Validation of a 10-Color Acute Myeloid Leukemia Minimal Residual Disease Flow Cytometry Assay

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Abstract

Currently, measurable residual disease (MRD) flow cytometry assays identify residual leukemia in treated patients. Specifically, MRD occurs when the level of leukemic cells in a patient is below the detectable limits of morphological methods. The following study is a validation of a new acute myeloid leukemia (AML) MRD flow cytometry panel developed at Allina Abbott Northwestern for their new BD FACSLyric flow cytometer (BD Biosciences, San Jose, California). Various aspects of the AML MRD assay were investigated. They include cell loss due to processing, intra-assay precision, carryover, limit of detection (LOD), and inter-assay agreement with Abbott Northwestern's previous residual AML panel on their old BD FACSCanto II flow cytometer (BD Biosciences, San Jose, California). Overall, cell loss due to processing was dependent on the total number of processed white blood cells (WBCs) where processing volumes containing a higher total WBCs having greater cell loss compared to processing volumes containing lower total WBCs. Precision and carryover were acceptable per clinical guidelines, and the LOD was lower than morphological methods. Lastly, comparison between the Lyric and the Canto panels revealed comparable % myeloblast frequency in bone marrow samples. Although, the Lyric panel is better able to accurately detect the presence of residual disease qualitatively. Given these aspects, the new Lyric AML MRD flow cytometry assay is better able to detect the presence of residual AML than the Canto Residual AML panel. To further improve MRD status determination with the new panel, further display changes improving AML MRD detection is suggested.

Introduction

In most cases, current acute myeloid leukemia (AML) treatments result in clinical remission; however, many patients still experience disease recurrence. As a result, more sensitive methods to detect residual leukemic cells are needed. Potential solutions include using flow cytometry to evaluate measurable residual disease (MRD), a prognostic tool for determining disease relapse. Specifically, a positive MRD status correlates to a five-fold increase in AML relapse (Peters and Ansari 2011). MRD occurs when the presence of leukemic cells is between $1:10^4$ and $1:10^6$ (WBCs) which is below the detection capabilities of morphologic based methods. In contrast, the number of leukemic cells that morphologic review can detect is only 1:20 WBCs (Schurrhusi et al. 2018).

When using flow cytometry to assess acute myeloid leukemia (AML) MRD, the following strategy helps to successfully identify residual leukemic cells. First, some cell markers must differentiate between normal hematopoiesis and leukemic stem cells. To do so, the progenitor cell population, identified by CD45 vs side scatter, are evaluated for asynchronous or aberrant markers in AML. Second, a selection of markers must evaluate the bone marrow for a monocytic population to detect AML with a monocytic component. Thirdly, a different from normal immunophenotype approach when analyzing flow scatter plots is best for MRD evaluation (Figure 1). A different from normal approach looks for differences in the shape of cell distribution of the resulting flow plots compared to normal distribution patterns. Finally, if a diagnostic immunophenotype is obtained, it guides the different from normal approach since it narrows down possible differences (Wood 2020).

Clinically, implementation of new AML MRD assays must be validated. Validation parameters include specificity, intra-assay precision, limit of detection (LOD), carryover, and inter-assay agreement. Specificity in flow cytometry is the assay's ability to measure only the cellular population of interest. Typically, the antibody panel design and processing procedures address specificity. Intra-assay precision is degree of similarity between repeated measures of a single sample under the same conditions, such as analysis by the same flow cytometer. The LOD, a sensitivity parameter, is the lowest number of leukemic cells detected by the flow assay. Carryover is when cells from a high cellular sample interfere with a low cellular sample causing potential false positives. It is detrimental to accurate detection of rare populations such as residual leukemic cells in MRD. Most importantly, inter-assay agreement compares a new assay or analyzer against the previous assay or analyzer analyzing the same cell population (Selliah et al. 2019).

At Allina-Abbott Northwestern in Minneapolis MN, a new AML MRD panel for their new 3 laser BD FACSLyric Flow Cytometer (BD Biosciences, San Jose, CA) was developed. In the following validation study, the detection capability for residual leukemic cell populations by the new Lyric MRD panel is evaluated against the current residual AML panel using the BD FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA), both qualitatively and quantitatively. In addition, intrinsic assay parameters such as LOD, precision, and carryover were evaluated according to acceptable guidelines.

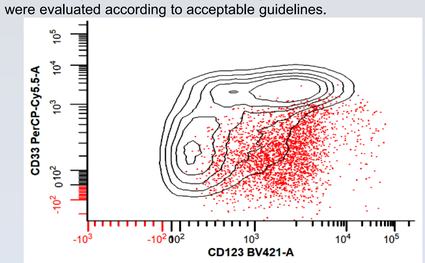

Figure 1: Different from Normal Gating Strategy Example. *The contour lines in the scatter plot represent the normal distribution of myeloblasts. Contour lines only appear on the Lyric panel and not the Canto panel. The red dots represent the phenotype of the various myeloblasts in an MRD positive post-treatment bone marrow, B22-1151. Most of these cells fall outside the normal distribution indicating a different from normal phenotype for CD33 and CD123.*

Materials and Methods

Three different experiments were performed to validate various aspects of the new AML MRD panel for Abbott Northwestern. The first experiment assessed the cell loss that occurs during specimen processing using peripheral blood dilutions in 1% bovine serum albumin (BSA) phosphate buffered saline (PBS) expanding the expected range of samples submitted for AML MRD testing. A hemocytometer count determined the WBC count of the 1st dilution, and the WBCs counts of the remaining dilutions were determined using the dilution factors. Each dilution was acquired in triplicate. The volume analyzed for each dilution was dependent on the WBC count of the dilution. The triplicates were processed according to the Allina Immunophenotyping Procedure. Processing volumes were dependent on the following guidelines: dilute to $20x10^9$/L and use 100 uL of sample if WBC count is greater than $20x10^9$/L, use 100 uL of sample if WBC count is between $10x10^9$/L and $20x10^9$/L, and use 200 uL of sample if WBC count less than $10x10^9$/L, 200 uL of sample. For the lowest WBC dilution, a second set of triplicates were created using a specimen volume of 300 uL. No antibodies were added to the replicates. 2.5 mL of Pharm Lyse (BD Biosciences, San Jose, CA) were added to each tube which were then vortexed. Tubes were incubated at room temperature for 4 minutes to allow for red blood cell (RBC) lysis. The tubes were then centrifuged for 4 minutes at 1250 RPM. After centrifugation, the RBC lysate was removed, and the tubes vortexed to resuspend the WBC pellet. Next, 3.5 mL of Wash Solution (0.1% Sodium Azide PBS solution) was added followed by centrifugation for 4 minutes at 1250 RPM. The samples were aspirated to a final volume of ~500 mL and then vortexed to resuspend the cells. The tubes were acquired manually on the Lyric flow cytometer on high flow until the entire volume was aspirated leaving a remaining 30 µL dead volume. The raw data was analyzed using a sequential gating strategy described in the Allina Data Analysis and Reporting procedure. Air bubbles or other interruptions to acquisition stability were excluded using time (FTIM) vs forward scatter width (FSC-W). Cell doublets were excluded using forward scatter height (FSC-H) vs FSC-W. Dead cell and debris was then excluded using forward scatter area (FSC-A) vs side scatter area (SSC-A) (Figure 2).

The remaining experiments were adapted from Selliah et al. 2019 and the CLSI Document H2: Validation of Assays Performed by Flow Cytometry. The second experiment assessed various assay characteristics including precision, carryover, and the limit of detection using a 1:5 serial dilution method of an AML bone marrow sample spiked into a normal bone marrow sample. Tubes were set up by adding 100 uL to a tube containing pre-cocktailed M4 antibodies (Table 1) and processed according to the steps described in experiment one. Tubes were set to acquire 300,000 total events automatically using a worklist programed with wash solution blanks to simulate acquisition conditions for the clinical assay. were run before each dilution for 30 seconds. The same gating strategy used in experiment one was repeated followed by the addition of flow plots to determine the number of leukemic cells in the dilutions. The additional plots include CD45 vs SSC, FSC-A vs CD34, then CD123 vs CD45RA for progenitor cell population identification, myeloid blast population identification, and leukemic cell population identification, respectively (Figure 2). Precision of the assay was assessed by calculating the %CV between replicates using the percentage of blasts detected of total nucleated cells for each dilution. The %CV at each dilution was compared to acceptable %CV limits. Carryover was assessed by running the triplicates of the undiluted abnormal bone marrow followed by triplicates of undiluted normal bone marrow. The following calculation where H3 was the number of leukemic blasts of the 3rd AML bone marrow replicate and L1 and L3 was the number of leukemic blasts in the 1st and 3rd normal bone marrow replicates, respectively: $Carryover\ (\%) = \frac{(L1-L3)}{(H3-L3)} X100$. The LOD was determined to be the average observed % positive events of the lowest level dilution where leukemic cells were identified.

The third experiment was a method comparison study to ensure inter-assay reliability between the current Canto Residual AML flow cytometry panel (Table 1) and the new Lyric AML flow cytometry panel. 8 bone marrow specimens submitted for follow-up AML evaluation were analyzed on both instruments accordingly to previously described procedures except that the leukemic cell population was determined by evaluating multiple additional flow plots. Qualitative (positive or negative for leukemic phenotype) and quantitative (blast frequency comparisons) were made between assays. For 3 of the samples, molecular NPM1 MRD PCR results were also available for comparison.

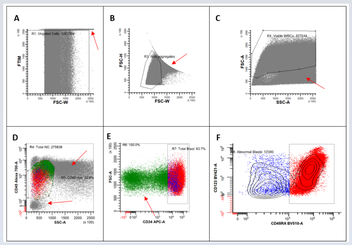

Figure 2: Gating Strategy and Flow Scatter Plots for Experiments One and Two. *Experiment one uses plots A through C. Experiment two uses plots A through F. A. FTIM vs FSC-W. Gate is set to exclude air (red arrow) and other interruptions to acquisition stability B. FSC-H vs FSC-W. Gate is set to include singlets and exclude doublets (red arrow). C. FSC-A vs SSC-A. Gate is set to include total nucleated cells and exclude unlysed RBCs and debris (red arrow). D. CD45 vs SSC-A. Gate is set to include progenitor cell population and exclude all other nucleated cells (red arrows). E. FSC-A vs CD34. Gate is set to include myeloblasts (CD34 positive) and exclude non-myeloblasts (CD34 negative). F. CD123 vs CD45RA. Gate is set to include CD45RA positive events, the leukemic cell population, which showed the most defined different from normal phenotype.*

Table 1. Lyric and Canto Antibody Panel with Fluorochromes

Panel Name	Tube	FITC	PE	PCP5.5	PC7	APC	AF700	H7	BV421/ V450/PB	BV510	BV605
AML MRD	M1	65	117	15	13	34	45	DR	33 BV421	10	38
	M2	64	300e	56	34	11b	45	14	DR V450	117	16
	M4	19	371	33	117	34	45	DR	123 BV421	45RA	38
Residual AML	A3	65	117	33	13	34	45	DR	----	----	----
	A4	15	34	56	117	11b	45	64 (APC-AF750)	----	----	----
	A5	38	371	34	117	123	45	14	----	----	----

Results

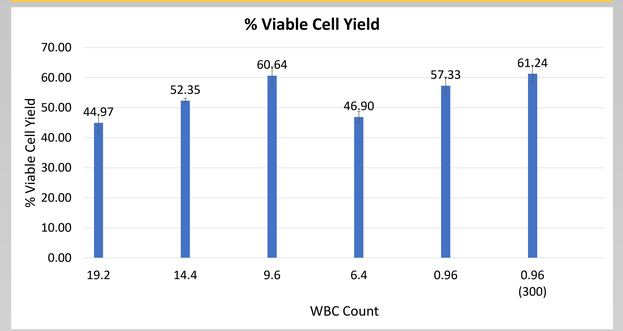

Figure 3: Average % Viable Cell Yield Due to MRD Flow Cytometry Sample Processing. *The dilutions of varying WBCs were made using peripheral whole blood samples in 1% BSA PBS solution. The WBC count of each dilution was calculated from the WBC count of the original sample using dilution factors. Processing volumes were determined after obtaining a WBC estimate by determining the average of number of cells in a single square of a hemocytometer. The following volumes from each dilution were used: WBC 19.2, 100 uL; WBC 14.4, 100 uL; WBC 9.6, 100 uL; WBC 6.4; 200 uL; WBC 0.96, 200 uL; WBC 0.96 (300), 300 uL. There replicates of each dilution were analyzed. For the 14.4 WBC dilution, only two replicates were used to calculate the average following Z-score analysis to remove outliers outside one standard deviation from the mean. Standard deviation error bars were used.*

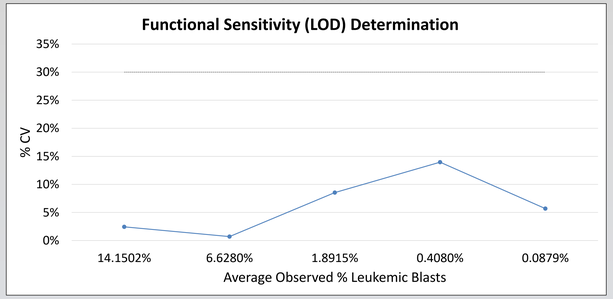

Figure 4: Lyric MRD Panel Leukemic Blast Functional Sensitivity (LOD) Determination. *The following plot compares the % CV to the average observed % leukemic blasts out of total nucleated events for each dilution. The following dilutions are represented from left to right: 1:5, 1:25, 1:125, 1:625, 1:3125. Functional LOD is the lowest detectable signal that can be reliably detected. The lowest average observed % leukemic blasts with a %CV below the laboratory set acceptable %CV threshold (30% for Allina Abbott Northwestern) is the reliable LOD for the Lyric MRD panel.*

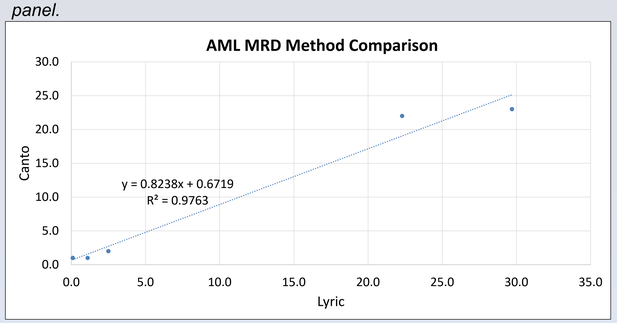

Figure 5: Lyric and Canto Method Comparison Using % Blast Frequency. *Blast frequency was used to compare the Lyric MRD panel to the Canto Residual AML panel. Both panels determine blast frequency using the FSC-A vs CD34 scatter plots. The Lyric panel is the test method, and the Canto panel is the reference method.*

Discussion

Leukemic cells are a minor population in samples submitted for MRD assays. As a result, the sample processing procedure for MRD assays is optimized to detect as many cells in the sample as possible. Based on the cell loss study of this MRD assay validation, dilutions that result in a larger number of total processed cells correlate with a lower % viable cell yield (Figure 3). If not corrected, the loss of potential leukemic cells could result in a false negative when interpreting the final MRD status for the patient. Potential causes of the WBC loss when processing a higher total number of WBCs includes the loss of cells when pipetting since any accidental transfer loss such as from poor pipetting technique is more significant given the larger number of cell present. Possible solutions include expanding the WBC count range for using only a 100 uL processing volume in addition to diluting high WBC count specimens in this range.

The assay characteristics such as precision, carryover, and the limit of detection for the Lyric MRD Assay are acceptable for MRD analysis. For cell-based assays with a rare population such as MRD flow cytometry assays, the acceptable precision criteria are under 30% CV (Selliah et al. 2019) which the Lyric MRD assay at Allina Abbott Northwestern clearly demonstrates (Figure 4). Likewise, carryover was found to be 0% which is also ideal for a high sensitivity assay such as MRD assays. For the limit of detection, there are no acceptance criteria. Although, the LOD for the Lyric Panel, 0.0879% (Figure 4), was lower than the detection capabilities of morphological methods, 1:20 WBCs or 5% (Schurrhusi et al. 2018).

When comparing the Lyric MRD panel to the Canto Residual AML panel, the Lyric panel is better able to detect the presence of leukemic cells qualitatively. Out of three cases with available molecular NPM1 MRD PCR results, the Lyric panel was able to correctly detect MRD for the two positive molecular MRD cases contrasting the Canto panel which was unable to detect MRD for these two cases. Differences between the two panels that most contribute this are the new markers in the Lyric panel that are not the Canto panel. The new marker that has the greatest contribution to this is CD45RA (Figure 2). It is a marker used to differentiate normal hematopoiesis from leukemic blasts where the marker is typically negative on normal hematopoietic stem cells and positive on leukemic blasts (Kersten et al. 2016). Another possibility is the inclusion of contour lines on the Lyric panel compared to the Canto panel. By using the contour lines, bias reduction occurs when evaluating the plots for residual disease. Along with the qualitative improvements of the Lyric panel over the Canto panel, the quantitative blast frequency comparison between the two panels was acceptable (Figure 5). A possible cause for the slight difference between the two panels includes different voltage settings between the two instruments causing more PMNs grouped into the blast gate on the Canto compared to the Lyric.

Conclusion

Overall, the Lyric AML MRD panel is better able to detect the presence of residual acute myeloid leukemia than the Canto Residual AML panel. One limitation to the current validation is that dilutions in the serial dilution study all showed the presence of leukemic cells. To get a better idea of the actual LOD, the serial dilution study can be repeated with lower dilutions. In addition, improvement to the flow scatter plots used to evaluate MRD is recommended based on hematopathologists' preferences and useful cell markers.

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