

Original Article

## Titration of monoclonal antibodies and stability of tandem dyes in cocktail preparation for flow cytometry processing

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### ABSTRACT

**Objectives:** Tandem dyes are routinely used in multicolor flow cytometry (MFC) and consist of two fluorochromes, one donor and one acceptor. Antibody cocktails for various panels are prepared by combining the pre-titrated volumes of each fluorochrome-conjugated antibody. As the performance and stability of tandem dyes may deteriorate over time, this study aimed to optimize antibody titration volumes and assess the stability of pre-titrated tandem dyes in cocktail preparations.

**Materials and Methods:** This was a quantitative study in which titration was performed, and the prepared antibody cocktail comprised cytoplasmic myeloperoxidase-Fluorescein Isothiocyanate (FITC), cytoplasmic cluster of differentiation (cCD) 79a-Phycoerythrin (PE), CD19-energy coupling dye (ECD), CD34-phycoerythrin-cyanin (PC) 5.5, cCD3-PC7, CD45-allophycocyanin (APC) 700, CD7-APC750, sCD3-pacific blue (PB), and human leucocyte antigen - D region related (HLA-DR isotype) tagged with krome orange (KO). CytoComp™ lyophilized cells were used to evaluate tandem dye stability daily for five days and weekly for four weeks. The stain-lyse-wash procedure was used, and data acquisition and analysis were performed in the Navios™ flow cytometer.

**Statistical analysis:** The mean fluorescent intensity and percentage of positive cells were recorded for most of the antibodies in the cocktail. A coefficient of variation (CV) value of >5% was considered statistically significant, indicating the degradation of tandem dyes.

**Results:** Antibody titration reduced the final antibody volume by 77%, reducing the cost per test. CV values remained below 5% during all assessments, confirming cocktail stability for up to four weeks when tested with lyophilized cells.

**Conclusions:** The pre-titrated antibody cocktail was cost-effective and stable for up to four weeks, including tandem dyes, making it suitable for routine flow cytometric applications.

**Keywords:** Antibody cocktail, Monoclonal antibodies, Stability and multicolor flow cytometry, Tandem dyes, Titration

### INTRODUCTION

Multicolor flow cytometry (MFC) helps measure the physical properties of the cells based on light scatter and the antigenic properties of the cells with the help of antibodies labeled with fluorochrome. Antibodies are the key components of MFC analysis.<sup>[1]</sup> For reproducible analysis, high-quality antibody performance is essential. To use these antibodies judiciously in routine practice, the appropriate volume should be determined through titration experiments. The

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individual antibodies should be checked for their stability in cocktail preparation after titration as a part of the validation procedure.<sup>[2-4]</sup>

Antibody titration is the process of identifying and determining the correct concentration of antibody. It helps establish the optimal antibody volume that provides the best separation of cell populations and the most accurate measurement of expression levels. By optimizing the concentration and amount of antibody needed for a specific test, non-specific antibody binding (noise) can be minimized, reducing background interference.<sup>[2-5]</sup> However, not all antibodies can be assessed by simple signal-to-noise ratio (SNR) or signal-intensity graphs, as internal negative or unreactive cell populations are not always present.<sup>[6]</sup>

The next step is to prepare antibody cocktails for different panels by combining the pre-titrated volumes of each fluorochrome-conjugated antibody. The performance and stability can be impaired when stored as antibody cocktails. Moreover, this problem gets exaggerated when using tandem dyes, which comprise two fluorochromes, namely one donor (phycobiliprotein) and one receiver/acceptor (such as Alexa Fluor or cyanine dye). In clinical practice, commonly used phycobiliproteins include phycoerythrin (PE), allophycocyanin (APC), and the peridinin–chlorophyll–protein complex. Frequent re-compensation may be required due to the possible spectral spillover between tandem dyes, which can reduce the robustness of clinical MFC panels. Since tandem dyes are essential for standard 8–10 color or higher-order MFC, several studies have emphasized the importance of assessing their stability in cocktail preparations.<sup>[7]</sup> Therefore, the primary aim was to evaluate the optimal titration volume and the stability of the pre-titrated volume of tandem dyes in cocktail preparations.

## MATERIALS AND METHODS

The present quantitative study was carried out after obtaining approval from the Institute's Ethics Committee from November 2021 to August 2022. The inclusion criteria were that all monoclonal antibodies in the acute leukemia orientation tube (ALOT) were used for titration and for checking the stability of tandem dyes in the ALOT cocktail. Clotted/lysed samples, samples received in the wrong anticoagulant tube, outdated monoclonal antibodies, and samples from acute leukemia patients on chemotherapy or

follow-ups were excluded from the study.

This study was conducted in a phased manner, in which a titration experiment was carried out in part 1 and stability testing for tandem dyes was performed in part 2. A convenience sampling technique was used, and peripheral blood samples (2 mL) received in ethylenediaminetetraacetic acid were screened for normal white blood cell (WBC) count and distribution. Ten such samples were used for the titration experiment (part 1), and CytoComp™ lyophilized cells were used for stability testing (part 2). Finally, the validation of the antibody cocktail was performed using acute leukemia cases for routine practice.

### Antibody panel and sample processing

The pre-defined ALOT antibody panel, which was adapted and modified from the EuroFlow Consortium recommendations, was used [Table 1].<sup>[3,4]</sup> All the reagents and consumables were procured from Beckman Coulter India Private Limited, and the occasional antibody (nTdT – nuclear terminal deoxynucleotidyl transferase) was obtained from Becton, Dickinson Biosciences Private Limited.

For all antibodies in the ALOT tube, either surface or intracytoplasmic staining procedures were followed using the stain-lyse-wash method. Staining was performed with the fluorochrome-tagged surface or intracellular antibodies. Initially, the staining with surface antibodies was performed with 100 µL of the sample. This step was followed by vortexing and incubating the mixture in the dark for 20 minutes. Washing to remove excess unbound antibodies was performed by adding 2 mL of phosphate-buffered saline, vortexing for a few seconds, centrifuging for five minutes at 698 relative centrifugal force per minute, and discarding the supernatant.

Formaldehyde was used for fixation, and saponin for the permeabilization step for the intracytoplasmic or nuclear antibodies (Intraprep R1 and R2™). The addition of intracytoplasmic or nuclear antibodies, incubation, and a washing step followed the permeabilization step. In the case of surface antibodies only, Optilyse™ was used to lyse the red blood cells after the staining step, followed by a washing procedure.

An unstained tube was processed along with each panel to check for autofluorescence separately for surface and

**Table 1:** Panel of antibodies and their fluorochrome used in the ALOT tube.

| Fluorochrome | FITC | PE     | ECD  | PC5.5 | PC7  | APC  | APC 700 | APC 750 | PB   | KO     |
|--------------|------|--------|------|-------|------|------|---------|---------|------|--------|
| ALOT tube    | cMPO | cCD79a | CD19 | CD34  | cCD3 | nTdT | CD45    | CD7     | sCD3 | HLA-DR |

ALOT: Acute leukemia orientation tube, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, ECD: Energy coupling dye, PC: Phycoerythrin-cyanin, APC: Allophycocyanin, PB: Pacific blue, KO: Krome orange, CD: Cluster of differentiation, MPO: Myeloperoxidase; c: Cytoplasmic, s: Surface n: Nuclear staining, TdT: Terminal deoxynucleotidyl transferase, HLA-DR: Human leucocyte antigen–DR isotype

intracellular antibodies. The diluent (IsoSheath Fluid™) was used to resuspend the cells for acquisition. A proteolytic enzyme solution (Cleanse™) was used to remove unwanted debris from the instrument at the end of the procedure.

### Acquisition and analysis

Samples were acquired on a three-laser, ten-color flow cytometer (Navios, Beckman Coulter™) comprising two fluorescent channels from the violet (405 nm) laser, five channels from the blue (488 nm) laser, and three channels from the red (638 nm) laser. The optical alignment, electronic standardization, and sensitivity/linearity of the MFC were calibrated at the time of installation, with re-calibration performed annually.

Flow cytometer performance was monitored daily using commercially available fluorescent beads (Flow-Check Pro™). Photomultiplier tube voltages and compensation settings were standardized using both cell-based methods and calibration beads, plotted on the Levey–Jennings chart, and verified on a day-to-day basis. For all the samples, at least 50,000 events per tube were acquired. Data were analyzed using Kaluza software (version 2.1).

### Titration experiment (part-1)

In principle, antibodies were serially diluted (20, 10, 5, 2.5, 1.25, and 0.625 µL) and used in a single-staining assay using normal peripheral blood samples for most antibodies. However, B/T-lymphoblastic leukemia samples were spiked with normal peripheral blood and diluted to obtain a WBC count within the normal range for testing different markers: Cluster of differentiation (CD) 34 tagged with PE-cyanin (PC5.5), cytoplasmic CD3 (cCD3) tagged with phycocyanin (PC7), and nTdT tagged with APC.

Mean fluorescence intensity (MFI) was obtained by plotting fluorescence intensity on the x-axis and side scatter on the y-axis. The MFI values were then plotted against antibody concentration to generate the titration curve. Using the MFI, the SNR was calculated. Another parameter, the stain index (SI), was calculated using the formula:  $SI = (MFI [signal] - MFI [noise]) / 2 * \text{standard deviation (SD) of noise}$ .

Titration curves were constructed for each antibody-fluorochrome conjugate, with the x-axis representing antibody concentration and the double y-axis showing SNR and SI, respectively. For visual interpretation, histograms were examined to compare signal and noise MFI. In this study, the best titrated volume was defined as the lowest volume of antibody that produced optimal separation between signal and noise in the visual interpretation of histograms and the best SNR and/or SI values.

### Stability testing for tandem dyes (part-2)

Pre-titrated volumes of monoclonal antibodies were kept in aliquots as cocktail preparations and stored at 2–8°C. The commercially available CytoComp™ lyophilized cells were used daily for five days to assess cocktail stability during the first week, and on a specified day (Wednesday) of each subsequent week for up to four weeks. The manufacturer's (Beckman Coulter) recommendations were strictly followed for reconstitution and storage conditions. Granulocytes, lymphocytes, and monocytes in this lyophilized preparation were stable, and MFI was calculated using the appropriate cells for each antibody under study. Subsequently, the pre-titrated volumes of antibodies in the cocktail preparation were validated in diagnostic acute leukemia cases and also monitored for stability during routine diagnostic practice.

### Statistical method used

The MFI values were recorded to assess the stability of tandem dyes. The mean, SD, and CV were calculated for each respective week. A CV value >5% was considered significant, indicating deterioration of the tandem dyes, while a  $p < 0.05$  was considered statistically significant.

## RESULTS

### Titration experiment (part-1)

The titration experiment was carried out for all the antibodies included in the ALOT tube. Tables 2 and 3 summarize the titration results for CD19 energy coupling dye (ECD) and sCD3-pacific blue (PB), and their graphical interpretations are shown in Figure 1. The final titrated volume of each antibody and the corresponding cost–benefit analysis have been shown in Table 4.

### Stability testing for tandem dyes (part-2)

Pre-titrated volumes of monoclonal antibodies were aliquoted to prepare antibody cocktails. The daily MFI values for granulocytes, lymphocytes, and monocytes were calculated, and their mean, SD, and CV have been shown in Table 5. The CV was consistently <5% during the initial 5 days. Subsequently, the weekly MFI values, along with mean, SD, and CV, have been shown in Table 6 and Figure 2. Similarly, the CV was consistently <5% throughout the four weeks, indicating that the tandem dyes were stable in the cocktail when tested with lyophilized cells. Finally, the validation of the cocktail was performed using acute leukemia cases during routine diagnostic practice. The compensation matrices used for analyzing the ALOT cases during days 1-5 and during the four weeks have been shown in Figures 3 and 4, respectively.

**Table 2:** CD19-ECD titration.

| Antibody    | Conjugate | Clone  | Isotype | Species | Vendor  | Cat. no | Lot no  |
|-------------|-----------|--------|---------|---------|---------|---------|---------|
| CD19        | ECD       | J3-119 | IgG1    | Mouse   | Beckman | A07770  | 200131  |
| VOLUME (µl) | MFI +     | MFI -  | MFP-MFN | SD NEG  | S/N     | SI      | % gated |
| 10          | 18982     | 312    | 18670   | 157     | 60.78   | 59.6    | 23.01   |
| 5           | 16740     | 301    | 16440   | 146     | 55.71   | 56.5    | 23.09   |
| 2.5         | 12466     | 292    | 12174   | 137     | 42.72   | 44.5    | 22.56   |
| 1.25        | 7210      | 293    | 6917    | 138     | 24.63   | 25.2    | 23.52   |
| 0.625       | 3979      | 286    | 3693    | 139     | 13.90   | 13.3    | 22.82   |
| 0.31        | 2277      | 288    | 1989    | 138     | 7.91    | 7.2     | 23.41   |

ECD: Energy coupling dye, CD: Cluster of differentiation, Cat no: Catalog number, Ig: Immunoglobulin, MFI: Mean fluorescent intensity, MFP: Mean fluorescent intensity positive, MFN: Mean fluorescent intensity negative, SD NEG: Standard deviation of negative signal, S/N: Sound to noise ratio, SI: Stain index, µL: microliter

**Table 3:** sCD3-PB titration.

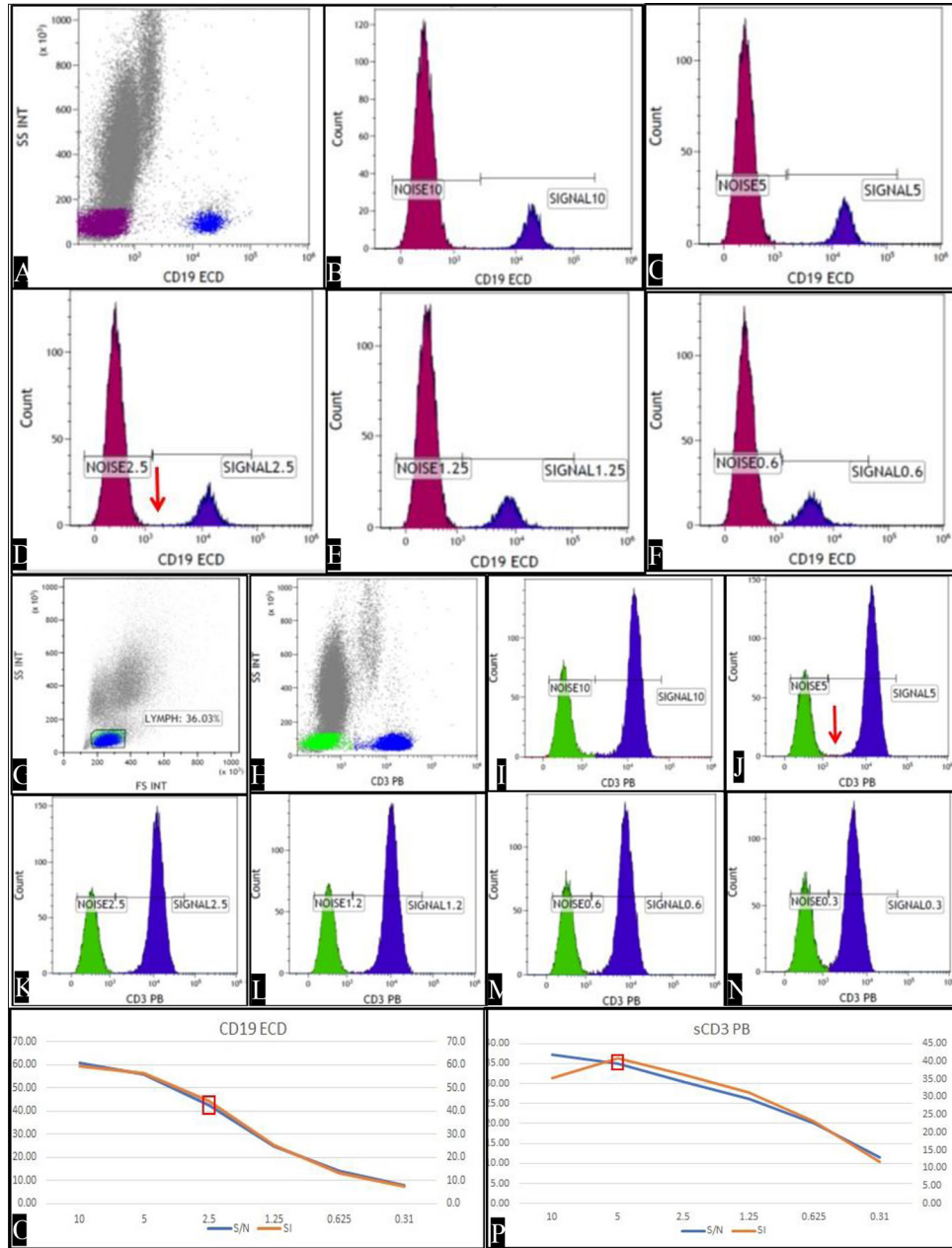
| Antibody    | Conjugate | Clone | Isotype | Species | Vendor  | Cat. no | Lot no  |
|-------------|-----------|-------|---------|---------|---------|---------|---------|
| CD3         | PB        | UCHT1 | IgG1    | Mouse   | Beckman | A93687  | 200033  |
| Volume (µL) | MFI +     | MFI - | MFP-MFN | SD NEG  | S/N     | SI      | % gated |
| 10          | 14924     | 400   | 14524   | 206     | 37.32   | 35.19   | 36.03   |
| 5           | 13827     | 396   | 13432   | 164     | 34.94   | 40.98   | 36.3    |
| 2.5         | 12043     | 396   | 11647   | 161     | 30.44   | 36.22   | 37.36   |
| 1.25        | 10144     | 386   | 9758    | 156     | 26.28   | 31.31   | 36.17   |
| 0.625       | 7838      | 390   | 7448    | 161     | 20.11   | 23.17   | 36.54   |
| 0.31        | 4788      | 414   | 4375    | 188     | 11.58   | 11.60   | 36.55   |

PB: Pacific blue, 's' for surface processing, CD: Cluster of differentiation, Cat no: Catalog number, Ig: Immunoglobulin, MFI: Mean fluorescent intensity, MFP: Mean fluorescent intensity positive, MFN: Mean fluorescent intensity negative, SD NEG: Standard deviation of negative signal, S/N: Sound to noise ratio, SI: Stain index, µL: Microliter

**Table 4:** Final titrated volume and cost-benefit analysis for each antibody.

| Antibody-conjugate | Cost per vial (rupees) | Number of tests/volume per test as per the manufacturer | CPT as per manufacturer (rupees) | Titrated volume (µL) | Cost per test after titration (rupees) | Percentage reduction in CPT (%) |
|--------------------|------------------------|---|----------------------------------|----------------------|--|---------------------------------|
| cMPO-FITC          | 11,750                 | 100/20  | 118                              | 10                   | 59                                     | 50                              |
| cCD79a-PE          | 43,853                 | 100/20  | 439                              | 5                    | 110                                    | 75                              |
| CD19-ECD           | 33,747                 | 100/10  | 338                              | 2.5                  | 84                                     | 75                              |
| CD34-PC5.5         | 58,000                 | 100/10  | 580                              | 0.6                  | 36                                     | 84                              |
| cCD3-PC7           | 47,493                 | 100/10  | 475                              | 2.5                  | 119                                    | 75                              |
| nTdT-APC (BD)      | 41,076                 | 100/5   | 411                              | 2.5                  | 205                                    | 50                              |
| CD45-APC700        | 36,276                 | 100/10  | 363                              | 0.6                  | 23                                     | 84                              |
| CD7-APC750         | 45,448                 | 50/10   | 909                              | 2.5                  | 227                                    | 75                              |
| sCD3-PB            | 19,638                 | 50/10   | 393                              | 5                    | 196                                    | 50                              |
| HLA-DR-KO          | 51,912                 | 50/10   | 1,038                            | 1.25                 | 130                                    | 88                              |
| Total              | 3,89,193               |   | 5,064                            |                      | 1,189                                  | 77                              |

µL: Microliter, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, ECD: Energy coupling dye, PC: Phycoerythrin-cyanin, APC: Allophycocyanin, PB: Pacific blue, KO: Krome orange, CD: Cluster of differentiation, MPO: Myeloperoxidase, c, s, n indicates cytoplasmic, surface and nuclear staining, TdT: Terminal deoxynucleotidyl transferase, HLA-DR: Human leucocyte antigen-DR isotype, BD: Becton, Dickinson biosciences company and all the other antibodies from Beckman Coulter company, CPT: Cost per test



**Figure 1:** (A-N) Histogram, and (O) double Y-plot showing titration experiment for cluster of differentiation (CD) 19 energy coupling dye (ECD); (P) double Y-plot showing titration experiment for surface CD3 pacific blue (PB)

## DISCUSSION

The major pre-analytical variable that should be addressed in MFC is the validation of monoclonal antibodies. The initial step in this process includes titration of each antibody, as endorsed by the EuroFlow Consortium for standardization in MFC.<sup>[3]</sup> Most manufacturers recommend a higher concentration of the antibody for MFC testing; however, following these recommendations directly may not be cost-effective.

The need to titrate the antibody is to optimize signal intensity, achieve staining saturation, and conserve reagents. This is usually done using objective methods such as SNR or SI, or using a subjective method such as visual interpretation of histograms or plots to determine the lowest effective antibody concentration.<sup>[3-6]</sup> In this study, both of these approaches were used, as both of them were found to be complementary to each other, thereby negating the disadvantages associated with using either method alone. Titration experiments not

**Table 5:** Stability of tandem dyes in cocktail using CytoComp™ lyophilized sample for the initial 5 days.

| Antibody-conjugate | Day 1 MFI value | Day 2 MFI value | Day 3 MFI value | Day 4 MFI value | Day 5 MFI value | Mean  | SD   | CV (%) |
|--------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-------|------|--------|
| cMPO-FITC          | 3.2             | 3.0             | 6.6             | 3.0             | 2.1             | 3.6   | 1.7  | 0.48   |
| cCD79a-PE          | 5.8             | 5.1             | 12.1            | 5.1             | 7.8             | 7.2   | 3.0  | 0.41   |
| CD19-ECD           | 10.6            | 10.9            | 8.5             | 8.1             | 10.5            | 9.7   | 1.3  | 0.13   |
| CD34 – PC5.5       | NA              | NA              | NA              | NA              | NA              | NA    | NA   | NA     |
| cCD3-PC7           | 1.9             | 1.7             | 2.4             | 2.2             | 1.8             | 2.0   | 0.3  | 0.14   |
| nTdT-APC           | NA              | NA              | NA              | NA              | NA              | NA    | NA   | NA     |
| CD45-APC700        | 112.9           | 92.3            | 92.6            | 195.1           | 170.7           | 132.7 | 47.4 | 0.35   |
| CD7-APC750         | 13.8            | 24.6            | 21.7            | 21.6            | 15.4            | 19.4  | 4.6  | 0.23   |
| sCD3-PB            | 11.2            | 13.2            | 12.3            | 13.0            | 12.9            | 12.5  | 0.8  | 0.06   |
| HLA-DR-KO          | 16.5            | 14.9            | 26.8            | 30.4            | 34.4            | 24.6  | 8.6  | 0.34   |

FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, ECD: Energy coupling dye, PC: Phycoerythrin-cyanin, APC: Allophycocyanin, PB: Pacific blue, KO: Krome orange, CD: Cluster of differentiation, MPO: Myeloperoxidase, c, s, n indicates cytoplasmic, surface and nuclear staining, TdT: Terminal deoxynucleotidyl transferase, HLA-DR: Human leucocyte antigen-DR isotype, MFI: Mean fluorescent intensity, SD: Standard deviation, CV: Co-efficient of variation, NA: Not available

**Table 6:** Stability of tandem dyes in cocktail using CytoComp™ lyophilized sample on every Wednesday for a continuous 4 weeks.

| Antibody-conjugate | Week 1 MFI value | Week 2 MFI value | Week 3 MFI value | Week 4 MFI value | Mean  | SD   | CV (%) |
|--------------------|------------------|------------------|------------------|------------------|-------|------|--------|
| cMPO-FITC          | 11.14            | 14.16            | 16.31            | 11.07            | 13.17 | 2.5  | 0.19   |
| cCD79a-PE          | 3.73             | 4.98             | 4.12             | 4.17             | 4.25  | 0.52 | 0.12   |
| CD19-ECD           | 4.06             | 5.65             | 4.44             | 4.55             | 4.67  | 0.59 | 0.13   |
| CD34 – PC5.5       | NA               | NA               | NA               | NA               | NA    | NA   | NA     |
| cCD3-PC7           | 4.61             | 5.70             | 6.44             | 3.70             | 5.11  | 1.04 | 0.20   |
| nTdT-APC           | NA               | NA               | NA               | NA               | NA    | NA   | NA     |
| CD45-APC700        | 69.18            | 125.25           | 81.32            | 107.99           | 95.93 | 21.9 | 0.23   |
| CD7-APC750         | 15.01            | 17.95            | 14.48            | 15.28            | 15.68 | 1.3  | 0.08   |
| sCD3-PB            | 10.41            | 11.66            | 10.39            | 10.23            | 10.67 | 0.57 | 0.05   |
| HLA-DR-KO          | 18.14            | 17.88            | 16.94            | 18.10            | 17.76 | 0.54 | 0.03   |

FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, ECD: Energy coupling dye, PC: Phycoerythrin-cyanin, APC: Allophycocyanin, PB: Pacific blue, KO: Krome orange, CD: Cluster of differentiation, MPO: Myeloperoxidase, c, s, n indicates cytoplasmic, surface, and nuclear staining, TdT: Terminal deoxynucleotidyl transferase, HLA-DR: Human leucocyte antigen-DR isotype, MFI: Mean fluorescent intensity, SD: Standard deviation, CV: Coefficient of variation, TM: Trademark, NA: Not available

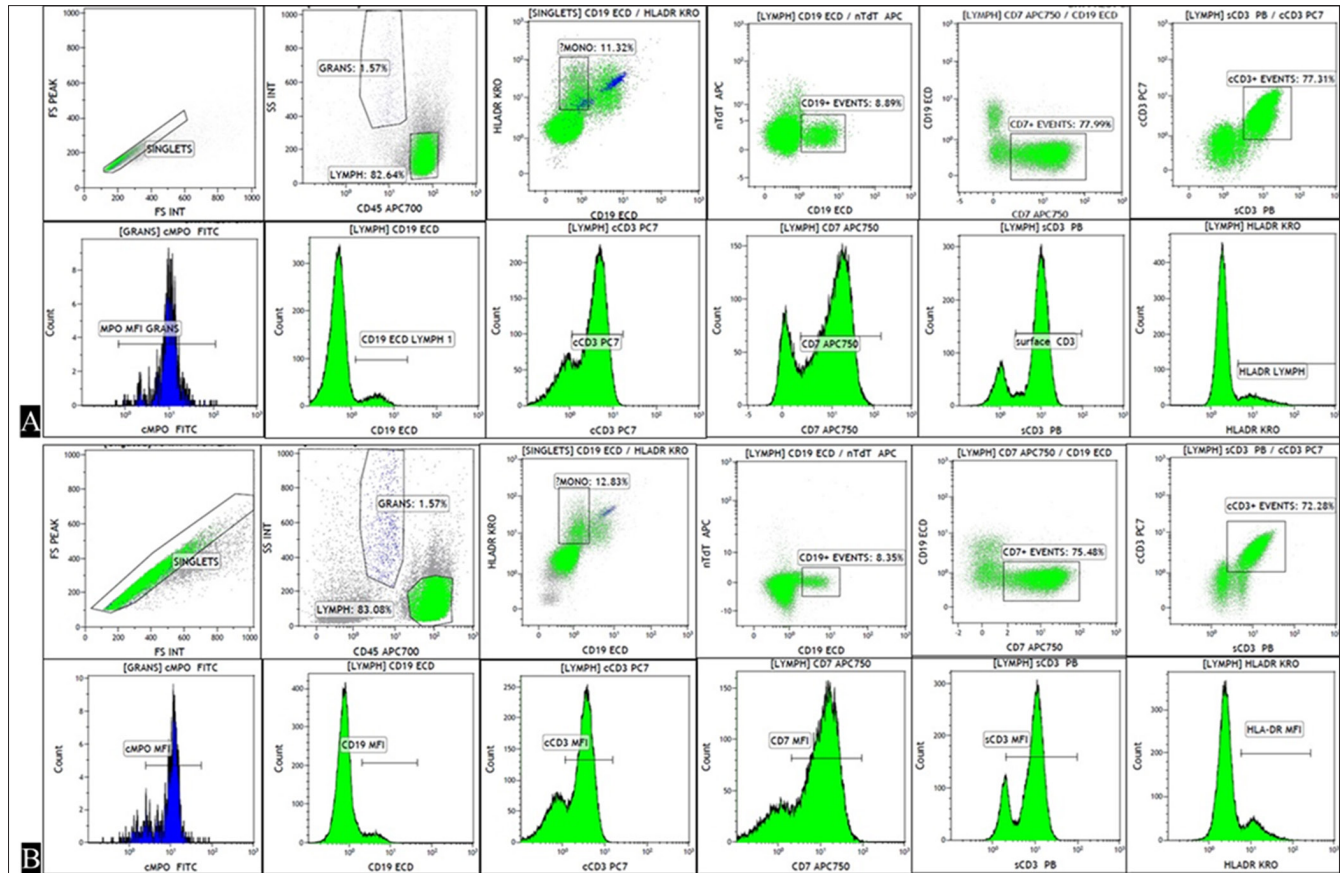
only improve multicolor panel experiments but also optimize fluorescent staining in MFC.<sup>[4]</sup>

As part of the initial phase of the study, the individual antibodies in the ALOT tube were titrated. It was found that there was a greater reduction in the final antibody concentrations required for MFC testing. This optimization proved to be highly cost-effective in our laboratory, resulting in approximately a 77% reduction in the cost of ALOT tube testing alone. We also extended the same titration experiment to the other panels for the sub-categorization of acute leukemias.

In the second phase of the study, the pre-titrated volumes of monoclonal antibodies were combined to prepare antibody cocktails before adding them to the test samples.

The initial titration experiment also reduced the workforce requirements by decreasing the time spent on each test and minimizing non-conformities related to the omission or incorrect addition of individual antibodies, although this was not objectively evaluated.<sup>[3-5]</sup>

In general, it is recommended to store antibody cocktails for the expected maximum number of MFC tests, typically for 1–2 weeks.<sup>[3-5]</sup> In our laboratory, the prepared antibody cocktails are stored for a period of 7–10 days. The major concern is whether tandem dyes conjugated to monoclonal antibodies remain stable during prolonged storage. This stability varies within and between laboratories.<sup>[7-11]</sup> This was the stimulus for us to check for the stability of tandem dyes in antibody cocktail preparations in our laboratory.



**Figure 2:** (A and B) week 1 and 4 stability check with CytoComp™ lyophilized sample on every Wednesday.

There are several studies that have discussed and debated this issue.<sup>[7-15]</sup> We conducted this study like that of Johansson and Macey<sup>[9]</sup>, using both fresh peripheral blood samples during the initial five days to check for the stability and CytoComp™ lyophilized cells for up to four weeks. The study results were in agreement with those of these authors. We had tested for the maximum of only four weeks, beyond which the utility of maintaining antibody cocktails was minimal; therefore, further evaluation was not performed.

The main practical difficulty encountered in this study was the unavailability of acute leukemia cases daily for five days during the stability analysis. Consequently, objective evaluation of CD34-PC5.5 and nTdT-APC was not performed in the daily testing, as CytoComp™ lyophilized samples predominantly showed mature cells for weekly stability assessment. However, this specific difficulty was addressed by validating the same antibody cocktail in routine diagnostic practice using acute leukemia samples, which demonstrated clinically acceptable stability for 10–14 days.

Rawstron *et al.*<sup>[16]</sup> tested two separate antibody cocktails, each containing six antibodies (including APC-H7- and PE-Cy7-conjugated antibodies), over 28 days. They observed

no loss of PE-Cy7 or APC-H7 signal intensity during this time; however, there was an increase in background APC-H7 intensity on APC-positive cells. In a similar study, Le Roy *et al.*<sup>[10]</sup> showed that APC tandems may undergo degradation depending on the cell types present in the sample. The effect occurred relatively immediately (within 30 min) and was also observed in single antibody-stained samples. However, it was difficult to determine how the results were influenced by cell type and whether the APC tandem dyes were stored individually or as part of a cocktail.

Hulspas *et al.*<sup>[11]</sup> studied the photosensitivity of PE-based tandem dyes, particularly PE-Cy7. They were stored as antibody cocktails in light-protective bottles and subjected to a single daily light exposure of 10 min (for preserved cells) or 20 min (for fresh bone marrow), reflecting the average time spent on the bench for each type of cocktail. This level of light exposure did not appear to cause tandem degradation. In this study, we did not analyze the tandem degradation after light exposure.

O'Donahue *et al.*<sup>[12]</sup> studied the validation of antibody cocktails for MFC. They observed that to improve laboratory efficiency, they recommend using the antibody cocktails

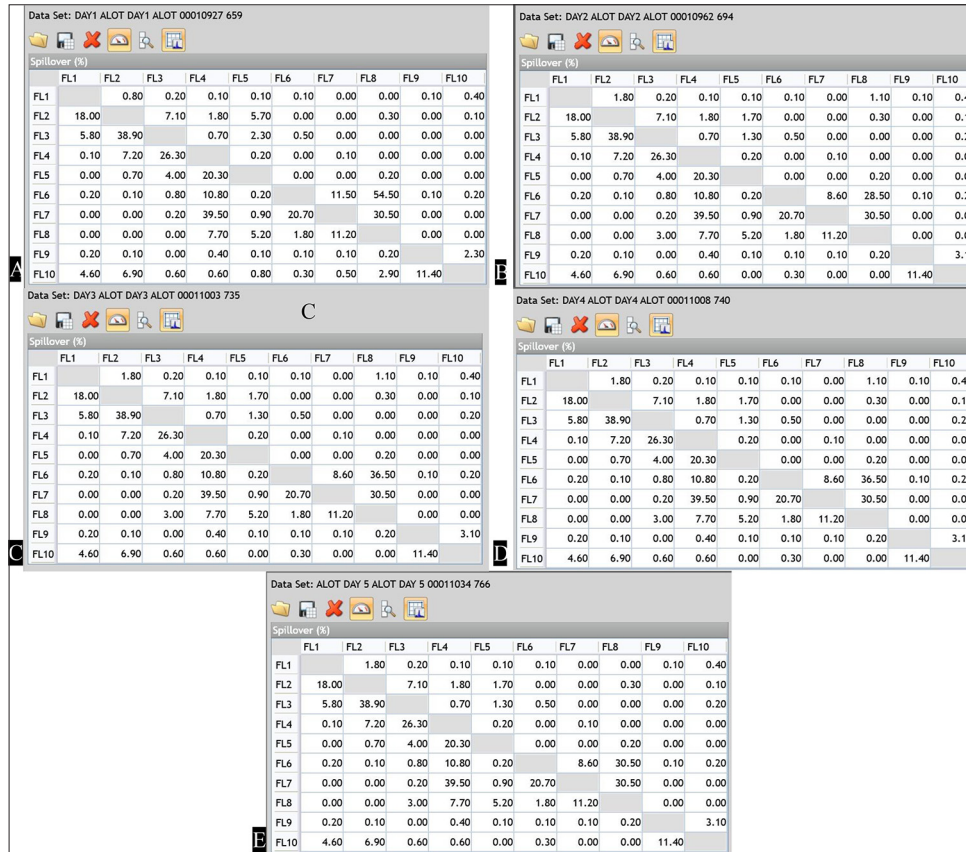


Figure 3: (A-E) Compensation matrix from day 1 to day 5 for the acute leukemia orientation tube.

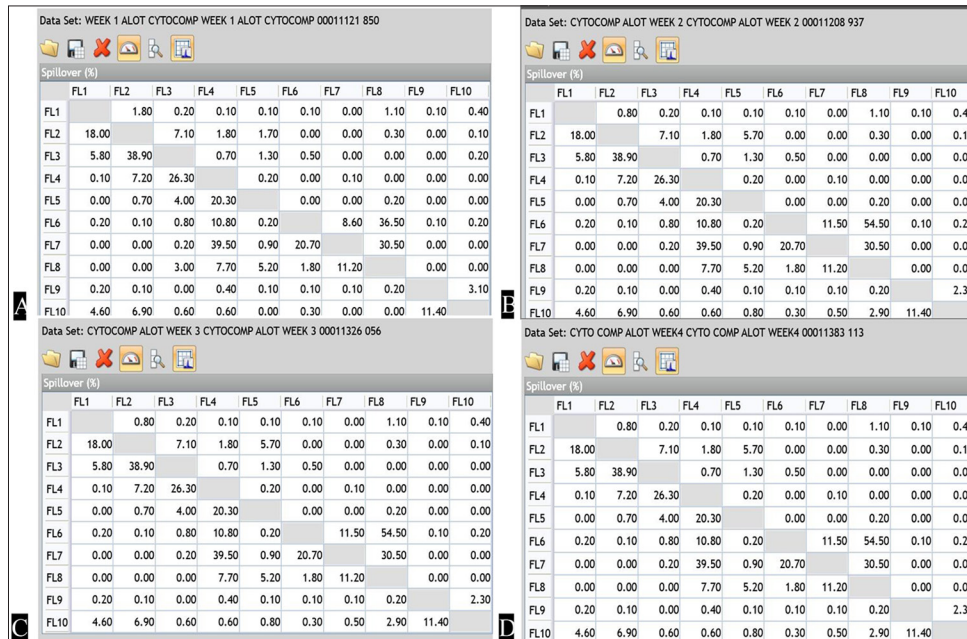


Figure 4: (A-D) Compensation matrix from week 1 to week 4 for acute leukemia orientation tube using CytoComp™ lyophilized cells.

rather than adding individual antibodies before testing. They found that pipetting errors were the most common problem during cocktail preparation. To avoid these issues, they recommend using electronic pipettes and regular calibration, to be performed and monitored at least twice per year. They concluded that antibody cocktails, when performed with proper standardization and appropriate quality control, enhance the accuracy of the results and improve overall performance in the MFC laboratories.

## CONCLUSIONS

Titration of monoclonal antibodies resulted in a substantial reduction in the final volume of antibodies required for the cocktail preparation compared to the manufacturer's recommendation. This experiment led to an approximate 77% reduction in cost per test, representing a significant improvement in resource utilization. Although not objectively measured, there was also an observed improvement in the need for manpower, including reduced time per test and fewer non-conformities related to the omission of individual antibodies. The pre-titrated antibody cocktail remained stable for the initial five days and demonstrated tandem dye stability for four weeks when tested using CytoComp™ lyophilized samples. This stability was further validated in acute leukemia samples during routine daily reporting.

**Author's contributions:** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SK and PM. The first draft of the manuscript was written by SK and PM commented and corrected the previous versions of the manuscript. All authors read and approved the final manuscript.

**Ethical approval:** The research/study was approved by the Institutional Review Board at JIPMER, Puducherry, approval number JIP/IEC/20fiv1/093, dated 26th April 2021.

**Declaration of patient consent:** Patient's consent not required as there are no patients in this study.

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