding portion of human FCRIII receptor or CD16, detectable by this clone

sample in media was added directly to their dry reagent tube consisting of the same antibody clones used for manual cytometry, though these were spectrally optimized for the Accellix. All samples were run in triplicate to assess repeatability of the assays run on the platform. Figure 1 shows a representative comparison of the different gating strategies for a PBMC sample on the two platforms.

In order to assess dilutional linearity, proportional frequencies of each endpoint need to be generated and tested. To this end we used spiked known proportions of ACTR T-cell product into cells that had gone through a similar manufacturing process but had not been transduced with the construct (MOCK cells) as a diluent to generate known concentrations of transduced cells. Briefly, MOCK cells and a representative T-cell product were normalized to the same concentration and a two-fold serial dilution series of six samples was generated by diluting ACTR drug product cell suspension into the MOCK cell suspension.

The resulting data from these experiments were converted to FCS files and manually gated in FlowJo™ (Treestar Software) flow cytometry analysis software in order to obtain relative frequency of the CD3, CD4 and CD8 population to assess precision by repeatability, and to obtain frequency of ACTR

DOI: 10.18609/cgti.2019.125

in serially diluted samples to assess linearity. The automated analysis function of the Accellix will be evaluated in future work with the system. Dead cells were identified in each assay platform when fixed cells were not used and eliminated from the analysis. Table 1 outlines the panel used in the Accellix cartridges vs. our current flow cytometry procedure.

RESULTS

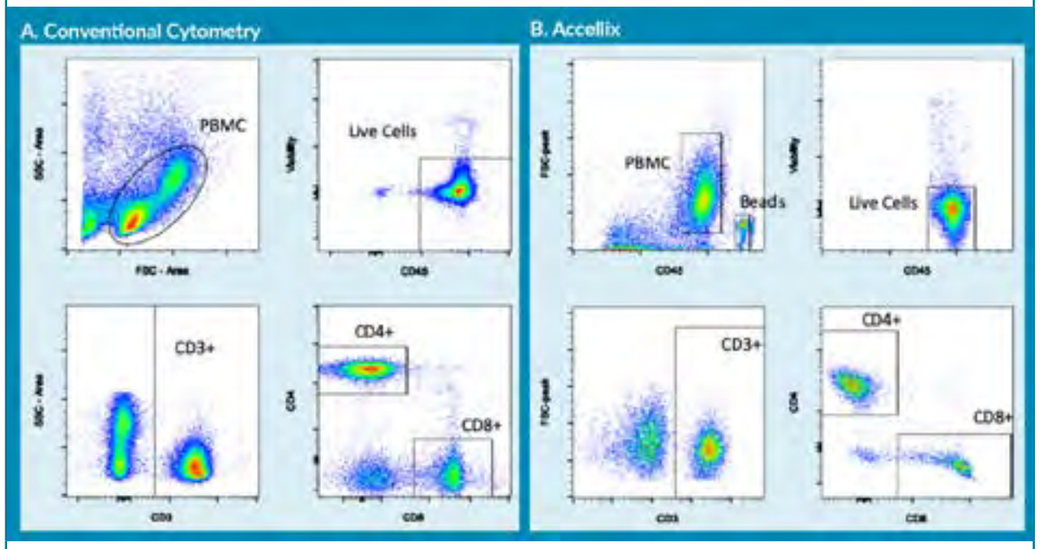
Repeatability

When comparing frequencies of T-cell populations in the Cyto-trol™ cells, Figure 2 shows both platforms were able to meet the manufacturer's specifications for the cell ranges expected in the control product for CD3 and CD8. Measured CD4 cells were slightly above this range; however, when compared to our conventional cytometry data, the Accellix data closely agreed for all measured populations.

When T-cell population frequencies obtained by each of the two methods are compared by linear regression, the slope of the correlation curve for CD4 and CD8 indicate a bias between the technologies; however, a high correlation is observed over the range of samples (Figure 3). The range of T cells in these samples represents as low as 50% of viable lymphocytes in the PBMC samples to nearly 100% of cells representative of our T-cell product. CD3 shows good correlation over this range ($R2 = 0.9020$; $p < 0.0001$) and both CD4 ($R2 = 0.9339$; $p < 0.0001$)

FIGURE 1

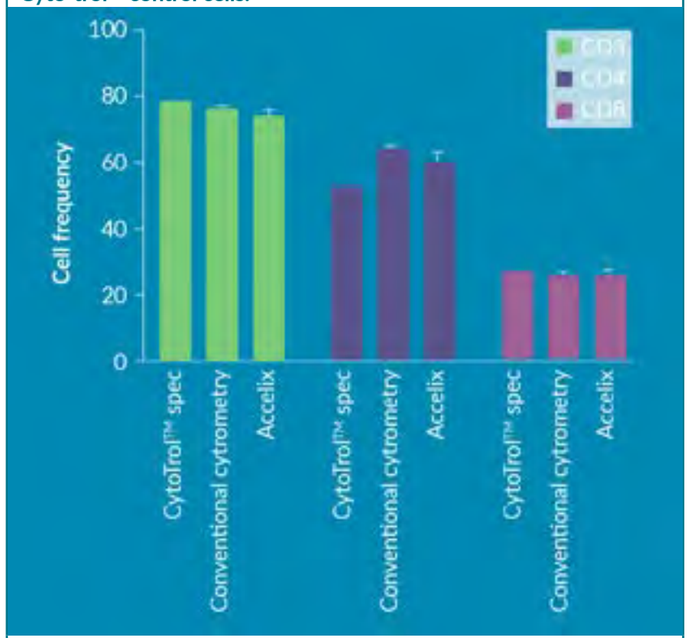
Comparison of gating strategies.



Differences in gating strategies between the Accellix and Conventional Cytometry for identifying T-cell subsets from a PBMC sample. In Conventional Cytometry (A), after doublet discrimination PBMCs are identified on a Forward Scatter (FSC) vs. Side Scatter (SSC) plot. Lice cells are identified as CD45+ and Viability dye (7AAD)-. T cells are subsequently Identified as CD3+ and CD4+ and CD8+ are gated as subsets of CD3+. On the Accellix (B), Cells are Identified as CD45+ and discriminated from QC beads by their higher FSC and lower fluorescence in the same CD45 channel. Live cells are then similarly identified as CD45+ and Viability dye (DiYO-3™negative). Similar subsequent gating strategy is then followed to identify T cells, and CD4+/CD8+ subsets respectively.

FIGURE 2

Comparison of Accellix with conventional cytometry using Cyto-trol™ control cells.



Cyto-trol™ cells were tested in a conventional flow cytometry assay and Accellix. Good repeatability was observed and cell populations were generally in line with the Cyto-trol™ manufacturer's specification. Slightly higher CD4 numbers than Cyto-trol™ spec were reproduced on both platforms.

and CD8 (0.9644 $p < 0.0001$) show excellent correlation over a range of as low as 20% to up to 80% of T-cells.

In order to assess repeatability, the coefficient of variation (CV) was calculated from the replicate samples run on each platform. Figure 4 shows excellent repeatability on both platforms and comparable results. It should be noted that a benchmark of acceptable variability for a flow cytometry assay is 20% [2]. Agreement between the two methods, as measured by relative percent difference (RPD) between the values calculated from each instrument, are summarized in Table 2. Good reproducibility was observed with values generally under 30% and average RPD for all samples at 5%, 13% and 19% for CD3, CD4 and CD8 respectively. RPD values above 30% were observed for two out of ten samples for CD8, for samples three and ten, which represent samples with the lowest initial cell concentration and as result, the lowest number of events acquired on the Accellix.

Linearity

When comparing ACTR frequency measurements determined by the Accellix to expected concentrations, data presented in Figure 5 demonstrate excellent linearity with an R^2 value of 0.9987 ($p<0.0001$) for ACTR T cells and RPD values (Table 3) of less than or equal to 12.1% throughout the entirety of the dilution series. These data represent good dilutional linearity of ACTR T-cell frequencies as low as 1.5%.

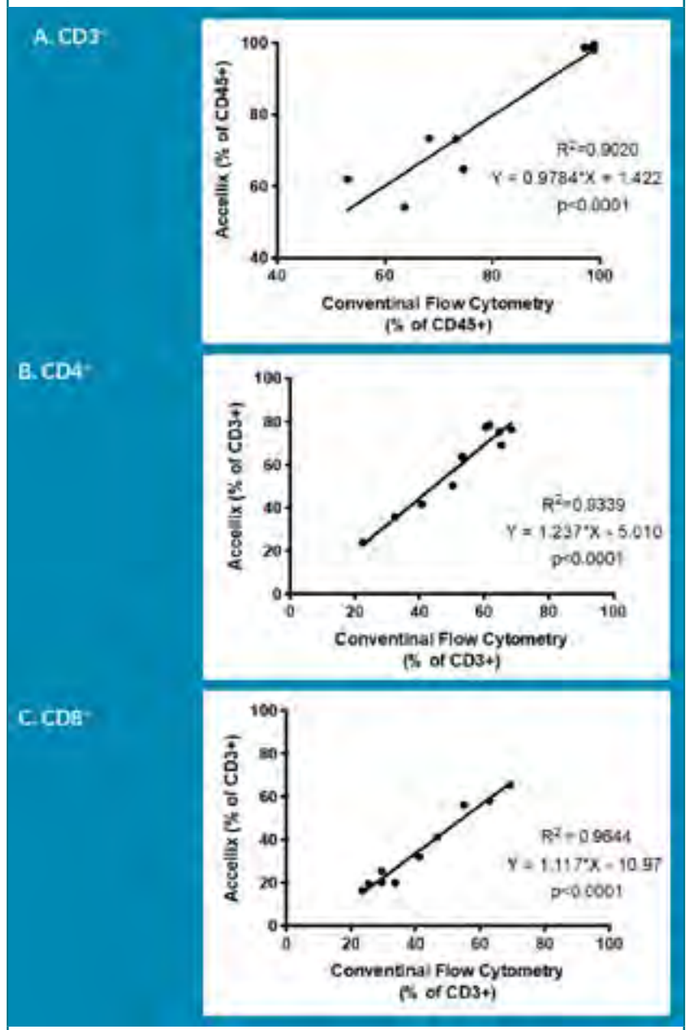
TRANSLATION INSIGHT

The data from these studies taken together show an initial proof-of-concept for this platform, which can be further evaluated for use in GMP manufacturing assays. Manual analysis of the results shows a platform with the potential for providing data to the regulated environment of T-cell manufacturing. Further evaluation would be needed to fully validate this platform, particularly in regard to is performance on extended data sets showing intermediate precision, reproducibility, range, and quantitation limits. Particular attention should be paid to assessing optimal cell input; this should be evaluated and appropriately controlled for each assay migrated onto the Accellix cartridge. Absolute cell counting and automated data analysis are also available features of this platform, though they are not assessed in the current study.

The Accellix Platform is best suited to provide automated, in-process, single cell insights on individual samples that can be encountered in the typical cell therapy manufacturing QC lab. To provide these insights, the current assay configuration on Accellix requires a preparation/execution/analysis cycle of approximately 35 minutes for a single sample as opposed to 3 hours or more estimated for a typical GMP sample requiring instrument startup and QC, samples preparation/staining and analysis. For extensive studies conducted in basic research, where testing multiple conditions in a single experiment may be desired, this combination of duration and single-cartridge throughput may make the Accellix platform less suitable than other flow cytometric platforms. Accellix

FIGURE 3

Comparability to conventional flow cytometry.

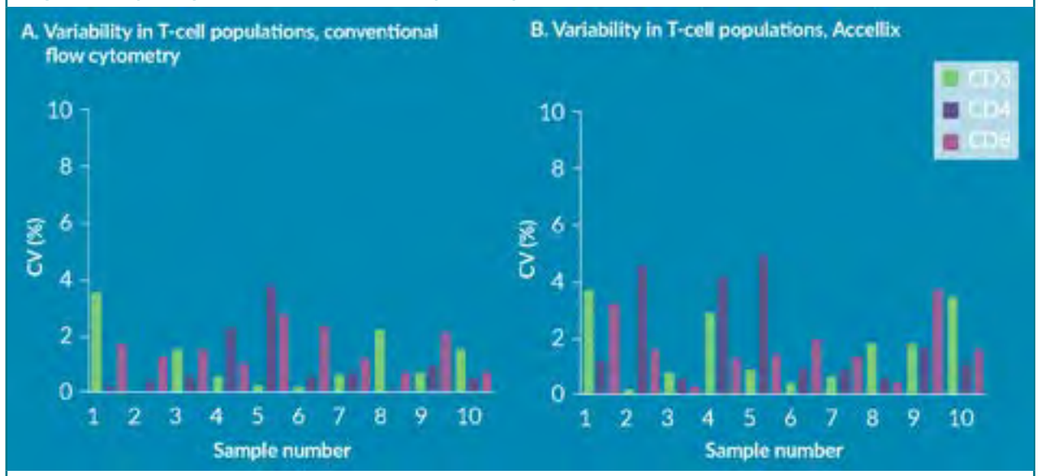


Linear regression analysis comparing T-cell specific targets, CD3, CD4 and CD8, frequency in 10 samples of PBMC and drug product. R2 values of 0.9020 for CD3, 0.9339 for CD4 and 0.9644 for CD8 demonstrate good correlation between the two technologies.

DOI: 10.18609/cgti.2019.125
Cell & Gene Therapy Insights - ISSN: 2059-7800

FIGURE 4

Repeatability comparison of conventional cytometry to Accellix.



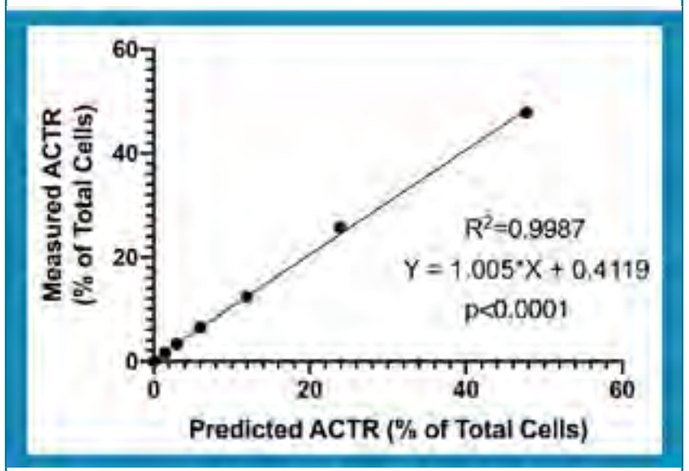
Samples run in triplicate on each platform were assessed for repeatability using Coefficient of Variability (CV). Both platforms showed excellent repeatability of their respective assays with CV values under 5%. Accellix was comparable to conventional cytometry in this regard.

will not, and apparently does not aim to, replace traditional flow cytometry in this regard. To fully evaluate the Accellix Platform, a more extensive validation study (with a large number of samples) would require a significant amount of time, assuming the current one-cartridge-at-a-time throughput of the Accellix Platform. It is noted that a higher throughput could be obtained by operating multiple Accellix instruments in parallel, or by ensuring that the next generation of the instrument can process multiple cartridges simultaneously. It is not clear at this time if this feature is under development for future versions.).

Potential gains in the simplification and standardization of flow cytometry assays make the Accellix System worth further evaluation for use as an assay platform in the GMP manufacturing environment. We plan to perform further evaluations in regard to its performance in additional assay qualification endpoints, as well as to assess the Accellix's capability to automate data analysis and provide absolute counts for single platform enumeration of cell subsets.

FIGURE 5

Linearity of ACTR+ cells in a controlled mixed dilution series.



Linear dilution series analyzed on the Accellix platform. A linear regression analysis showing excellent dilutional linearity when the measured frequency of ACTR+ cells is compared against the expected concentration of each dilution. An R2 value of 0.9987 was observed.

TABLE 2

Reproducibility between Accellix and conventional cytometry.

Sample number	CD3				CD4				CD8			
	Conventional cytometry (% CD3)	Accellix (% CD3)	RPD* (%)		Accellix (% CD4)	Conventional cytometry (% CD4)	RPD* (%)		Conventional Cytometry (% CD8)	Accellix (% CD8)	RPD* (%)	
1	68.2	72.7	6.5		78.3	61.7	26.9		23.5	16.4	30.4	
2	98.9	98.7	0.2		24.0	22.4	7.0		69.2	65.4	5.4	
3	53.1	61.0	14.8		77.7	60.6	28.1		33.7	20.0	40.7	
4	98.4	98.8	0.4		35.9	32.4	10.8		62.9	58.1	7.7	
5	98.9	99.6	0.7		41.8	40.7	2.8		54.9	56.1	2.2	
6	97.1	98.7	1.6		69.0	65.2	5.8		29.4	25.6	12.9	
7	98.9	98.5	0.4		50.3	50.3	0.1		46.7	41.4	11.4	
8	63.7	54.2	14.9		63.7	53.2	19.8		41	32.1	21.8	
9	74.6	64.8	13.1		76.4	68.4	11.7		25.5	19.6	23.0	
10	73.3	73.3	0.0		75.4	64.7	16.5		29.4	20.4	30.6	
Mean RPD (%)			5.3				12.9				18.6	

Direct comparison of the two technologies. Matching samples were run via conventional flow cytometry and on the Accellix using their T-cell cartridge. This table represents good reproducibility between the platforms with RPD values generally under 30% and average RPD of 5%, 13% and 19% for CD3, CD4 and CD8 respectively. RPD of above 30% were observed in two out of ten samples for CD8.
RPD: Relative percent difference.
*RPD = (Accellix result - conventional cytometry result)÷ conventional cytometry result

DOI: 10.18609/cgti.2019.125

TABLE 3

Comparison of measured to predicted values of the dilution series

Fold dilution	Measured frequency (%)	Predicted frequency (%)	RPD (%)
Neat ACTR T-cells	47.8	47.8	N/A
2×	25.7	23.9	7.6
4×	12.4	11.9	3.9
8×	6.5	6.0	9.4
16×	3.3	3.0	9.6
32×	1.7	1.5	12.1

An initial frequency from Neat ACTR T-Cells was used to extrapolate predicted frequencies of the dilution series. These values were used to determine RPD of the measured to the predicted frequency for each dilution.
RPD: Relative percent difference.

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *A Gassman and A Trent are employees of Accellix, Inc. (formerly LeukoDx, Inc.), which is the company which markets and sells the instrument under evaluation in the mauscript. S Kunitake was previously and employee of LeukoDx Inc.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: Aug 17 2019; Revised manuscript received: Sep 17 2019; Publication date: Oct 4 2019.

AFFILIATIONS

Kevin W Johnson
Unum Therapeutics, Cambridge, MA USA

Conor O'Malley
Unum Therapeutics, Cambridge, MA USA

Steve Kunitake
Accellix Inc., San Jose, CA, USA

Amanda Trent
Accellix Inc., San Jose, CA, USA

Aaron Gassman
Accellix Inc., San Jose, CA, USA

Jyostna Ivatury
Unum Therapeutics, Cambridge, MA USA

Anna Brown
Unum Therapeutics, Cambridge, MA USA

Geoff Hodge
Unum Therapeutics, Cambridge, MA USA

REFERENCES

1. International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH), Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (Q6B), March 1999 [online] www.ich.org/products/guidelines/quality/article/quality-guidelines.html
2. Wu DY, Patti-Diaz L, Hill CG. Development and validation of flow cytometry methods for pharmacodynamic clinical biomarkers. *Bioanalysis* 2010; 2(9): 1617–1626.