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A Brighter Future in Flow

ED7747 Monocyte Blocking Buffer: A reagent designed to inhibit tandem-dye-induced non-specific binding of monoclonal antibodies to monocytes

Abstract

Tandem dye-to-monocyte non-specific binding is a widely documented undesirable phenomenon observed in multi-color flow cytometry. Tandem dye non-specific binding of monocytes complicates rare cell analysis and monocyte antigen analysis by increasing background of monocytes, virtually making monocytes falsely positive. This requires the use of additional monocyte markers and specific antibodies to be used in order to exclude falsely positive monocytes from analysis.

We hereby present the importance and possibilities of prevention of such non-specific binding. EXBIO Praha a.s. has developed ED7747 Monocyte Blocking Buffer, a reagent intended to prevent non-specific monocyte-tandem dye interactions entirely. Monocyte blocking function has been confirmed by series of experiments. We also demonstrate that Monocyte Blocking Buffer does not affect binding of monoclonal antibodies reactive towards antigens expressed by monocytes.

Introduction

With increasing number of detectors available in modern cytometers, more complex multi-color panels are desirable in order to increase information output from a single tube. However, complexity of such panel increases exponentially with each antigen to be detected by labelled monoclonal antibody. Fluorescence spillover, background and fluorescence intensity are the most important factors when building a panel. Non-specific binding of a tandem dye by monocytes may have serious negative impact on otherwise delicately selected combination of antibodies and fluorochromes by increasing background fluorescence of monocytes which may interfere with cell populations of interest. This phenomenon, typical for myeloid lineage, is mainly caused by abundant Fc-receptors. Fc receptors are mainly presented on specific markers for monocytes – CD16, CD64 (Nimmerjahn et al., 2008; Bruhns et al., 2012). As was observed, non -specific binding to CD64, could be protected by using phosphorothioate-oligodeoxynucleotides –the mechanism of the effect was not yet elucidated (Jahrsdörfer et al., 2005). Significantly strong non-specific binding of murine IgG1 and IgG2a isotypes to monocytes was confirmed, however effective protection could be provided by a so-called Fc-block (Andersen et al., 2016). Similar background-decreasing effect may be observed by staining on ice or by pre-fixing using formaldehyde (pre-fixing may affect antibody binding).

Kristensen et al. were also suspecting tandem dye PE-Cy™7 from non-specific binding of CD206 marker despite it is not expressed on non-macrophage monocytes (tested in the absence of a blocking reagent). The effort was made to review papers from last 5 years (2015 to 2019). As a result, only 20% of all research groups using tandem dyes in multi-color flow cytometry panels actually had added blocking reagent, nor mentioning about non-specific binding caused by tandem dyes. Their study

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is comparing "Fc block" – 100 µg/ml human IgG (Anderesen et al., 2016), True-Stain Monocyte Blocker (BioLegend) and phosphorothioate oligondeoxynucleotides – called "Oligo-Block" (Sigma-Aldrich, St. Louis, MO – already described by Jahrsdörfer et al., 2005). Surprisingly Fc block was insufficient on its own in comparison with unstained control. Nevertheless, increasing concentration (to 5 µg/ml) of Oligo-Block successfully decreased non-specific binding of all tested Cyanine tandem dyes (PE–Texas-Red®, PE-Cy™7, PE-Cy™5). Similar level of decreased non-specific binding was achieved when True-Stain Monocyte Blocker™ (5 µl to 100 µl of final volume as recommended?) was used. To be noted is that the blocking effect decreased only false positive signal of CD56 "positive" monocytes, yet specific staining (CD56 positive NK cells) was not affected (Kristensen et al., 2020).

Developing Monocyte Blocking Buffer aims to overcome misinterpretation in the evaluation of the monocyte population, as supported by the available literature. Here we present how Monocyte Blocking Buffer affects non-specific binding of tandem dyes by monocytes on rigorously selected antibody panels.

Materials and Methods

All mAbs listed were produced by the company EXBIO Praha, a.s.

Testing ED7747 Monocyte Blocking Buffer on CD19 conjugates

To evaluate non-specific binding, anti-Hu CD19 monoclonal antibody was chosen as a non-monocytic marker antibody in all available colors (more information listed in Table 1). For comparison, non-tandem dye staining profiles were included (APC, PE, Pacific Blue™). To exclude debris and non-lysed cells, CD45 Pacific Blue™ and CD45 APC (in case of CD19 Pacific Blue™) were added. Variation of tubes with corresponding volume of Monocyte Blocking Buffer (EXBIO, Cat. No.: ED7747, 10 µl/100 µl - following technical datasheet) and tubes without Monocyte Blocking Buffer were prepared. Table 1 shows labelled monoclonal antibodies used. Blood samples from three healthy donors were analyzed. The measurement was performed on DxFLEX (Beckman Coulter) flow cytometer. Data were evaluated using FlowJo 10 Windows (FlowJo™, LLC) software.

Table 1: List of tested CD19 conjugates and backbone markers CD45.

mAb	Fluorochrome	Clone	Cat. No	Concentration in testing tube (mg/ml)	Volume (µl)
anti-CD19	APC-Cy™7	LT19	ED7135	0,0017	10
anti-CD19	PE-Cy™7	LT19	ED7133	0,0026	10
anti-CD19	PE-Cy™5	4G7	T8-663	0,0026	10
anti-CD19	PerCP-Cy™5.5	LT19	ED7172	0,0043	10
anti-CD19	PE-DyLight®594	LT19	ED7227	0,0035	10
anti-CD19	PB	LT19	PB-305-T100	0,0035	10
anti-CD19	APC	LT19	ED7134	0,0030	10
anti-CD19	PE	LT19	ED7518	0,0035	10
anti-CD45	APC	2D1	ED7267	0,0026	5
anti-CD45	Pacific Orange™	2D1	ED7094	0,0052	5

Testing fluorescent spectra in Monocyte Blocking Buffer for fluorescence quenching

To confirm that the Monocyte Blocking Buffer reagent does not affect labeled antibody fluorescence spectra and the fluorescence intensity itself, CD19 antibodies (except for Pacific Orange™, listed in Table 2) were diluted into Monocyte Blocking Buffer and common antibody dilution buffer (1× PBS, 0,2% BSA, 15mM NaN_3) to achieve the final concentration in cuvette (see Table 2), the volume was preserved. Measurement was performed on Agilent Varian Cary Eclipse 60 fluorescence spectrophotometer. Concentrations are described in Table 2 below. Data were analyzed in Varian – Scan app.

Table 2: List of tested conjugates.

mAb	Fluorochrome	Clone	Cat. No	Concentration in cuvette (mg/ml)
anti-CD19	Pacific Blue™	LT19	PB-305	0,060
anti-CD45	Pacific Orange™	2D1	ED7094	0,060
anti-CD19	FITC	LT19	ED7517	0,020
anti-CD19	PE	LT19	ED7518	0,020
anti-CD19	PerCP	LT19	PC-305	0,100
anti-CD19	PE-DyLight®594	LT19	ED7227	0,040
anti-CD19	PE-Cy™5	4G7	T8-663	0,040
anti-CD19	PE-Cy™7	LT19	ED7133	0,015
anti-CD19	APC-Cy™7	LT19	ED7135	0,010
anti-CD19	AF700	LT19	ED7099	0,025
anti-CD19	APC	LT19	ED7134	0,0175

Antibody Cocktailing

To observe the decrease of non-specific background fluorescence in the presence of Monocyte Blocking Buffer and exclude an effect on specific bonds MIX 1 was composed from specific markers for monocyte populations: anti-CD14 FITC, anti-CD16 APC and from non-specific marker anti-CD19 PE-Cy™7, as backbone marker CD45 Pacific Orange™ was chosen.

Combinations of commonly used markers were designed in cocktails (presented in tables below). Selected antibodies were added as per technical datasheet: 5 µl of labeled antibody per 100 µl of peripheral human blood (except for CD19 PE-Cy™7 pipetted in 10 µl volume), see Table 3 and 4. Total amount of MIX 1 and MIX 2 was 25 µl. Tubes containing Monocyte Blocking Buffer (10 µl/100 µl - following Instruction for Use, EXBIO, Cat. No.: ED7747) and without Monocyte Blocking Buffer were prepared. In the case of MIX 2, compensation tubes for antibody cocktail were prepared also in variation with and without Monocyte Blocking Buffer. Table 3 shows labelled monoclonal antibodies used.

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Table 3: Composition of cocktail MIX 1.

mAb	Fluorochrome	Clone	Cat. No	Concentration in testing tube (mg/ml)	Volume (µl)
anti-CD45	Pacific Orange™	2D1	ED7094	0,0044	5
anti-CD19	PE-Cy™7	LT19	ED7133	0,0022	10
anti-CD14	FITC	MEM-15	ED7127	0,0030	5
anti-CD16	APC	3G8	ED7192	0,0022	5

Table 4: composition of cocktail MIX 2.

mAb	Fluorochrome	Clone	Cat. No	Concentration in testing tube (mg/ml)	Volume (µl)
anti-CD45	Pacific Orange™	2D1	ED7094	0,0044	5
anti-CD19	PE-Cy™7	LT19	ED7133	0,0022	10
anti-CD14	PE	MEM-15	ED7128	0,0037	5
anti-CD16	APC	3G8	ED7192	0,0022	5

The measurements were performed using DxFLEX (Beckman Coulter), Navios EX (Beckman Coulter), BD FACSLyric™ (BD Biosciences), BD FACSCanto™ II (BD Biosciences) and XF-1600 (Sysmex) flow cytometers. Confirmation of stability after 24hod was performed only on DxFLEX flow cytometer. Data were evaluated in Flowjo 10 Windows (FlowJo™, LLC) software.

Testing the effect of pre-lysed anti-coagulated blood on non-specific binding

Anti-Hu CD19 monoclonal antibody conjugated to tandem dyes: CD19 APC-Cy™7, PE-DyLight®594, PerCP-Cy™5.5, PE-Cy™7, PE-Cy™5 were tested on fresh EDTA anticoagulated peripheral blood samples and on EXCELLYSE Live ammonium chloride based pre-lysed blood samples in the presence and absence of Monocyte Blocking Buffer (10 µl of reagent per 100 µl of blood - following technical datasheet, EXBIO, Cat. No.: ED7747).

Preparation of pre-lysed blood

1. In 50ml centrifuge tube 2ml of fresh EDTA blood was mixed with 45 ml of diluted 1× EXCELLYSE Live (EXBIO, Cat. No. ED7068)
2. Followed by 10min incubation in dark in room temperature.
3. 5 ml of 10× PBS was added and the suspension was mixed by hand.
4. The tube was centrifuged (at 250×g for 5 min), supernatant was discarded.
5. 1,8 ml of antibody dilution buffer (1× PBS, 0,2% BSA, 15mM NaN_3) and 0,2 ml of 10% BSA were added to the pellet.
6. Before staining, suspension was mixed by pipette.

Table 5 shows labelled monoclonal antibodies used. Backbone marker CD45 PB (EXBIO, Cat. No.: PB-222-T100; 0,030 mg/ml; 5 µl/100 µl) was included. Blood samples from two donors were analysed. The measurement was performed on flow cytometer DxFLEX (Beckman Coulter). Data were evaluated in Flowjo 10 Windows (FlowJo™, LLC) software.

Table 5: List of tested anti-CD19 conjugates.

mAb	Fluorochrome	Clone	Cat. No	Concentration in testing tube (mg/ml)	Volume (µl)
anti-CD19	APC-Cy™7	LT19	ED7135	0,0017	10
anti-CD19	PE-Cy™7	LT19	ED7133	0,0026	10
anti-CD19	PE-Cy™5	4G7	T8-663	0,0026	10
anti-CD19	PerCP-Cy™5.5	LT19	ED7172	0,0043	10
anti-CD19	PE-DyLight®594	LT19	ED7227	0,0035	10

Volumetric cell counting

In order to exclude the possibility that the reagent Monocyte Blocking Buffer affects cell count samples were acquired using Sysmex XF-1600™ flow cytometer with the option of volumetric cell counting. Four healthy patient blood samples were treated with Monocyte Blocking Buffer (EXBIO, Cat. No.: ED7747 10 µl of reagent per 100 µl of blood) and True-Stain Monocyte Blocker™ (Biolegend, Cat. No.: 426102 5 µl of reagent per 100 µl of blood). As a control untreated blood samples were included. Backbone marker CD45 Pacific Orange™ mixed with CD19 PE-Cy™7 was chosen to track non-specific binding. Standard instructions given in chapter Sample Preparation were followed. To analyse exactly the same volume of each sample, the measurement was performed on flow cytometer XF-1600, volume was preserved to 150 µl. Data were evaluated in Flowjo 10 Windows (FlowJo™, LLC) software.

Sample Preparation

For all experiments fresh EDTA anticoagulated peripheral whole blood samples from healthy donors were used (24 h stored blood samples were included for stability comparison only).

1. Test tubes in replicates containing diluted mAbs in antibody dilution buffer (1× PBS, 0,2% BSA, 15mM NaN_3) were prepared.
2. 10 µl of Monocyte Blocking Buffer or 5 µl of True-Stain Monocyte Blocker were added.
3. 100 µl of blood was added.
4. The mixture was incubated for 20 min in dark at room temperature.
5. 2ml of diluted 1× EXCELLYSE Easy (EXBIO, Cat. No. ED7066, diluted 1:10) was added and incubated 10min.
6. Tubes containing clear red solution were centrifuged (300×g, 5 min).
7. Supernatant was discarded and 100 µl of antibody dilution buffer (1× PBS, 0,2% BSA, 15mM NaN_3) was added. Tubes were vortexed prior to flow cytometry analysis.

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Results

The main purpose of this study was to verify the function of the Monocyte Blocking Buffer: suppression of non-specific binding and the exclusion of a negative effect on specific binding of the antibody to the target antigen.
These hypotheses were confirmed by the following experiments using different approaches demonstrating a defined function of the Monocyte Blocking Buffer.

EXP1 aim:
To confirm that Monocyte Blocking Buffer decreases non-specific background fluorescence of mAb anti-CD19 conjugated to tandem dyes on monocytes in comparison with standard staining profile without the addition of Monocyte Blocking Buffer.
Results:

This hypothesis was confirmed. An overlay of staining profiles of samples containing Monocyte Blocking Buffer (red) and samples w/o Monocyte Blocking Buffer (black) in Fig. 1 shows decrease in non-specific monocyte background fluorescence of anti-Hu CD19 mAb (clone LT19). Fluorochromes APC, Pacific Blue™ and PE do not show any staining profile difference upon addition of Monocyte Blocking Buffer.

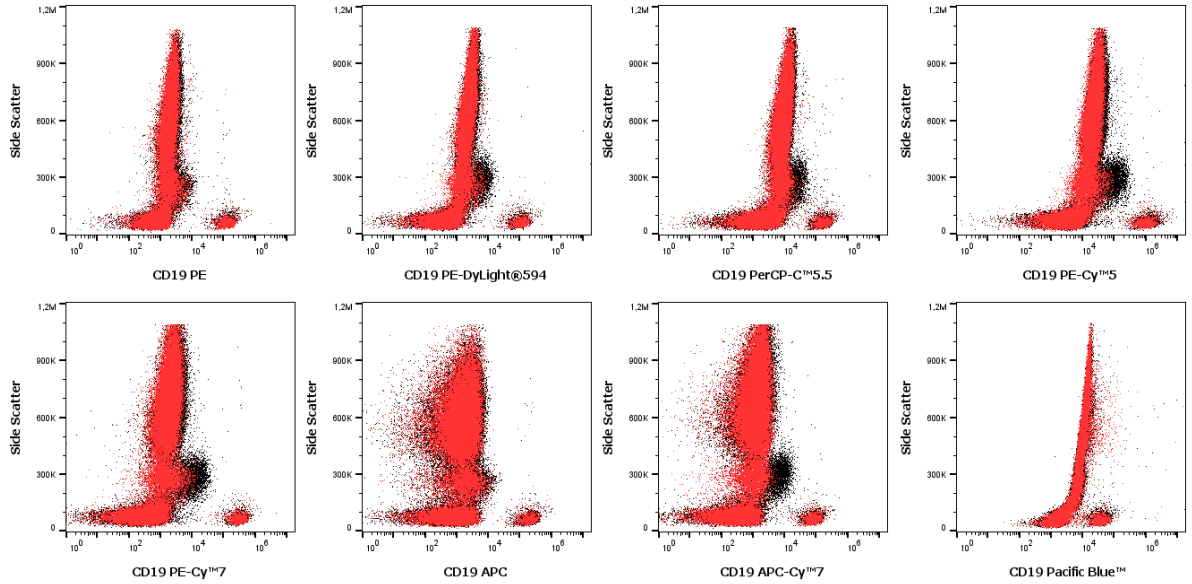


Figure 1: An overlay of staining profile of tested anti-CD19 conjugates in variant with Monocyte Blocking Buffer (red) and without Monocyte Blocking Buffer (black).

EXP2 aim:
To confirm that Monocyte Blocking Buffer does not influence fluorescence spectra of fluorochromes.
Results:

Wide range of measured wavelengths were set to capture eventual changes, see in graphs below. For each fluorochrome, the value of fluorescence intensity was measured at the indicated excitation

wavelengths. For the Pacific Orange™ and Pacific Blue™ fluorochromes, additional dilutions with different concentration of Monocyte Blocking Buffer were prepared to confirm that the measured difference between the sample with and without Monocyte Blocking Buffer is only due to measurement variability. This hypothesis was also confirmed, and any recorded differences were only below the limit of measurement variability.

Table 6: Comparison of percentage values of labelled antibody fluorescence Intensity$_{max}$ (a.u.)

		Intensity$_{max}$ (a.u.)		
	Wavelength (nm)	w/o Monocyte Blocking Buffer	with Monocyte Blocking Buffer	% difference compared to w/o Monocyte Blocking Buffer
FITC	519	173.9	169.5	103%
PE	574	393.7	366.4	117%
PE-DyLight®594	614	301.6	303.8	107%
PerCP	675	199.7	190.9	107%
PE-Cy™5	667	132.5	123.8	120%
PE-Cy™7	775	12.04	12.18	99%
APC	660	141.9	128.4	111%
AF700	716	20.33	19.63	97%
APC-Cy™7	776	6.977	6.215	112%
Pacific Blue™	452	575.1	490.1	105%
Pacific Orange™	548	75.10	62.51	99%

EXP3 aim:

To confirm that adding Monocyte Blocking Buffer to a cocktail of antibodies (MIX 1 and MIX 2; see above chapter Materials and Methods – EXP3), will retain its function, and the specific binding of mAb anti-CD14 to monocytes would not be affected.

Results:

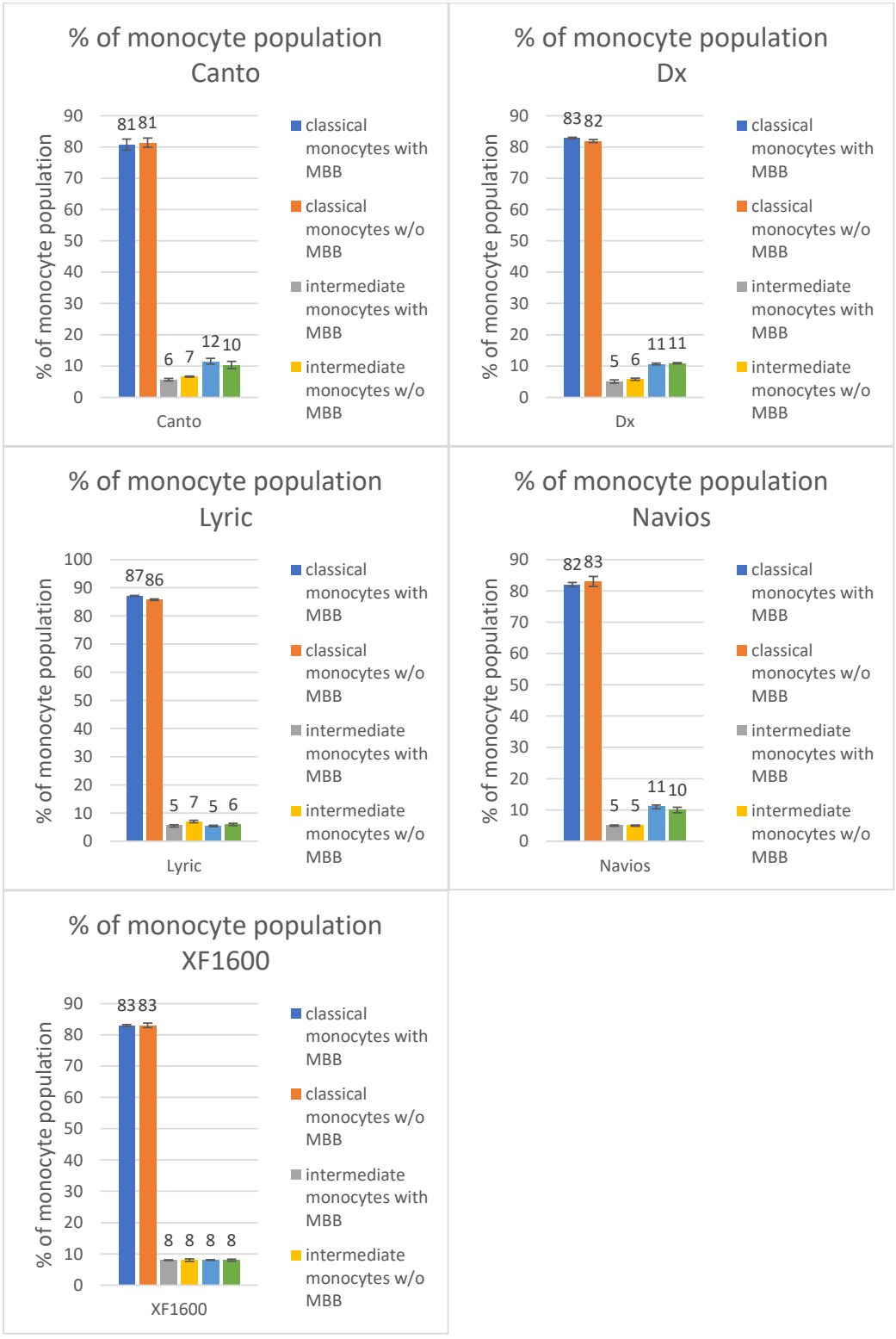
By evaluation of MFI of CD14+ and CD16+ monocytes (see Graph 11 and 12) and percentages of monocytes and their subpopulations (classical, intermediate, non-classical; see Graphs 13 - 19) no significant differences were observed between samples with and without Monocyte Blocking Buffer. As can be seen in Table 7 and 8, the differences between the sample with Monocyte Blocking Buffer treatment and the sample without Monocyte Blocking Buffer are smaller on individual cytometers than the differences measured on different cytometers. Based on this, it can be concluded that the mentioned differences when using Monocyte Blocking Buffer are only caused by measurement variability. This hypothesis was also confirmed.

Table 7: MFI of CD14 FITC labelled monocytes and % difference.

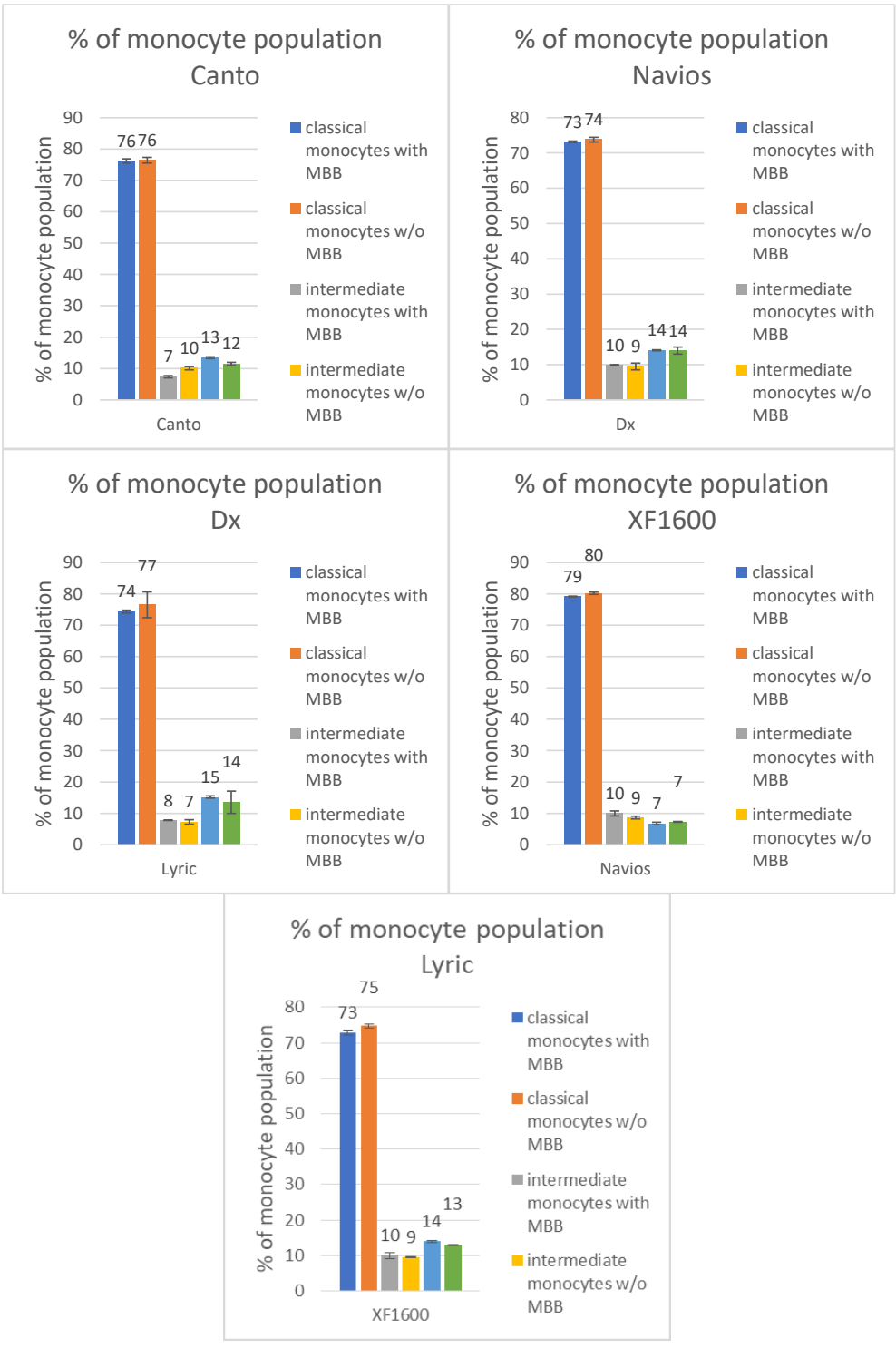
	MFI w/o Monocyte Blocking Buffer	MFI with Monocyte Blocking Buffer	% difference compared to w/o Monocyte Blocking Buffer
BD FACSCanto™ II	38240	36678	95.9%
DxFLEX	553333	544000	98.3%
BD FACSLyric™	48356	46934	97.1%
Navios EX	204264	196129	96.0%
XF-1600	3234	3120	96.5%

Table 8: MFI of CD14 PE labelled monocytes and % difference.

	MFI w/o Monocyte Blocking Buffer	MFI with Monocyte Blocking Buffer	% difference compared to w/o Monocyte Blocking Buffer
BD FACSCanto™ II	83399	89525	107.4%
DxFLEX	1340000	1350000	100.8%
BD FACSLyric™	17072	15772	92.4%
Navios EX	5217	5521	105.8%
XF-1600	4353	4483	103.0%



Graphs 1-5: Percentage representation of monocyte subpopulations on different cytometer platforms (MIX 1).



Graphs 6-11: Percentage representation of monocyte subpopulations on different cytometer platforms (MIX 2).

Table 9: Comparison of percentage values of $CD14^+$ FITC monocyte population.

	CD14+ FITC monocyte population with MBB	CD14+ FITC monocyte population w/o MBB	percentage of CD14+ FITC monocyte population with MBB
BD FACSCanto™ II	7.88%	8.10%	102.79%
DxFLEX	7.78%	7.74%	99.44%
BD FACSLyric™	8.00%	7.91%	98.83%
Navios EX	8.44%	8.47%	100.36%
XF-1600	7.83%	7.74%	98.81%

Table 10: Comparison of percentage values of $CD14^+$ PE monocyte population.

	CD14+ FITC monocyte population with MBB	CD14+ FITC monocyte population w/o MBB	percentage of CD14+ FITC monocyte population with MBB
BD FACSCanto™ II	9.62%	9.53%	99.03%
DxFLEX	9.40%	9.05%	96.31%
BD FACSLyric™	9.25%	8.11%	87.74%
Navios EX	7.04%	6.93%	98.48%
XF-1600	8.94%	8.81%	98.51%

EXP4 aim:

To confirm that pre-lysed peripheral EDTA anticoagulated blood is affected by Monocyte Blocking Buffer the same way as whole blood.

Results:

Pre-lysed blood samples shows a slight suppression of non-specific signal compared to a whole blood lacking Monocyte Blocking Buffer. This suppression of non-specific binding to monocytes is very weak compared to Monocyte Blocking Buffer-supplemented samples. This experiment demonstrates that Monocyte Blocking Buffer fulfils its intended purpose of use. This hypothesis was not confirmed.

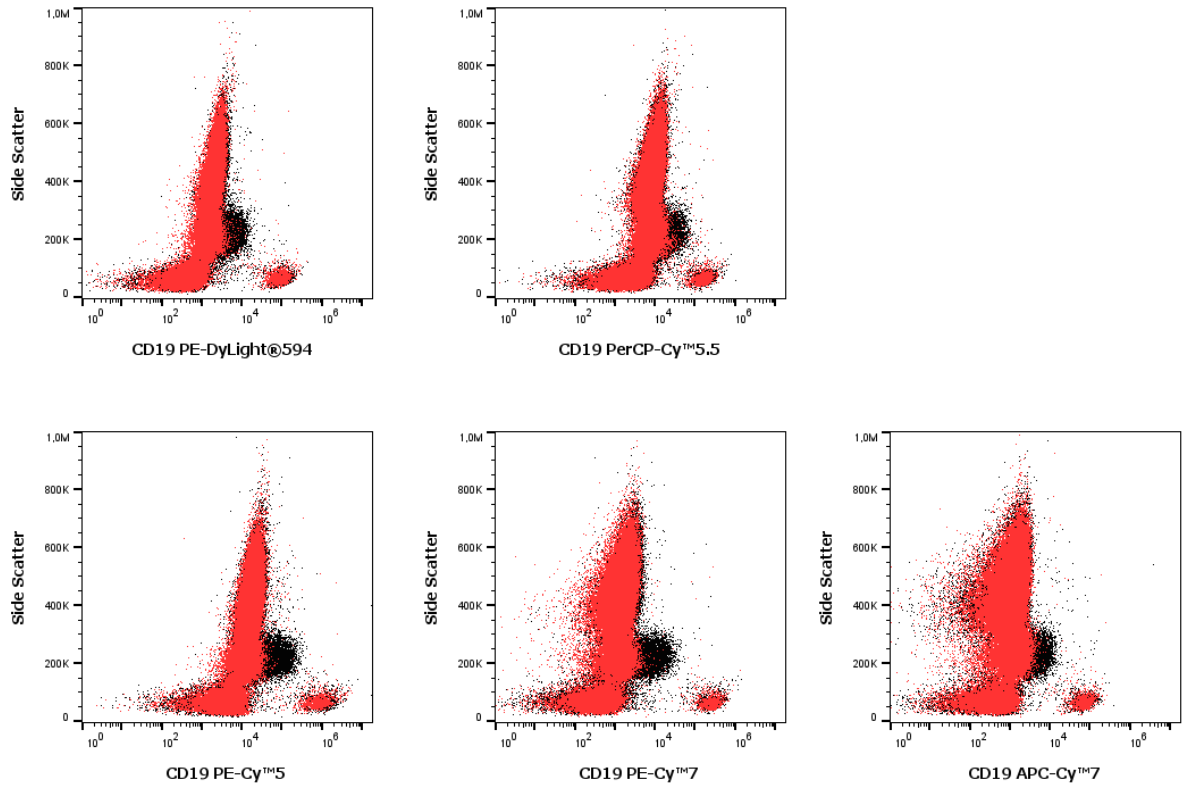


Figure 2: An overlay of staining profile of tested anti-CD19 conjugates on pre-lysed blood sample (2) in the presence (red) and absence of Monocyte Blocking Buffer (black).

Conclusion

Non-specific binding of tandem-dye-labeled monoclonal antibodies to monocytes may be safely inhibited by using Monocyte Blocking Buffer in multi-color flow cytometry panels. Affinity of used antibodies is not affected and pre-lysing blood sample also does not affect the ability to decrease tandem dye background on monocytes.

By using Monocyte Blocking Buffer credibility and reliability of a multi-color panel containing tandem-dye-labelled monoclonal antibodies are enhanced and increase confidence in test results.

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