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Validation of Manufacturers Recommended Working Volumes of Fluorochrome-conjugated Monoclonal Antibodies for Flowcytometric Analysis of Lymphocytes

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Authors' contributions

This work was carried out in collaboration among all authors. Author MDL designed the study, performed the experiments and produced the first draft of the manuscript. Authors MDL, GID and TSV performed literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Flow cytometry is a robust and rapidly growing technique used to analyse multiple parameters concurrently on a single cell basis. Cell populations can be characterised using a combination of both surface and intracellular antigens. The act of generating the best research data under given circumstances begins with a well-designed reagent optimisation protocol. Applying flow cytometric analysis to obtain reliable and dependable research data requires establishing the best working volumes of the monoclonal antibodies.

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Aim: This study aimed to provide practical illustrations on the approach for determining optimal working volumes (concentrations) of fluorochrome-conjugated monoclonal antibodies for flow cytometry.
Methods: Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were stained using three different volumes of respective fluorochrome-conjugated monoclonal antibodies: one volume below, a volume above and the volume recommended by the manufacturers. The antibodies analysed include CD3-FITC, CD4-APC-Cy7, CD19-Alexa Fluor 700, CD45-AmCyan, CD28-PE, and CD45-AmCyan isotype control. Depending on the availability of cells, a total of 10,000 to 30,000 events were acquired for analysis. Combinations of the mean fluorescence intensity (MFI), the number of events in the population of interest, and the clearance of the Isotype control histogram peak from the positive population were used to determine the best working volumes of the mAbs used.
Results: The study reported optimum working volumes of 10µL for CD45-Amcyn, 20µL for CD3-FITC, 10µL for CD4-APC-Cy7, 10µL for CD19-Alexa Fluor 700, 20µL for CD28-PE. We confirmed the recommended volumes provided by the manufacturer for CD3-FITC and CD28-PE. However, higher volumes of CD45-Amcyn, CD4-APC-Cy7 and CD19-Alexa Fluor 700 were found more optimal than the recommended volumes supplied by manufacturers.
Conclusion: The application of a simple validation experiment for the working volumes (concentrations) of fluorochrome-conjugated monoclonal antibodies, like the one described here, is recommended as an integral part of the optimisation protocol for flow cytometry.

Keywords: Fluorochrome conjugated; flow cytometry; working volume; monoclonal antibodies; lymphocytes, validation.

1. INTRODUCTION

Flow cytometry is a powerful and widely used research tool that can be used to identify, separate, characterise and count various subpopulations of immune cells in a heterogeneous fluid mixture as they pass through the laser [1,2]. The technique measures particles' physical and chemical characteristics in a stream of fluid flowing in a single file [3]. The general principle of flow cytometry depends on measuring individual cells' optical and fluorescence characteristics of particles pre-labelled with fluorescent dyes [4]. As they pass through a light source, the pre-labelled molecules (fluorochromes) are excited to a higher energy state [4,5]. On returning to their resting states, the fluorochromes emit light energy at a higher wavelength detected by a computer, and the software translates the signal to electronic data [5]. Multiple fluorochromes can be used, each with similar excitation wavelengths and different emission wavelength to allow several cell characters to be measured simultaneously [2]. A multicolour flow cytometric analysis is a powerful and fast-growing technology that enables researchers to perform complex cellular analysis promptly and effectively by analysing several parameters concurrently [6]. This technique can analyse thousands of cells in seconds by employing multiple fluorescent markers to identify and characterise the subtypes of cells [7,8].

The differences in cells size and internal complexity, such as DNA or RNA content, cytoplasmic features and other membrane-bound and intracellular proteins, can be distinguished by light scattering at different angles, while light emitted from fluorescently conjugated antibodies can help in identifying a wide array of cell surface and cytoplasmic antigens [2,5]. The technique is a robust research platform that can be used for immunophenotyping of cells, cell sorting, cell cycle analysis, analysis of apoptosis, as well as cell proliferation assays and intracellular calcium flux [9-11]. This approach has become the most puissant research tool for quantitative and qualitative analysis of various subtypes of cells involved in immune responses to several diseases [12,13].

Cells and specific particles can be analysed from various specimens such as whole blood, peripheral blood mononuclear cell (PBMC), bone marrow, cerebrospinal fluid, urine, fluids from the serous cavity and solid tissues [2,14,15]. Therefore, this study was designed to provide a working protocol that can help young researchers to determine the best working volumes of fluorochrome-conjugated antibodies for flow cytometric analysis.

2. MATERIAL AND METHODS

2.1 The Study Design

Three different volumes (concentrations) of the six fluorochrome-labelled monoclonal antibodies were prepared in a three-tube design, as shown in Table 1 below. The analysis of cell acquisition and the quality of histograms obtained from the three tubes were used to determine the optimal working volumes of the monoclonal antibodies for flow cytometric analysis of cells and particles.

2.2 The Fluorochrome-conjugated Monoclonal Antibodies (mAbs)

As part of a bigger study that evaluated the effect of CMV infection on the circulating lymphocytes in follicular lymphoma (FL) patients (unpublished data), the working volumes of 26 fluorochrome-conjugated monoclonal antibodies selected for the study were determined using a similar approach used for this experiment. We determined the best working volumes by immunophenotyping of equal amounts of peripheral blood mononuclear cells (PBMCs) stained in three different volumes of fluorochrome labelled monoclonal antibodies. In this study, the optimum working volumes of the following fluorochrome-conjugated monoclonal antibodies (mAbs) were determined; CD3-FITC (BD Biosciences, Oxford, UK), CD4-APC-Cy7 (BD Biosciences, Oxford, UK), CD19-Alexa Fluor 700 (BD Biosciences, Oxford, UK), CD45-AmCyan (BD Biosciences, Oxford, UK), CD28-PE (BD Biosciences, Oxford, UK), and isotype control, CD45-AmCyan (BD Biosciences, Oxford, UK).

2.3 Sample Collections and PBMCs Isolation

The whole blood samples collected from healthy volunteers (HV) who consented to donate blood to optimise the flow cytometric technique used to evaluate lymphocytes in FL patients were used for this study. The density centrifugation technique was used to process the anticoagulated blood samples, and the peripheral blood mononuclear cells (PBMCs) were isolated and used for the study.

For PBMCs isolation, in a biological class II Safety Cabinet, Lymphoprep (Product #: 1114740, Axis-Shield, Alere Ltd., Stockport-UK) was pipetted into prelabeled tubes in a 2:1 ratio of blood to Lymphoprep. Blood was gently layered on top of the Lymphoprep using a serological pipette. The tubes were centrifuged at 800 g for 30 minutes at room temperature (RT) with the brake setting at low. Following centrifugation, mononuclear cells in the buffy coat layer were carefully removed into a tube, which was filled up with Roswell Park Memorial Institute (RPMI)-1640 medium (LM-R1641/500, Labtech International Ltd, East Sussex, UK) and centrifuged at 550 × g for 10 minutes to wash and pellet the cells with the brake on. The supernatants were discarded, and cells were resuspended in the appropriate volume of freezing solution A (10% foetal calf serum (FCS) in RPMI 1640 media. Cells were counted to determine live and dead cells using ChemoMetec Nuclear Counter in the GCP Lab facility, and an equal volume of medium B (10% FCS, 20% Dimethyl sulfoxide (DMSO in RPMI) was added slowly. Cells were aliquoted in vials and placed in Nalgene cryofreezing containers to -80 oC freezer for a minimum of 12 hours before moving to -150oC freezer in the GCP Lab Facility freezer room.

2.4 The PBMC Samples: Thawing and Recovery of Cells

When ready to use, the frozen specimens were thawed by being held in a closed fist before transferring the cells to pre-label Universals on ice. Each 1mL of thawed cell suspension was

Table 1. The design for the determination of working volumes of the fluorochrome-conjugated monoclonal antibodies

S/N	CONJ mAbs	TUBE#1 (µL)	TUBE#2 (µL)	TUBE#3 (µL)
1	CD545.Amcyan	2.5	5.0	10.0
2	CD3-FITC	10.0	20.0	30.0
3	CD4-APC-Cy7	2.5	5.0	10.0
4	CD19-Alexa Fluor 700	2.5	5.0	10.0
5	CD28-PE	10.0	20.0	30.0
6	CD45-Amcyan (Isotype)	2.5	5.0	10.0

slowly diluted with 10mL of RPMI- 1640 medium supplemented with 10% foetal calf serum (FCS). The RPMI 1640 solution was added to cells gradually in a drop-wise manner with constant and careful agitation throughout the process. The Universals were centrifuged at 500 × g for 5 minutes, and the supernatants were discarded. Then the cell pellets were resuspended, counted and assessed for viability using ChemoMetec NucleoCounter (ChemoMetec A/S, Denmark) by detecting total and dead cell counts. Cells were resuspended in BD FACSFlow Sheath Fluid (Cat #: 342003, BD Biosciences, Oxford UK) for lymphocyte immunophenotyping.

2.5 The Flow Cytometer-BD LSRFortessa™

The flow cytometer used for data acquisition is the BD LSRFortessa™ Special Order Research Product (BD Biosciences, Oxford, UK), an air-cooled multi-laser benchtop flow cytometer, equipped with Red, Yellow/Green, Blue, Violet and UV lasers and capable of supporting the analysis of up to 18 parameters. This equipment is housed in the Liverpool Good Clinical Laboratory Practice (GCLP) Facility, Liverpool.

2.6 The Cytometer Setup and Tracking (CS&T)

The CS&T is a fully automated BD FACSDiva software and reagent research system unique to BD digital cytometers designed to provide Characterisation, Setup, and Tracking for baseline settings. This system optimises and standardises cytometer setup and tracks cytometer performance, ensuring consistency and reproducibility of FACS data by offsetting the routine instrument variability. The BD FACSDiva CS&T Research Bead set (Cat #: 655051, BD Biosciences, Oxford UK) consists of uniform beads of different intensities (bright, mid, and dim beads) designed to characterise the flow cytometer fully. For daily performance checks, a CS&T passed result was considered essential for running the FACS experiment for the day.

2.7 Determination of the Working Volumes of the Conjugated mAbs

The optimal working volumes of the six fluorochrome-conjugated monoclonal antibodies were determined by single fluorescent staining of washed PBMCs from healthy volunteers (HV) using three different concentrations of fluorochrome-conjugated monoclonal antibodies as presented in Table 1. The recommended volume by the manufacturers was used as the starting point for the working volumes, and a value above and one below for each was used as in Table 1 above.

2.8 The Staining and Acquisition of Cells for FACS

Following the addition of antibodies (Table 1), 100µL containing about 2 × 105 to 1 × 106 well-mixed washed PBMCs were placed in the prelabelled FACS tubes. Tubes were incubated for 30 minutes in the dark, at room temperature, after which 2ml of CellWASH (Cat #:349524, BD Bioscience Oxford, UK) was added, and the tubes centrifuged at 300 × g for 5 minutes with low brake. The supernatants were discarded before repeating the washing process. Finally, the cells were resuspended in 500µL of BD FACSFlowTM Sheath Fluid before being gently vortexed. Depending on the availability of cells, the minimum acquisition target was set at between 10,000 and 30,000 events per tube.

2.9 Interpretation and the Design Used to Obtain the Optimal Values

The criteria used for the choice of optimum working volumes of the fluorochrome-conjugated antibodies was a combination of the mean fluorescence intensity (MFI) of the population of cells that stained positive for the fluorochrome-conjugated monoclonal antibody (P1 – P4), the number of events in the population of interest, and the clearance of the Isotype control histogram peak from the positive population. This process was carried out for the six monoclonal antibodies (mAbs).

The algorithm used for the choice of optimum working volumes of the flourochrome-conjugated monoclonal antibodies can be describes thus: values that showed a combination of higher MFI, higher per cent events with clearly distinct positive peaks were preferred. However, in some cases, we chose values with low MFI but with a clearer separation of the positive peaks as optimal as against values that showed higher MFI but with poor separation of the positive peaks from the negative.

3. RESULTS AND DISCUSSION

3.1 Results

The cells distribution are presented in quadrants. The cells that are positive to the respective

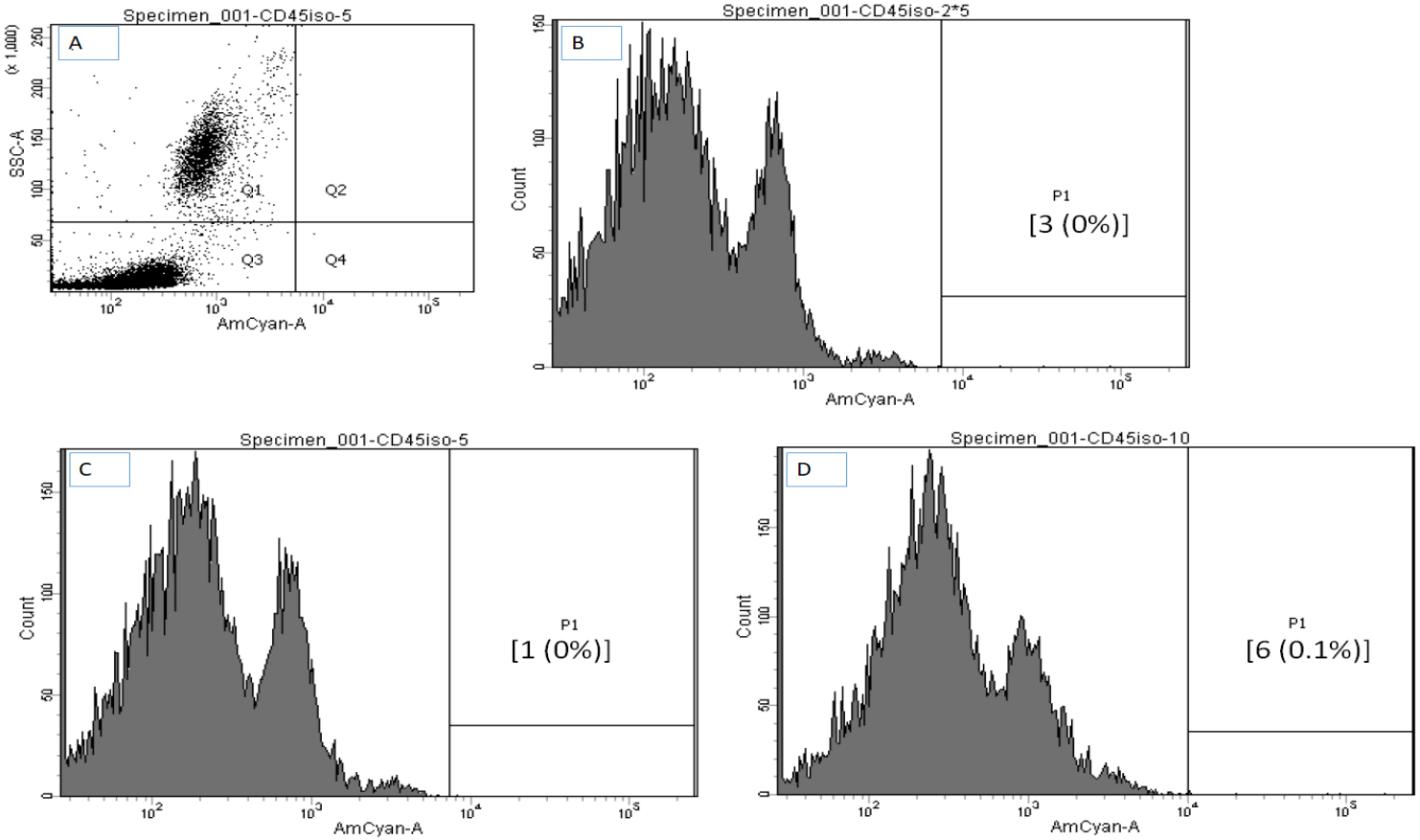

Fig. 1. CD45 –AmCyan (ISOTYPE) – A total of 10,000 cells were acquired. Panel A shows the distribution of cells in the quadrants. The positive cells to 2.5µL, 5.0µL and 10.0µL of anti-CD45- AmCyan (isotype control) were gated as shown in panels B, C and D, respectively. The figure shows that regardless of the volume of the isotype control used, the total events and the per cent parent cells in P1gating remains averagely low (1 to 6 events)

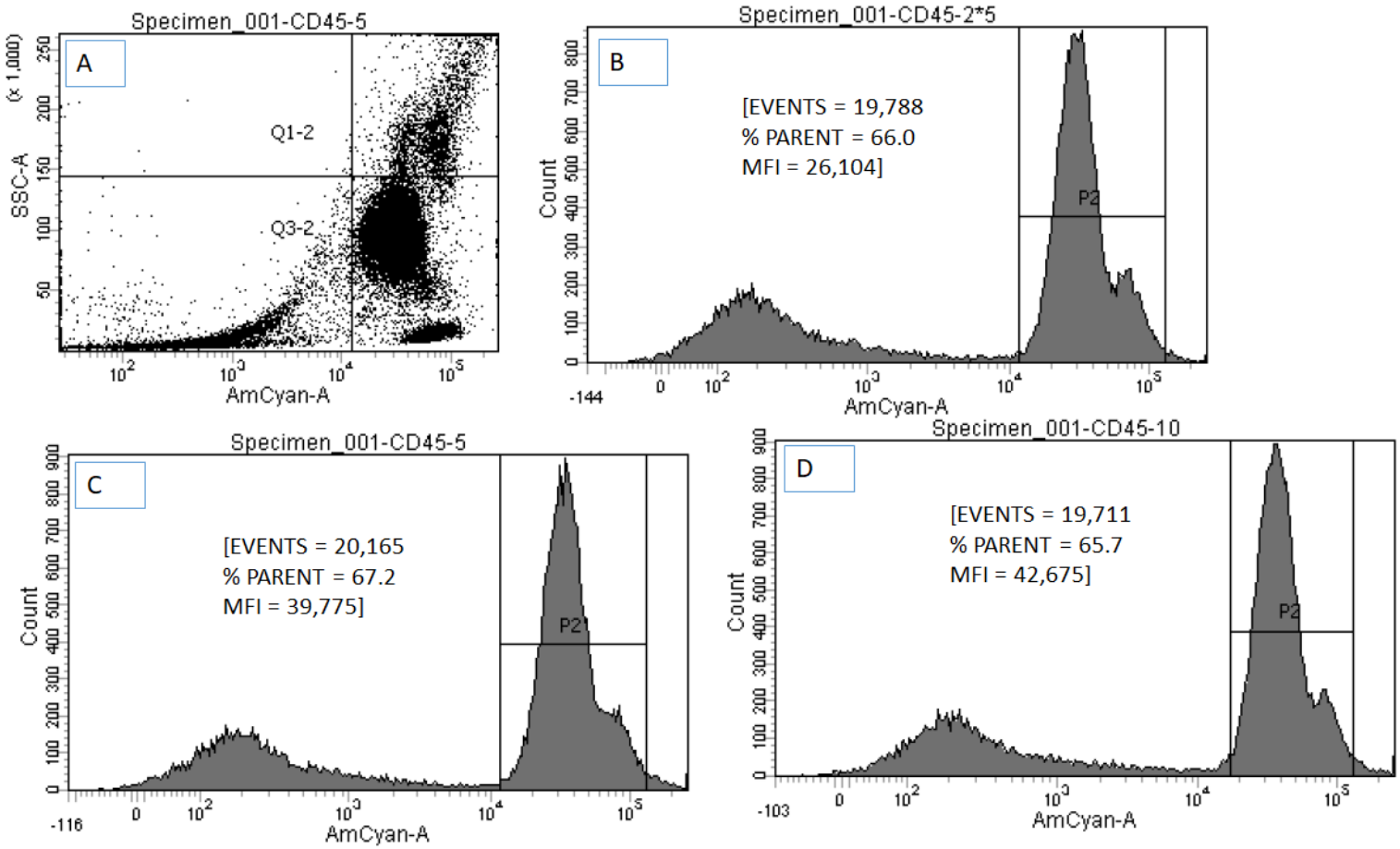

Fig. 1. CD45-Amcyn - A total of 30,000 events were acquired. Panel A shows the distribution of cells in the quadrants. The positive cells to 2.5µL, 5.0µL and 10.0µL of anti-CD45- AmCyan were gated as shown in panels B, C and D, respectively. The total events, per cent parents and MFI values for P2, which represents the population of positive cells for anti-CD45-AmCyan, are written in the respective panels B, C & D. The highest MFI was seen in panel D (the 10.0µL stained cells)

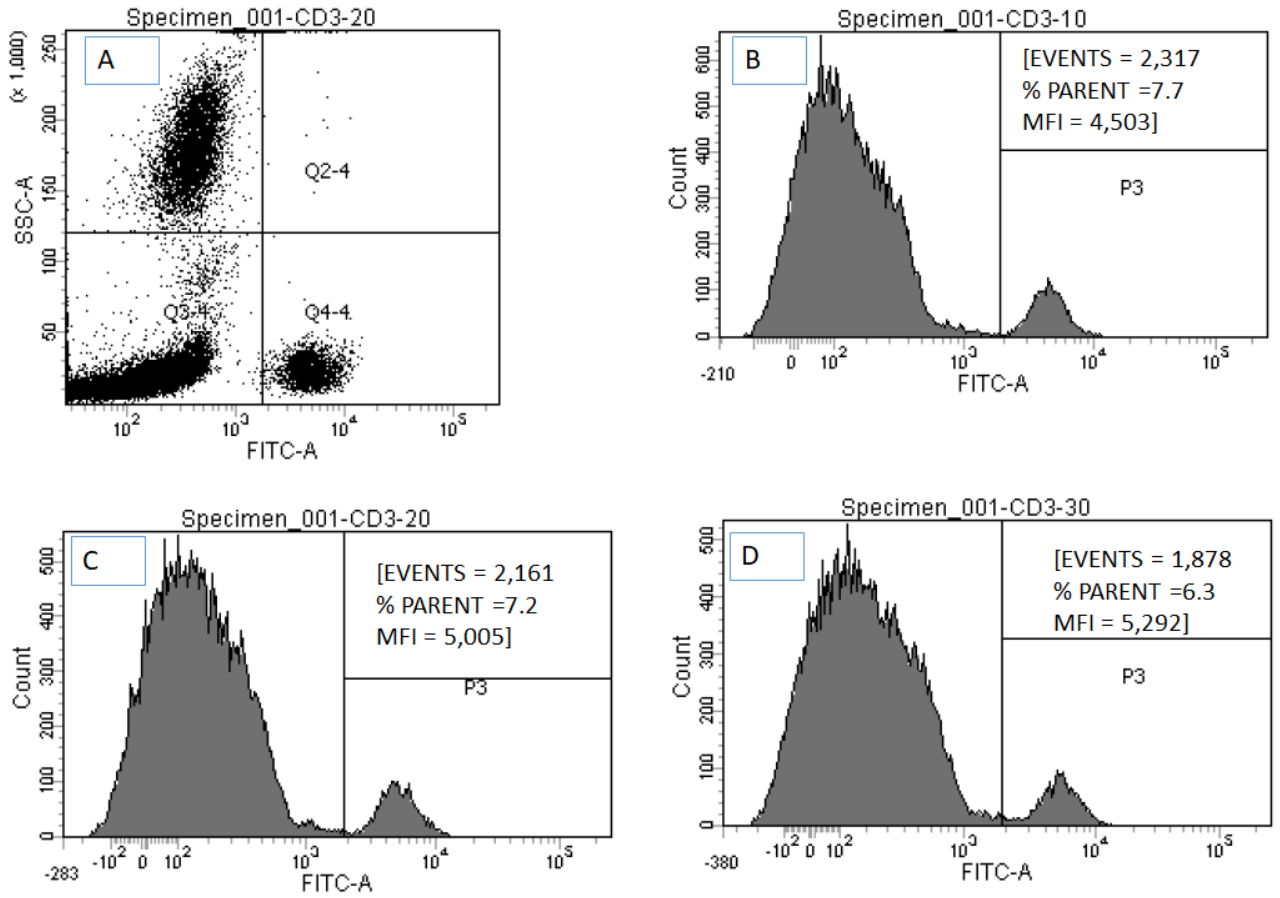

Fig. 2. CD3-FITC - A total of 30,000 events were acquired. Panel 'A' shows the distribution of events/cells in the quadrants. The positive cells to 10.0µL, 20.0µL and 30.0µL of anti- CD3-FITC were gated as shown in panels B, C and D, respectively. The events, the per cent parents and MFI values for P3, representing the population of positive cells for anti- CD3-FITC, are written in the respective panels B, C & D. The highest MFI value was seen in panel D (the cells that were stained with 10.0µL of anti-CD3-FITC)

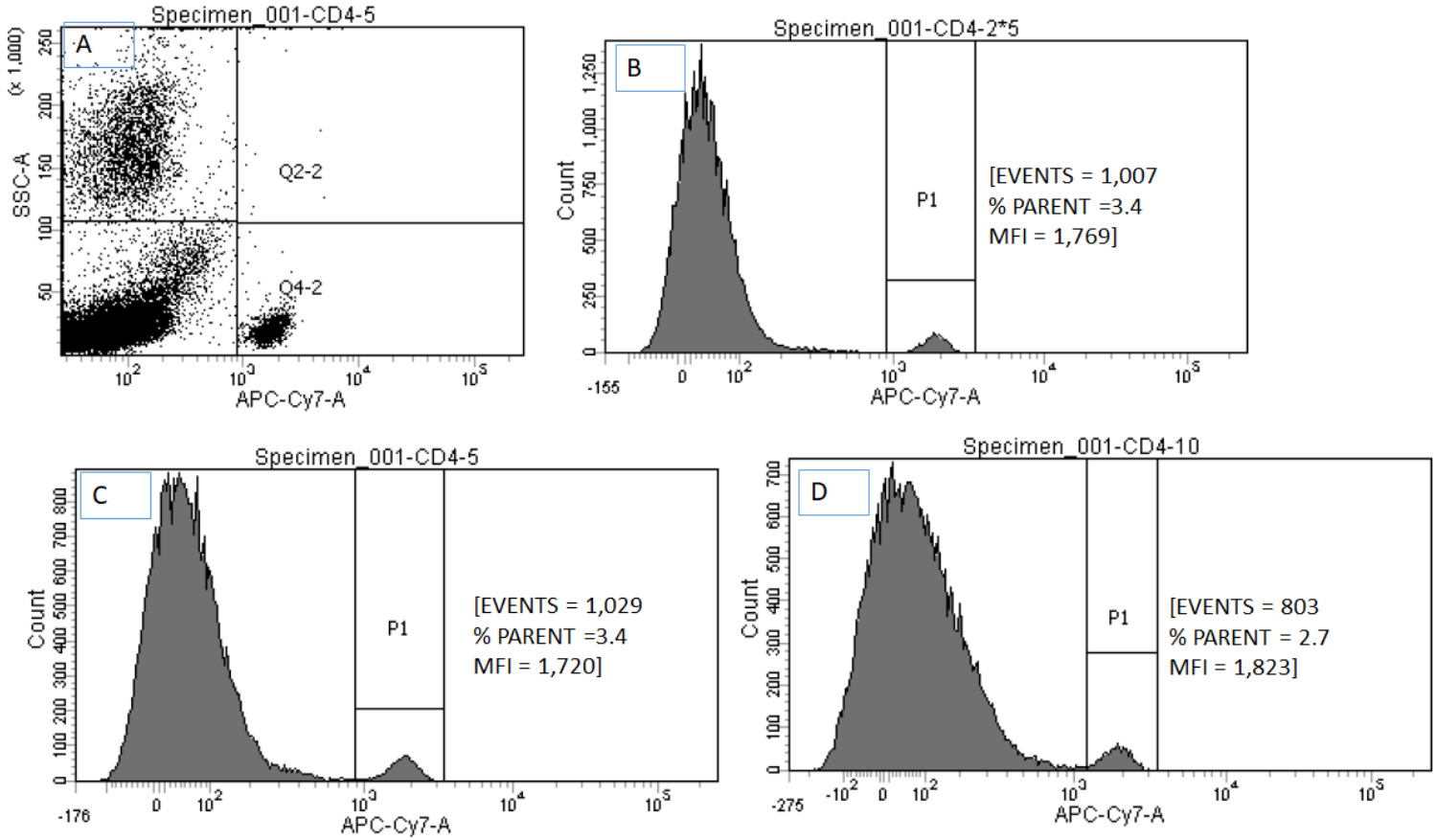

Fig. 3. CD4-APC-Cy7 - A total of 30,000 events were acquired. Panel 'A' shows the distribution of events/cells in the quadrants. The positive cells to 2.5.0µL, 5.0µL and 10.0µL of anti- CD4-APC-Cy7 were gated as shown in panels B, C and D, respectively. The events, the per cent parents and MFI values for P1, representing the population of positive cells for anti- CD4-APC-Cy7, are written in the respective panels B, C & D. The highest MFI value was seen in panel D (the cells that were stained with 10.0µL of anti-CD4-APC-Cy7)

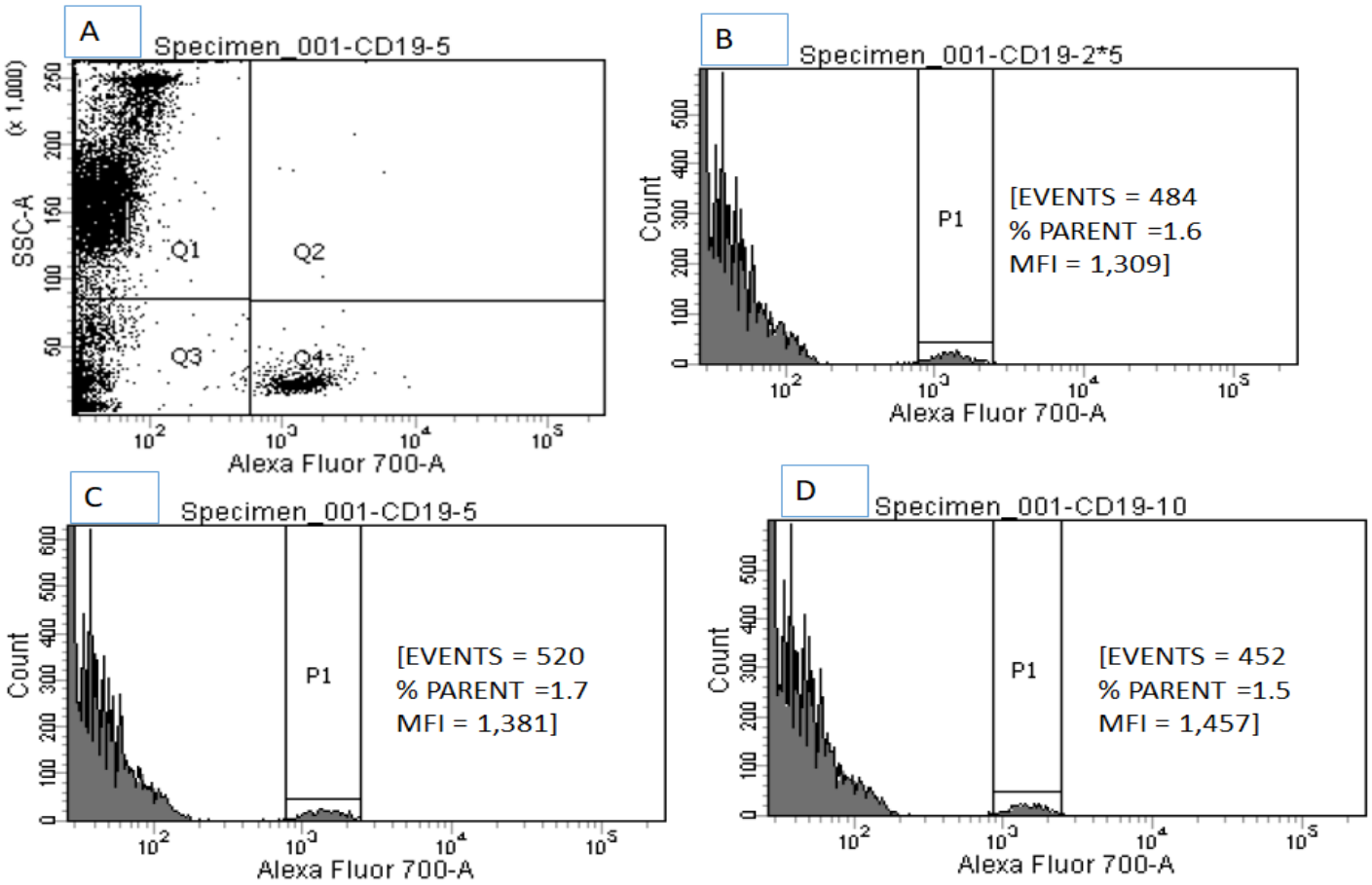

Fig. 4. CD19-Alexa Fluor 700 - A total of 30,000 events were acquired. Panel A shows the distribution of events/cells in the quadrants. The positive cells to 2.5.0µL, 5.0µL and 10.0µL of anti-CD19-Alexa Fluor 700 were gated as shown in panels B, C and D, respectively. The total events, the per cent parents and MFI values for P1, representing the population of positive cells for anti-CD19-Alexa Fluor 700, are written in the respective panels B, C & D. The highest MFI value was seen in panel D (the cells stained with 10.0µL of anti- CD19-Alexa Fluor 700)

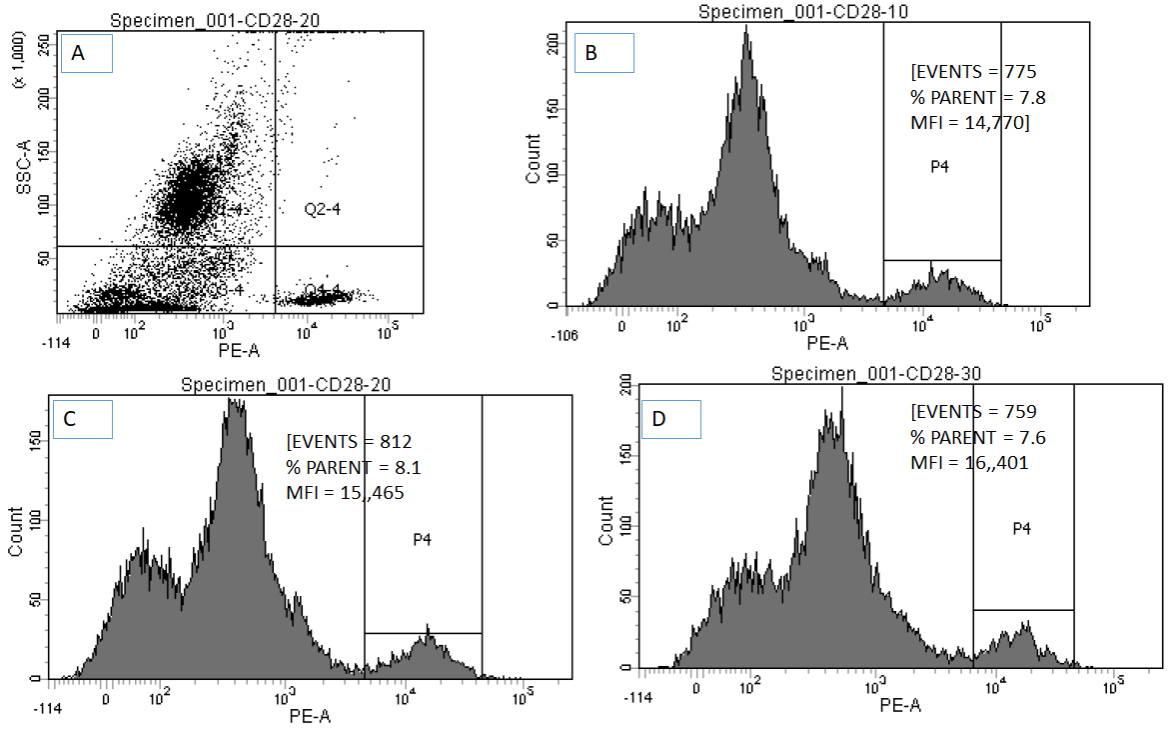

Fig. 5. CD28-PE - A total of 10,000 events were acquired. Panel A shows the distribution of events/cells in the quadrants. The positive cells to 10.0µL, 20.0µL and 30.0µL of anti-CD28- PE were gated as shown in panels B, C and D, respectively. The total events, per cent parents and MFI values for P4, representing the population of positive cells for anti-CD28-PE, are written in the respective panels B, C & D. The highest MFI was seen in panel D (the cells stained with 30.0µL of anti- CD28-PE). In contrast, the highest event and per cent parent were observed in panel C

fluorochrome-conjugated monoclonal antibodies are gated and presented in the histograms shown in Fig. 1 to 6. The three tubes for different volumes of CD45-AmCyan isotype control in the experiment are presented in Fig. 1. This has established that the cells gated in the subsequent fluorochrome-conjugated monoclonal antibody tubes are not artefacts. The figure shows that regardless of the volume of the isotype control used, the total events and the per cent parent cells in P1gating remains averagely low (1 to 6 events).

The optimum working volumes of the fluorochrome-conjugated antibodies were selected based on combinations of the mean fluorescence intensity (MFI) of the positive populations (P1-P4), the number of events (P1-P4), and the clearance of the Isotype control histogram peak from the positive population.

Data, as depicted in Fig. 2, shows that the optimum working volume of CD45-AmCyan is 10µL. The 10µL tube had the highest MFI value of 42,675, with a considerable number of events and the best clearance from the isotype control histogram peak. This observation is contrary to the 5.0µL recommended working volume by the manufacturer. For CD3-FITC (Fig. 3), 20µL was reported as the best working volume. The 20µL tube showed a considerable MFI of 5,005, with a sizable number of events and a good clearance of isotype control histogram peak compared to the other two concentrations. In this case, we confirm that the recommended working volume from the manufacturer is the same reported in our study. Fig. 4 shows the quadrant and histograms of events for CD4-APC-Cy7 positive cells. The 10µL tube was considered to be the optimum working volume. The statistics from this volume revealed the highest MFI of 1,823 with a reasonable number of events and a considerable clearance of isotype control histogram peak. This is against the recommended working volume of 5µL. For CD19- Alexa Fluor700, 10µL was considered the optimum working volume. It has the highest MFI of 1,457, with a considerable number of events and the best clearance of isotype control histogram peak, as against the recommended working dilution of 5µL as shown in Fig. 5. For CD28-PE, 20µL was considered the optimum working volume. It has a considerable MFI of 15,465, with the highest number of events and the best clearance of isotype control histogram peak from P4 compared with the other two dilutions, as shown in Fig. 6.

4. DISCUSSION

Flow cytometry is a laboratory technique that identifies and rapidly measures cells' physical and chemical properties [16]. Cells are labelled with fluorescent markers, referred to as fluorochrome-conjugated antibodies, injected into the flow cytometric machine to detect the target antigens directly. As they flow (one cell at a time) through the laser beam, a large population of cells are identified, characterized and categorized into groups by computer software [5]. Flow cytometry applications may include but not limited to Immunophenotyping, Cell Sorting, Cell Cycle Analysis, Apoptosis, Cell Proliferation Assays, and Intracellular Calcium Flux [5,17].

The major factors that can influence the results of flow cytometry have been grouped into preanalytical and analytical. The preanalytical factors may include pipetting, storage, centrifugation and washing steps. The quantity, quality, and specificity of the antibody and the labelled fluorescent dye have also formed part of the preanalytical factors capable of affecting the results of the technique [18,19]. The analytical factors have been shown to include statistical aspects, gating strategies and measuring principles. This study evaluated one of the preanalytical factors, the effect of the quantity (volume) of fluorochrome-conjugated monoclonal antibodies on flow cytometry results. The study was designed to determine the optimum working volumes of fluorochrome-conjugated antibodies for flow cytometric analysis in our laboratory. The approach was to compare the recommended working volume of the reagent by the manufacturers and two other volumes (one above and one below the recommended volume from the manufacturers) for the 6 fluorochrome-conjugated monoclonal antibodies.

The quadrants shown in 'A' panels of Figs. 1 - 6 were useful for gating and quantifying cells of interest. Generally, the lower left quadrant represents the negative cells population, while the upper left quadrant depicts a defined population of cells for one parameter [20,21]. The lower right quadrant shows the population of cells positive for mAbs (second parameter), and the upper right quadrant represents cells that coexpress both parameters (upper left and upper right) [21]. In the flow cytometric analysis, the principle of gating is employed. Gates and regions are placed around populations of cells with common characteristics, usually forward scatter, side scatter and marker expression, to

investigate and quantify these populations of interest [5].

Our data revealed that higher volumes (concentrations) of three fluorochrome-conjugated monoclonal antibodies (CD45-Amcyn, CD4-APC-Cy7 and CD19-Alexa Fluor 700) were required for higher MFI and increased events of the populations of interest compared to the recommended volumes provided by the manufacturer. However, this study confirms the manufacturer's recommended working volumes of two fluorochrome-conjugated monoclonal antibodies (CD3-FITC and CD28-PE).

5. CONCLUSION

This data shows that the recommended working volumes of three out of five monoclonal antibodies may change following a simple experiment to determine the optimum working volumes. It, therefore, means that the recommended working volumes for fluorochrome-conjugated monoclonal antibodies provided by the manufacturers might not necessarily be applicable in every laboratory. This could be due to environmental factors and other intrinsic factors, which are likely responsible for variation in optimum working volumes. Therefore, it is essential to revalidate the working volumes of all fluorochrome labelled antibodies before application for the flow cytometric analysis of lymphocytes.

CONSENT AND ETHICAL APPROVAL

The determination of the optimal working volumes of fluorochrome-conjugated monoclonal antibodies was part of a bigger study that evaluated T cell subtypes in Follicular lymphoma patients in the context of translational research (unpublished). As part of a trial looking at rituximab and chemotherapy as a treatment for follicular lymphoma in elderly patients (PACIFICO), this study had approvals of the European Union Drug Regulating Authorities Clinical Trials (EudraCT) on a unique number 2008-004759-31 and the International Standard Randomised Controlled Trial (ISRCTN) number ISRCTN99217456. We obtained written informed consent from healthy volunteers that donated blood for this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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