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A Novel Method for Performing Linearity Assessments in Whole Blood Prepared Using a Lyse/No Wash Procedure

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INTRODUCTION

Multiparametric flow cytometry has long been a powerful tool in laboratory medicine. With recent advancements in immunotherapeutics, vaccine technology, and cell-based therapies, flow cytometry has become an important platform for supporting drug development at almost every level from research and development to primary endpoint determinations. Despite this importance, no official guidance exists for validation of assays using flow cytometry. Industry consensus and recommendations have guided assay development, but applying generalized recommendations can be difficult when sample preparation varies widely between assays requiring manipulations such as red blood cell lysis, cell permeabilization, or tissue digestion for the preparation of single cell suspensions.

Here we present methods to perform the linearity assessments on low-abundance populations in fresh human whole blood in a lyse/no wash method of sample preparation. Current industry practice is often to combine stained/fixed/washed sample with increasing quantities of unstained sample, creating a linear dilution series of stained sample. However, with each manipulation of sample through washing and fixation there is the possibility of loss of cells impacting absolute counts. This is particularly troublesome when investigating low abundance populations, or populations that exist ≤1 event in 1000 of viable events in a sample. A lyse/no wash method is preferred when the desired analysis combines immunophenotyping and absolute counts, but this preparation can create challenges during method validation where excess antibody remains in the sample. Therefore, a method of sample preparation that prevents loss of cell, cross-staining, or interference from unstained sample is required for accurate linearity assessment.

METHOD

Four methods of linearity sample preparation (**fixed FMX**, **fixed unconjugated**, **fresh FMX**, and **fresh unconjugated**), shown in **Figure 1**, were compared to determine their impact on the linearity of T regulatory cells (Treg) and CD14-CD16+ monocytes, two low-abundance populations, in a lyse/no wash method of whole blood staining. Sample staining was performed by incubating 50.0 µL of whole blood sample with 25.0 µL of antibody cocktail for 30 minutes at room temperature; lysing and fixation were performed using 1X BD FACS Lysing Solution (BD Cat. No. 349202).

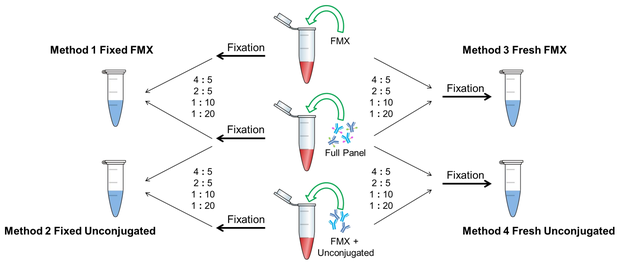

Figure 1. Linearity Sample Preparation

Whole blood sample from each donor was processed following the four methods in parallel. Prepared linearity samples were then acquired and recorded using a BD LSRFortessa™ Cell Analyzer. The gating strategy to select Treg population (CD127low CD25+) and to select CD14-CD16+ monocyte population is shown in **Figure 2**.

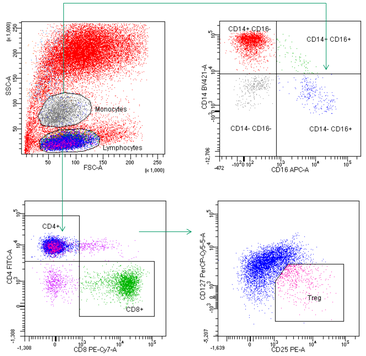

Figure 2. Flow Cytometry Gating Strategy

RESULTS AND DISCUSSION

Data from each preparation were analyzed using linear regression, as demonstrated in **Figure 3.** Linearity was assessed based on R^2 values, where an R^2 closer to 1 indicates good linearity among samples diluted at different ratios. In addition, accuracy of the method was assessed based on Y-intercept of the line of best fit, where a Y-intercept closer to 0 indicates good accuracy in diluting samples to the target ratio. Values for R^2 and Y-intercept of the line of best fit are reported in **Table 1**.

- For the Treg population, all four methods provided acceptable linearity ($R^2 \geq 0.980$). The fixed unconjugated method provided the best accuracy, where a Y-intercept of 0.6 indicated that this method is linear for the Treg population down to one event.
- For the CD14-CD16+ monocyte population, linearity samples prepared using fixed FMX and fixed unconjugated methods provided acceptable linearity ($R^2 \geq 0.980$). The fixed unconjugated method once again provided the best accuracy (Y-intercept = 4.0).

Overall, linearity samples prepared using the fixed unconjugated method demonstrated the best linearity and accuracy. The two factors at play—the addition of unconjugated anti-CD25 and anti-CD127 antibodies in FMX, as well as the fixation step prior to mixing fully stained samples with FMX stained samples—helped minimize any cross-staining from the excess antibodies that could not be removed due to the lyse/no-wash method.

Table 1. Statistical Values For Linearity Assessment

	Method	Population Reported	R^2	Y-Intercept
1	Fixed FMX	Treg	≥0.997	-3.2
		CD14-CD16+ Monocytes	≥0.996	-6.2
2	Fixed Unconjugated	Treg	≥0.995	0.6
		CD14-CD16+ Monocytes	≥0.993	4.0
3	Fresh FMX	Treg	≥0.980	22.5
		CD14-CD16+ Monocytes	≥0.598	573.9
4	Fresh Unconjugated	Treg	≥0.994	6.7
		CD14-CD16+ Monocytes	≥0.961	12.0

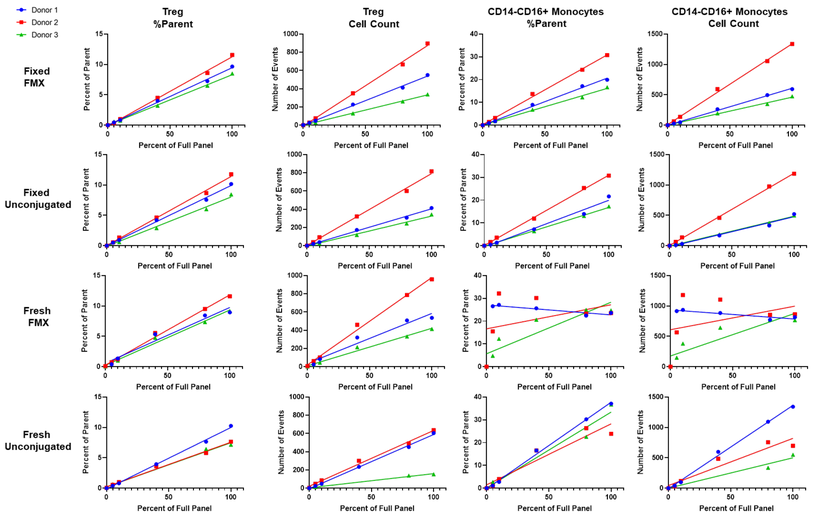

Figure 3. Linear Regression of Linearity Samples Prepared Using Different Methods

CONCLUSION

Assessing linearity is a major component of assay validation. Variations in sample preparation can make it difficult to prepare accurate and relevant dilutions in a lyse/no-wash method without interference. Here we demonstrate a method for preventing interference from cross-staining in sample preparation by including unconjugated antibodies.

Fixing samples prior to dilution drastically improves the quality of linearity assessments. In studies where samples cannot be fixed, the addition of unconjugated antibodies provides a major improvement compared to dilution using FMX stained samples. The fixed unconjugated method demonstrated superior results to the industry recommendation.

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