WHITE PAPER

Standardized Compensation Setup for the ClearLLab 10C Application

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IN THIS PAPER YOU WILL

Learn how to decrease multiple color compensation requirements in Flow Cytometry

Learn how to standardize the workflow in laboratories performing 10 color applications

Experience the robustness and utility of the ClearLLab Compensation Kit and ClearLLab Compensation Beads

Introduction

Standardization is essential for achieving consistent results and generating comparable data in multicolor flow cytometry. Standardization is ensured by utilizing a comprehensive setup that includes reagents, hardware, sample preparation, and analysis methods. The following three (3) reagents are used to setup the application:

- Flow-Set Pro Fluorospheres
- ClearLLab Compensation Kit
- ClearLLab Compensation Beads

ClearLLab 10C lot-specific Flow-Set Pro target values are provided for instrument standardization and the ClearLLab Compensation Kit and the Compensation Beads are provided to setup the compensation via AutoSetup Scheduler on the Navios and Navios EX Flow Cytometers.

The ClearLLab Compensation Kit was developed as an accessory for ClearLLab 10 color flow cytometry applications and consists of ten reagent tubes each comprised of one monoclonal antibody, CD3, CD4, or CD8, in a dry unitized format. Each antibody is labelled with one of the ten fluorochromes: FITC, PE, ECD, PC5.5, PC7, APC, APC-A700, APC-A750, Pacific Blue, and Krome Orange (Table 1). This reagent format eliminates the manual pipetting of liquid antibodies, thereby simplifying and standardizing the compensation setup workflow. Each compensation setup is performed by preparing a set of ClearLLab Compensation tubes staining the ClearLLab Compensation Beads.

ClearLLab Compensation Beads are to be used in conjunction with the ClearLLab Compensation Kit to establish compensation settings prior to multicolor analysis with the ClearLLab 10C Panels. Using the instrument Cytosettings a compensation matrix is generated on the Navios or Navios EX flow cytometer from the stained compensation beads.

Instrument standardization and compensation setup is not required daily, instead only when the Daily Quality Control fails, or after instrument service as needed, or when switching to a new lot of Flow-Set Pro. The compensation matrix is generated using the positive and negative bead populations and can be optimized and saved for each panel tube to accommodate the compensation variability of different lineages.

1. ClearLLab Compensation Kit

The ClearLLab Compensation Kit consists of 10 tubes each with a single-color antibody conjugated to the fluorochrome listed in the Table 1.

Table 1. ClearLLab Compensation Kit

	405 nm		488 nm					633 nm		
	PB (1)	Krome Orange	FITC	PE	ECD	PC5.5	PC7	APC	APC-A700 (2)	APC-A750 (3)
Specificity	CD4	CD8	CD4	CD4	CD3	CD4	CD4	CD4	CD4	4

One Compensation Kit contains five pouches, each pouch with the ten single color tubes for one compensation setup, each tube is used once.

2. ClearLLab Compensation Beads

ClearLLab Compensation Beads contain two vials of 3.0-3.4 μm beads in suspension. The Antibody Capture Negative Beads act as a negative control and do not bind antibodies. The Antibody Capture Positive Beads contain beads coated with an IgG-binding agent that will bind to mouse isotype antibodies, such as those contained in the ClearLLab Compensation Kit.

The Compensation Beads are designed to capture dye-conjugated antibodies to provide a fluorescent signal that can be detected by the Navios and Navios EX flow cytometers to generate a compensation matrix.

3. Compensation Reagents Performance

ClearLLab Compensation Beads were prepared by staining with ClearLLab Compensation Kit and analyzed on Navios and Navios EX flow cytometers. Figure 1a and Figure 1b illustrate the scatter gating of the single beads and an example fluorescence profile (FL1) plot of positive and negative beads, respectively.

(1) Pacific Blue* (2) APC-Alexa Fluor* 700 (3) APC-Alexa Fluor* 750

Figure 1. Stained ClearLLab Compensation Beads

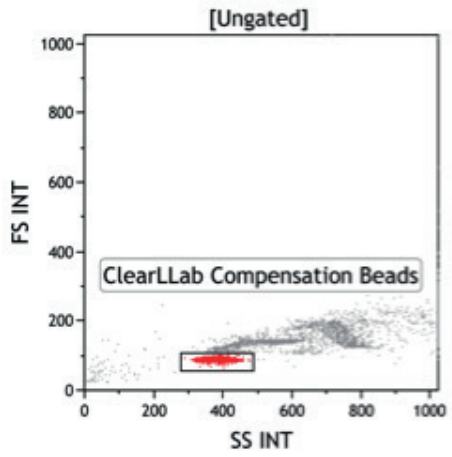


Figure 1a. Scatter Gating of Stained Beads

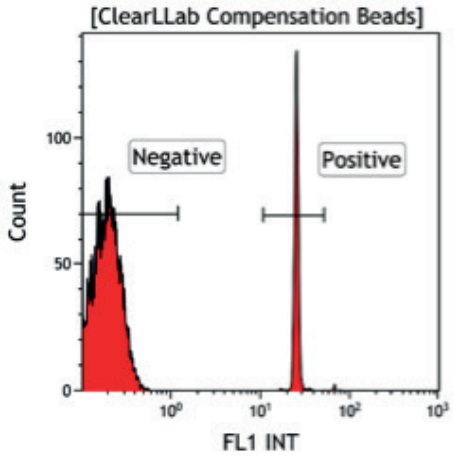


Figure 1b. Fluorescence profile of Stained Beads

The ClearLLab Compensation Kit can be stored at 18-30°C for up to 545 days and each pouch is for one compensation setup. The ClearLLab Compensation Beads can be stored at 2-8°C for up to 720 days with a 365 days in-use stability after the vial is open. The prepared bead sample can be stored at 2-8°C for up to 6 hours before sample analysis.

Four (4) lots of ClearLLab Compensation Beads and six (6) lots of ClearLLab Compensation Kit (Table 2), fresh and aged were tested in eight (8) combination lots. Prepared bead samples were tested immediately, 4 hours, 7 hours and 24 hours after preparation, each in triplicate.

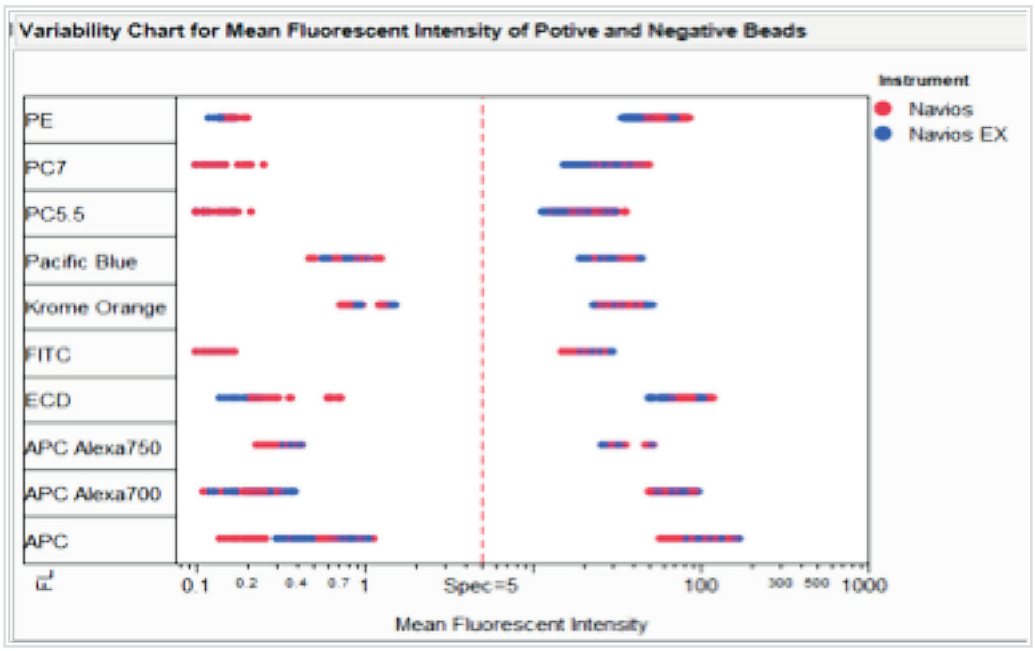
Table 2. ClearLLab Compensation Beads and Compensation Kit Lots tested for Stability

ClearLLab Compensation Kit	
Lot #	Closed Pouch Age
270317	29
190816	550
12_05_2016-2	565
10216	667

ClearLLab Compensation Beads			
Lot #	Closed Vial Age	Open Vial Age	Total Stability Staggering the Close and Open
4131004	739	0	739
BAF02 - S	908	0	908
BAG02 - W	753	0	753
BAG07	792	398	1190
4131009	770	398	1168
AH03	508	398	906

The MFI and signal to noise separation of each conjugate were monitored for each sample and no failure was observed (Figure 2).

Figure 2. Mean Fluorescent Intensity (MFI) of the Negative versus Positive Beads



The reproducibility of percent compensation between each primary and secondary channels was assessed from data collected using three (3) combination lots of Compensation Kit and Compensation Beads. Compensation bead samples were prepared in triplicate and each analyzed 0, 4, 7 and 24 hours after preparation on a Navios instrument. The average percent compensation and the corresponding reproducibility (%CV) are summarized in Table 3 and illustrated in Figure 3.

Table 3 is color coded based on the level of spillover from the primary to secondary channels:

- No spillover: highlighted in black
- Insignificant spillover (highlighted in grey): %Compensation ≤0.2%; %CV is relative high due to low values.
- The FL channels with significant compensation (>0.2% up to 76%) are highlighted in green(mean)/pink(%CV). The reproducibility %CV was <10% (0.4-9.3%) and showed consistency between different lots, across sample ages and between replicates.

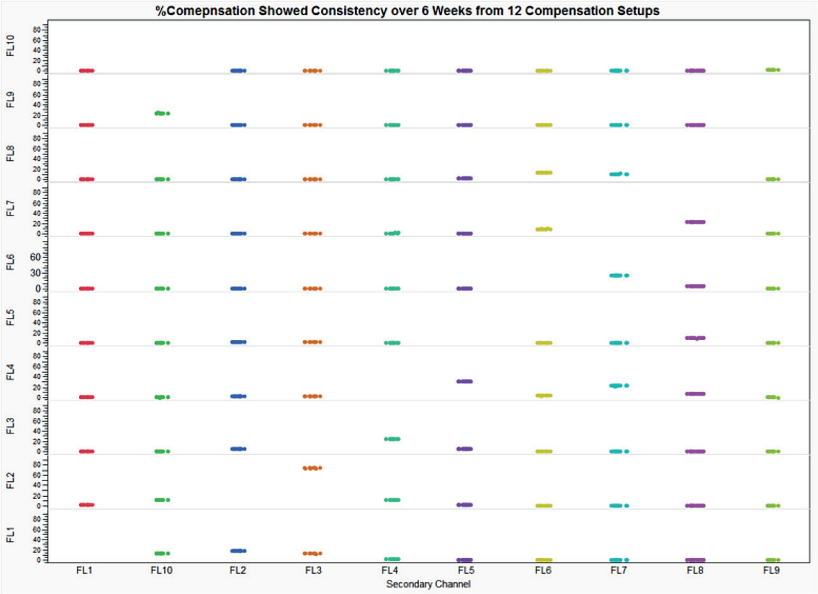
Table 3. Average Percent Compensation and the corresponding %CV of all Lots and Sample Ages

Secondary Channels	N	% Compensation	Primary Channels									
			FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1	36	Mean		0.9	0.1		0.1					
		%CV%		2.4	9.3		37.8					
FL2	36	Mean	20.4		5.7	1.4	1.1					
		%CV%	0.9		0.6	2.4	2.3					
FL3	36	Mean	14.4	76.0		1.2	0.9					
		%CV%	0.5	0.8		8.7	3.4					
FL4	36	Mean	1.7	10.7	21.5		0.3	0.5	0.9			
		%CV%	1.7	0.9	0.4		4.1	3.1	1.8			
FL5	36	Mean	0.3	1.6	4.0	28.0		0.1	0.5	2.0		
		%CV%	5.1	1.4	0.7	0.5		4.9	2.4	1.8		
FL6	36	Mean			0.1	1.8			7.6	7.8		
		%CV%			7.9	3.5			2.4	1.2		
FL7	36	Mean				16.3	0.5	41.8		12.1		
		%CV%				1.5	9.3	0.6		0.8		
FL8	36	Mean				4.8	5.9	7.6	20.4			
		%CV%				2.7	2.5	0.6	0.5			
FL9	36	Mean										2.4
		%CV%										3.4
FL10	36	Mean	13.9	20.6	0.9	0.2	0.2				16.5	
		%CV%	5.1	4.6	8.9	56.7	34.7				0.4	

Figure 3. Variability Chart of the Percent Compensation between Primary (y-axis) Channels and Secondary (x-axis) Channels: consistent between different lots, samples ages and replicates

The instruments were monitored by running the Flow-Set Pro using the Cytosettings established at Day 1 for up to six (6) weeks. A compensation matrix was generated twice a week (12 compensation setups) and showed consistency over the 6 weeks of testing.

Figure 4. Consistent Compensation is maintained for up to 6 weeks. Example from one Navios instrument (Primary Channels on the y-axis and secondary Channels are on the x-axis)



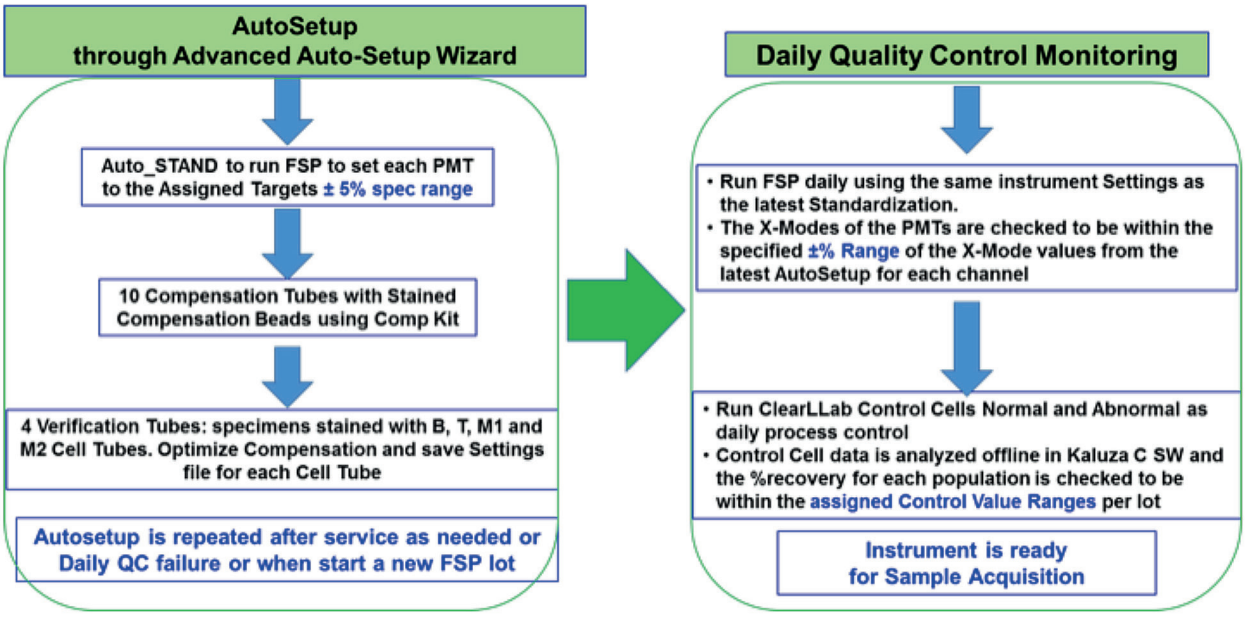
4. Workflow for Instrument Setup

The presented results clearly showed that compensation setup using the ClearLLab Compensation Beads and Compensation Kit is robust for ClearLLab 10C application on Navios and Navios EX flow cytometers. Navios and Navios EX flow cytometers have demonstrated stability during the six (6) weeks of testing and the compensation matrix showed consistency between lots and for the duration of testing and the claimed reagents stability.

Thus the workflow (Figure 5) was proposed and implemented for the ClearLLab 10C application including:

- AutoSetup (left panel) to standardize the instrument by running Flow-Set Pro to the assigned target ranges, establish the compensation matrix and verify the settings and compensation through AutoSetup Scheduler.
- Daily QC to monitor the instrument settings (right panel) by running the Flow-Set Pro and ClearLLab Control Cells using the Cytosettings established at AutoSetup.
  - o The X-mode values of Flow-Set Pro must fall within the daily QC ±% ranges of the X-mode values from the AutoSetup for each channel.
  - o Control Cells: the percent positive of each population must be within the Assay Ranges for the lot used.

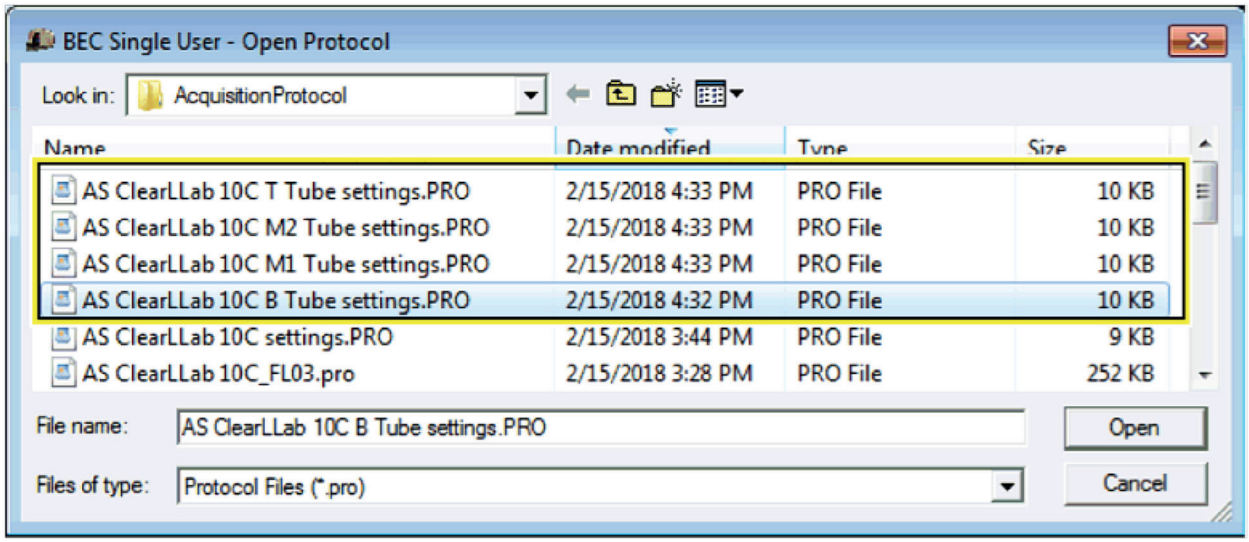
Figure 5. Setup workflow for ClearLLab 10C Application



The instrument standardization and compensation setup is performed at the initial setup of the application, is repeated only when the Daily Quality Control fails, or after instrument service as needed, or when switching to a new lot of Flow-Set Pro.

Compensation is always challenging for multicolor flow application, especially as there are four (4) ClearLLab 10C Panels available. The same compensation matrix may not always be suitable for all the panels, therefore, the software allows the user, at AutoSetup, to tailor and save the compensation matrix for each of four (4) ClearLLab 10C Panels to accommodate the variability of each lineage (Figure 6). This will reduce offline compensation adjustment and simplify the offline data analysis.

Figure 6. Cytosettings with tailored compensation matrix for ClearLLab 10C B, T, M1 and M2 Cell Tube



5. Conclusion

This study demonstrates that the ClearLLab Compensation Kit and ClearLLab Compensation Beads can be used for compensation setup as part of the AutoSetup for ClearLLab 10C Panels on Navios and Navios EX flow cytometers. This process can standardize and simplify the workflow for ClearLLab 10C application in a laboratory setting. The AutoSetup is not required daily. It is performed at initial application setup, and is repeated only when the Daily QC fails, or when the instrument is serviced as needed, or when starting a new lot of Flow-Set Pro, which allows for a more streamlined process of sample preparation and analysis. The compensation matrix can be tailored and saved for each of four (4) ClearLLab 10C Panels to accommodate the variability of each lineage, which would reduce offline compensation adjustment and simplify the compensation process.

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