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Relevance of sample preparation for flow cytometry

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Abstract

Introduction: Flow cytometry is a useful tool for diagnosis and minimal residual dis- ease (MRD) study of hematological diseases. Standard sample preparation protocols are characterized by stain-lyse-wash (SLW). To prevent nonspecific bindings and achieve high sensitivity in MRD studies, lyse-wash-stain-wash (LWSW) is required. To our knowledge, no comparison between the two methods has been performed.

Methods: We compared mean fluorescence intensity (MFI), stain index, signal-to- noise ratio, and percentage of positive cells of 104 antibodies and of 13 selected anti- bodies tested in 10 samples simultaneously prepared with the two methods.

Results: MFI and percentages of positive cells obtained by the two methods did not show significant differences and showed a very high correlation. Stain index and signal-to-noise ratio presented higher values for kappa and lambda surface chains in LWSW samples and a trend of higher values for the other antibodies in SLW samples.

Conclusions: We suggest to use LWSW method also at diagnosis to obtain more comparable antibody intensity expressions when samples from the same patient are processed for MRD evaluation after bulk lysis. Moreover, LWSW can prevent nonspecific bindings, shows no differences in the identification and quantitation of the populations of interest, and reduces acquisition of cell debris.

KEYWORDS

antibody binding, flow cytometry, lyse-wash-stain, sample preparation, stain-lyse-wash

1 | INTRODUCTION

Over the past decades, multiparameter flow cytometry has become the method of choice for the differential diagnosis of several hema- tological diseases, for the definition of prognostic factors, and for the identification of rare cell populations in peripheral blood (PB) and bone marrow samples (BM).[1-6]

Flow cytometry, through the presence or absence of specific sur- face antigens, detects abnormal cells and helps in identifying their lineage and maturation stage, detects abnormal cells through identifi- cation of antigen expression that differs from the normal. The wider ap- plication of flow cytometry in diagnostic and research fields increases the need to distinguish between real and artifact positive signals given that antigen-antibody interactions, characterized by weak bindings, may involve unexpected molecules. The presence of dead cells, cell doublets, and the nonspecific antibody binding can compete to gen- erate artifacts in the phenotype. In some cases, an antibody can bind similar antigens not of interest or an epitope can be shared by different antigens or the Fc of many antibodies can be bound by Fc-receptors expressed by different cells of the immune system.[7] Undesired anti- body's binding depends not only on the antibody's specificity but also on a wide variety of other interactions, as well as the physical and bi- ological conditions that the target cell population encountered during the assay.

The ability to reliably distinguish between positive and negative populations of cells is an essential aspect of clinical flow cytometry.

Sample preparation is a key phase to prevent nonspecific bindings or artifacts. Washing samples with a saline solution added with albu- min before staining or treating them with Fc-block reagents reduce interferences in antibody binding.[8]

A large number of guidelines suggest as standard preparation protocols for the immunophenotype study the stain-lyse-wash (SLW) methods to obtain best signal discrimination.[9-17] However, many reports on minimal residual disease (MRD) studies by flow cytome- try[18-22] recommend a bulk lysis of the sample, which consists on the lysis of a high number of cells in PB or BM samples followed by wash- ing and staining to increase sensitivity.

In this study, we compared the mean fluorescence intensity (MFI), stain index, signal-to-noise ratio, and percentages of positive cells tested with 104 surface antibodies before (SLW) and after lyse and wash (LWSW). Moreover, we compared the above-mentioned param- eters with 13 selected antibodies used to identify monocyte and lym- phocyte subpopulations.

2 | MATERIALS AND METHODS

2.1 | Samples collection

From August 2015 to January 2016, a total of 15 PB and 5 BM sam- ples, collected in vacutainers containing EDTA (Becton Dickinson (BD) San Josè, California, USA), were obtained from patients sent to our laboratory for routine flow cytometry analysis and tested.

2.2 | Ethics statement

The data collection and additional analyses, such as statistical tests, were performed anonymously. This study was carried out in accord- ance with Declaration of Helsinki and subsequent amendments.

2.3 | Lyse-wash-stain-wash (LWSW) procedure

1×10^6 of bone marrow cells or 100 μL of peripheral blood is incu- bated in the darkness at room temperature (RT) for 10 minutes with 3 mL of NH4Cl lysing solution (pH 7.3), centrifuged at 4°C for 5 min- utes at 500 *g*. The supernatant is discarded and the sample washed in 3 mL of phosphate saline buffer solution (PBS) + bovine serum albu- min (BSA) 0.5% and sodium azide 0.1% (1 mol L^{-1}), stained with the appropriate amount of antibodies in darkness at RT for 15 minutes and washed in PBS-BSA.

2.4 | Stain-lyse-wash (SLW) procedure

1×10^6 of bone marrow cells or 100 μL of peripheral blood is stained with the appropriate amount of antibodies in the darkness at RT for 15 minutes, incubated in the darkness at RT for 10 minutes with 3 mL of NH4Cl, centrifuged at 4°C for 5 minutes at 500 *g*. The supernatant is discarded and the sample is washed in PBS-BSA.

Mean fluorescence intensity (MFI), stain index, signal-to-noise ratio, and percentages of positive cells were assessed by correlating the population positive for a given antibody with white blood cells in the sample gated by physical cytometric parameters.

To compare the 2 preparation methods, the same amount of anti- bodies was employed, and the same compensation was applied.

The 104 antibodies were studied as single reagent in a total of five BM and five PB samples for one replicate for each reagent. Their fluorochromes, clones, manufacturers, and SLW/LWSW MFI ratio are reported in Table 1.

A 100.000 total events for each sample were acquired with Navios flow-cytometer Beckmann Coulter (BC) and data analysis was per- formed with Kaluza software (BC).

Unstained cells and single fluorochrome stained cells were used to set the instrument. Instrument performance was checked daily by recording fluorescence intensity with calibrating beads (Flow-Check Pro Fluorospheres BC, Beckman Coulter).

Moreover, 10 peripheral blood samples treated with both methods were tested with the following selected antibodies: CD45 krome or- ange (KO), CD14 phycoerythrin cyanine 5.5 (PC 5.5), HLA-DR fluores- cein isothiocyanate (FITC), CD3 phycoerythrin cyanine 7 (PC-7), CD4 pacific blue (PB), CD8 allophycocyanin (APC), CD16 + CD56 phyco- erythrin (PE), CD19 PC-7, CD20 APC-Alexa Fluor 750, CD5 PB, CD10 PC5.5 (BC), Lambda PE, Kappa FITC (DAKO).

In the analysis, the first step was to select white blood cells with- out debris by a FSC/SSC dot plot. The population well separated by all other cells, with a positive signal for an antigen (more than 10log of positivity), is selected as described in Figure 1.

The arithmetic MFI of each event for a given antibody was cal- culated by Kaluza software after the selection of the positive cell population.

Signal-to-noise ratio was calculated as follows: MFI of positive peak/MFI of negative peak and stain index as (MFI of positive peak - MFI of negative peak)/2*standard deviation of negative peak.

2.5 | Statistical analysis

Differences between means were studied by *T* test; correlation be- tween results was assessed by Pearson product-moment correlation coefficient, and agreement between two methods was determined by Bland-Altman plot.

3 | RESULTS

Means of MFI and of percentages of positive cells obtained by the two methods were not significantly different: mean MFI of SLW and LWSW were 27 and 29, respectively ($P = .224$), while mean % of SLW and LWSW were 20.4% and 20.7% ($P = .186$), respectively.

MFI and percentages obtained by the two methods showed a statistically significant correlation: $r^2 = 0.83$ $P < .001$ and $r^2 = 0.99$ $P < .001$, respectively (Figure 2A and B).

The Figure 2A shows some antibodies with a MFI higher in LWSW than in SLW samples (indicated as white circles) that were as follows: CD235a FITC, CD235a PE, CD25 APC, CD14 PC5.5,

TABLE 1 Antibodies, fluorochromes, and clones tested in the study

Antibody	Fluorochrome	Company	Clone	SLW/LWSW MFI	Antibody	Fluorochrome	Company	Clone	SLW/LWSW MFI
CD25	APC	BC	B9E9	0.03	CD4	PB	BC	13B8.2	1
CD235a	PE	DAKO	SC159	0.04	CD33	PE	BC	906	1
CD235a	FITC	DAKO	JC159	0.05	CD4	FITC	DAKO	MT310	1.01
Kappa/Lambda	FITC/PE	DAKO	Polyclonal	0.35/0.16	CD117	APC	CYTOGNOS	104D2	1.01
CD14	PC5.5	BC	RM052	0.36	CD64	PB	BC	22	1.03
CD11b	APC	BD	D12	0.42	CD43	FITC	DAKO	DF-T1	1.03
CD23	PC-7	BD	M-L233	0.47	CD8	APC	BC	SK1	1.04
CD66	FITC	SANQUIN	CLB-B13.9	0.49	CD45	APC-H7	BD	2D1	1.05
CD71	APC	BD	L01.1	0.55	CD5	PB	BC	BL1a	1.05
CD15	PerCP-Cy5.5	BD	HI98	0.56	CD38	PC-7	BD	HB7	1.05
CD52	PE	INVITROGEN	CF1D12	0.57	CD20	APC-A700	BC	B9E9	1.06
CD33	FITC	BC	D3HL60.251	0.6	CD14	APC	BD	Mϕ P9	1.07
CD13	PE	BC	SJ1D1	0.64	CD20	PC-7	BD	L27	1.07
CD38	APC-A750	BC	LS198-4-3	0.67	CD200	APC-A750	BC	OX-104	1.11
CD20	PE	DAKO	B-LY1	0.69	CD4	PC7	BD	SK3	1.13
CD10	PC5.5	BC	ALB1	0.71	CD45	V450	BD	HI30	1.13
CD16	PerCP-Cy5.5	BD	3G8	0.74	CD8	PC-7	BD	SK1	1.14
CD14	FITC	DAKO	TϋK4	0.74	CD22	PE	BD	S-HCL-1	1.16
CD14	PC-7	BD	M5E2	0.76	CD3	V450	BD	UCHT1	1.17
CD138	PB	BC	B-A38	0.77	CD8	PerCP	BD	SK1	1.17
CD64	FITC	BIOLEGEND	10.1	0.79	CD20	APC-A750	BC	B9E9	1.17
CD138	PC5.5	BC	B-A38	0.82	CD14	APC-H7	BD	Mϕ P9	1.18
CD33	PC5.5	BC	D3HL60.251	0.83	CD34	FITC	DAKO	BIRMA-K3	1.2
CD10	APC	BD	HI10a	0.84	CD71	FITC	DAKO	Ber-T9	1.2
CD33	APC	BC	D3HL60.251	0.84	CD20	FITC	BD	L27	1.22
CD4	APC-H7	BD	SK3	0.84	CD45	APC	BD	2D1	1.24
CD22	FITC	BD	S-HCL-1	0.85	CD4	PerCP-Cy5.5	BD	SK3	1.26
CD34	APC	BC	581	0.86	CD71	APC-A700	BC	YDJ1.2.2	1.26
CD45	KO	BC	J.33	0.88	CD5	PC-7	BD	L17F12	1.29
CD79B	PE	DAKO	SN8	0.88	CD34	PC-7	BD	8G12	1.29
CD16	PE	BC	3G8	0.9	HLA-DR	PE	DAKO	AB3	1.3
CD23	APC	BC	9P25	0.9	CD7	APC	BD	M-T701	1.31

(Continues)

Antibody	Fluorochrome	Company	Clone	SLW/LWSW MFI	Antibody	Fluorochrome	Company	Clone	SLW/LWSW MFI
CD59	PE	INVITROGEN	MHCD5904	0.9	CD20	PerCP-Cy5.5	BD	L27	1.33
CD4	V450	BD	RPA-T4	0.9	CD22	PC5.5	BC	SJ10.1H11	1.33
CD38	APC	BC	LS198-4-3	0.92	CD71	APC-H7	BD	M-A712	1.37
CD5	FITC	BC	BL1a	0.92	CD15	PE	BC	80H5	1.38
HLA-DR	APC	BC	IMMU-357	0.93	CD19	APC	BC	J3-119	1.4
CD45	V500	BD	HI30	0.94	CD3	APC	IMMUNOSTEP	145-2C11	1.4
CD56	PE	BC	N901	0.95	CD45	PerCP-Cy5.5	BD	2D1	1.42
CD117	PE	CYTOGNOS	104D2	0.95	CD3	PE	DAKO	UCHT1	1.44
HLA DR	FITC	BC	B8.12.2	0.96	CD14	PE	DAKO	TüK4	1.44
CD138	APC	BD	MI15	0.96	CD138	FITC	CYTOGNOS	B-A38	1.52
CD3	FITC	DAKO	UCHT1	0.97	CD8	APC-H7	BD	SK1	1.52
CD38	PB	BC	LS198-4-3	0.97	CD5	PE	BC	BL1a	1.53
CD5	PC5.5	BC	BL1a	0.97	CD34	PE	BD	8G12	1.54
CD20	APC	BC	B9E9	0.97	CD3	PC7	BC	UCHT1	1.58
CD55	PE	Southern Biotech	143-30	0.99	CD23	PE	DAKO	MHM6	1.59
CD56	APC	BC	N901	0.99	CD24	PE	BD	ML5	1.61
CD56	V450	BD	B159	1	CD33	PC-7	BD	P67.6	1.86
CD19	PB	BC	J3-119	1	CD2	PC-7	BC	39C1.5	1.89
CD19	PC-7	BC	J3-119	1	CD45	PB	DAKO	T29/33	2.47

BD: Becton Dickinson (San Josè, CA, USA); BC: Beckman Coulter (Brea, CA, USA); BIOLEGEND (San Diego, CA, USA); CYTOGNOS (Salamanca, Spain); DAKO (Golstrup, Denmark); IMMUNOSTEP (Salamanca, Spain); INVITROGEN (Waltham, MA, USA); SANQUIN (Amsterdam, The Nederlands); Southern Biotech (Birmingham, AL, USA).
APC, allophycocyanin; APC-A700, allophycocyanin-alexa 700; APC-A750, allophycocyanin-alexa 750; FITC, fluorescein isothiocyanate; KO, krome orange; PB, pacific blue; PC5.5, phycoerythrin cyanine 5.5; PC-7, phycoerythrin cyanine 7; PE, phycoerythrin.

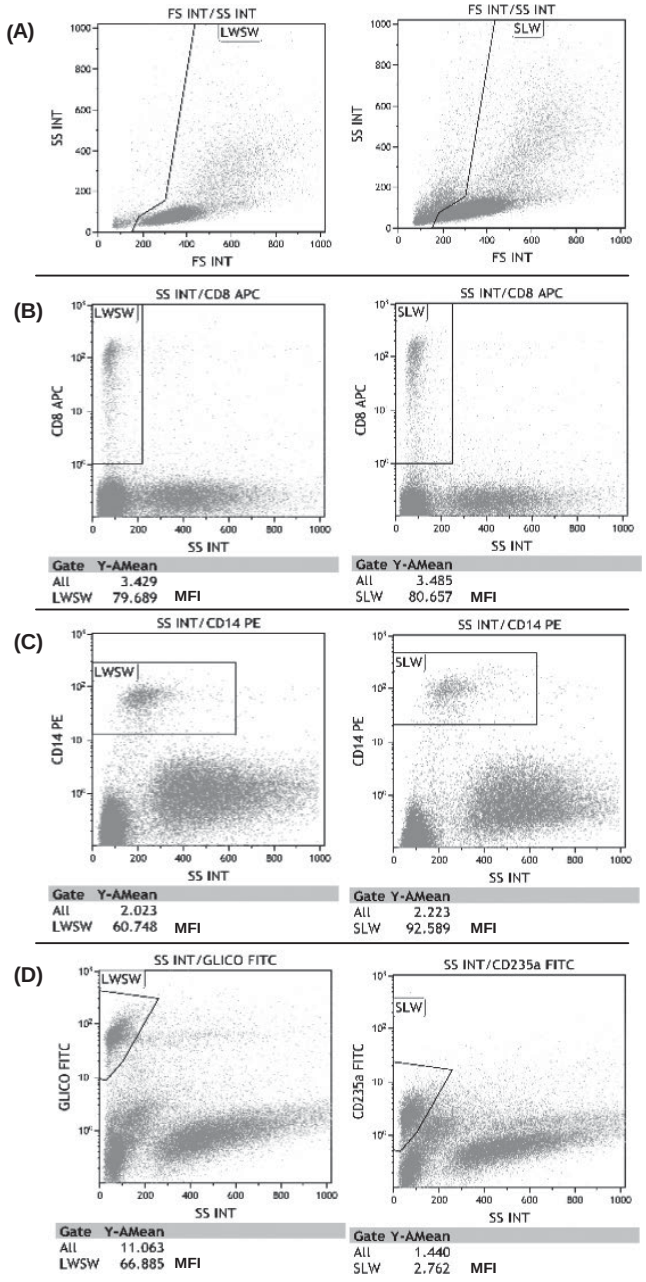

FIGURE 1 Gating strategy and exemplificative dot plots. (A) physical parameters obtained by the two methods. (B) antibody with similar MFI obtained by the two methods. (C) antibody with higher MFI obtained by SLW than LWSW method. (D) antibody with higher MFI obtained by LWSW than SLW method

CD11b APC, and CD71 APC, while the antibodies with higher MFI in SLW than LWSW sample were CD57 FITC and CD14 PE (indicated as white squares).

Bland-Altman analysis of percentages of positive cells showed mean difference of −0.20 with standard deviation of 2.04, and all differences between SLW and LWSW values were within a range of ±5% (Figure 3).

Bland-Altman analysis of MFI of positive cells showed mean of differences of −2.14 with standard deviation of 17.9 (data not shown).

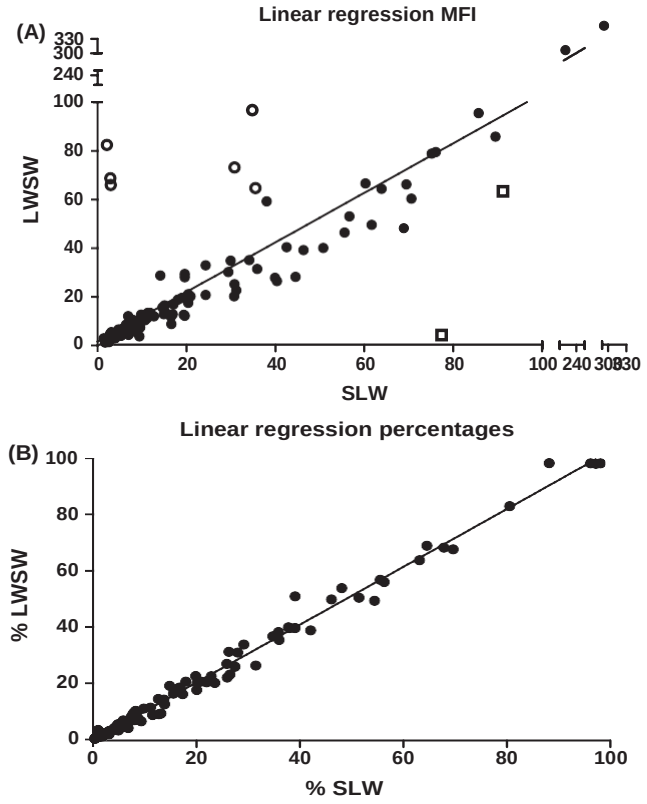

FIGURE 2 Linear regression of MFI and percentages values of 104 antibodies in samples simultaneously prepared with LWSW and SLW methods. (A) Linear regression of MFI values of 104 antibodies in samples simultaneously prepared with LWSW and SLW methods. White circles represent samples with visible higher MFI in LWSW than in SLW samples. White squares represent samples with visible higher MFI in SLW than in LWSW samples. (B) Linear regression of percentage of cells positive for 104 antibodies in samples simultaneously prepared with LWSW and SLW methods

Signal-to-noise ratio and stain index were higher in SLW than LWSW samples (59.8 vs 41.7 $P < .001$, 24.4 vs 17.8 $P = .001$, respectively).

Means of debris obtained by SLW (31.8%) and LWSW (19.3%) were significantly different $P < .001$) (Figure 4).

Analysis of 13 selected antibodies tested in 10 samples showed:

- no statistical significant differences in the percentages of positive cells;
- higher MFI for CD3 (means: 30.6 vs 23.6 $P < .01$) in SLW than LWSW samples and for HLA-DR (means: 12.3 vs 27.1 $P < .05$), CD16 + 56 (means: 74.4 vs 123.6 $P < .01$) in LWSW than SLW samples;
- higher signal-to-noise ratio in SLW samples for CD45 (means: 51.6 vs 36.6 $P < .05$), CD3 (means: 230.8 vs 36.9 $P < .05$), CD19 (means: 92.8 vs 46.8 $P < .01$), and CD10 (means: 49.7 vs 20.6 $P < .01$);
- higher stain index in SLW than LWSW samples for CD3 (means: 57.9 vs 20.2 $P < .01$), CD19 (means: 30.1 vs 20.9 $P < .01$), and CD10 (means: 28.4 vs 14.81 $P < .01$);
- higher MFI, stain index, and signal-to-noise ratio in LWSW samples

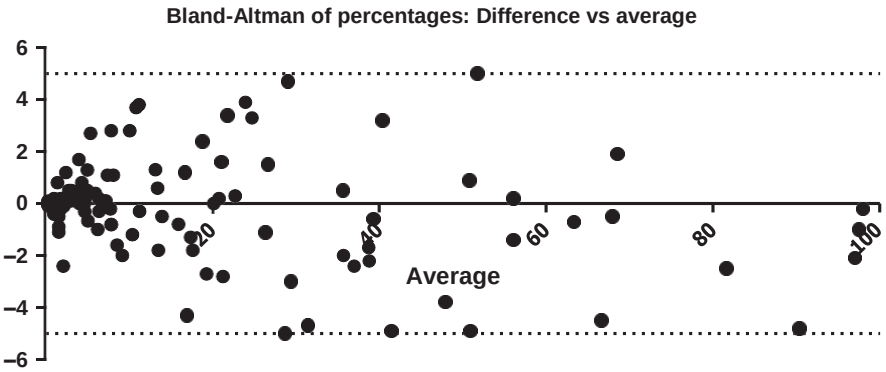

FIGURE 3 Bland-Altman analysis of percentages of cells positive for 104 antibodies in samples simultaneously prepared with LWSW and SLW methods

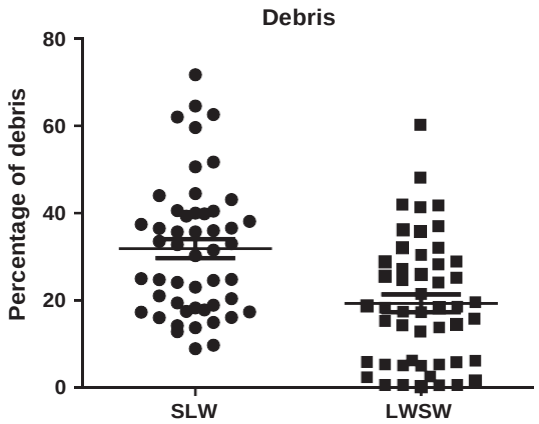

FIGURE 4 Percentages of debris obtained in LWSW and SLW samples

4 | CONCLUSION

Different methods to prepare samples for flow cytometry analysis have been described in a high number of study groups, and the SLW method is considered the standard one, because it provides the best signal output. However, in the antigen-antibody interactions, some difficulties can occur for the interactions with similar epitopes or for the interferences of different cells of immune system that can bind the Fc with a specific Fc-receptor.[7] A solution for this problem, in par- ticular for the study of surface kappa and lambda chains, is offered by some study groups that recommended the sample washing with PBS added with BSA to remove interfering free extracellular immu- noglobulins[23] or to avoid artifacts of cytophilic antibodies.[9] Therefore, lyse and wash of a sample before staining, as in LWSW method, can prevent artifacts and, in MRD studies, allows to obtain a higher

number of cells. To our knowledge, no comparison between these two methods has been described and it is not known whether a sec- ond wash gives significant differences in terms of antigens' binding and expression. We compared the expression of 104 antibodies as- sessed by both SLW and LWSW to identify differences between the two methods. Samples prepared with both methods did not show sig- nificant differences in terms of percentage and MFI of positive cells even though stain index and signal-to-noise ratio were significantly higher in SLW than LWSW samples. The antibodies with higher MFI in LWSW method, indicated as white circles in Figure 2A (CD235a FITC, CD235a PE, CD25 APC, CD14 PC5.5, CD11b APC, and CD71
APC), showed an MFI increase that overcame the slight increase of background. In two cases, indicated as white squares (CD57 FITC and CD14 PE), this phenomenon was observed in SLW samples. Moreover, the LWSW method increased both the intensity of background and of positive signal, but the stain index was reduced because the back- ground exceeded the signal. However, the selection of positive cell populations was not affected by these differences, and no different percentages were obtained.

Mean of differences between SLW and LWSW percentages was
−0.20, and all differences were within a range of ±5%. Moreover, SLW samples presented a significantly higher percentage of debris than LWSW samples that determined higher number of "useful events" with the same threshold setup.

We also analyzed the expression of 13 selected antibodies in 10 samples prepared with the two methods. No significant difference in terms of percentage of positive cells was observed and, except for sur- face kappa and lambda chains, all other antibodies have shown higher values of stain index and signal-to-noise ratio in SLW samples than LWSW.

Despite the high concordance of MFI between the two methods, the signal-to-noise ratios and the stain indexes obtained, confirmed that SLW gave the best signal discrimination as reported by some guidelines[9-17] and that the expression of kappa and lambda surface immunoglobulins became brighter after sample washing.[24]

However, to achieve a high sensitivity for MRD evaluation, LWSW should be performed as suggested by the Euroflow consortium.[22]

We suggest the use of LWSW also in samples from newly diag- nosed patients because, despite differences in stain index and signal- to-noise ratio, the percentages of positive cells were similar in the two

methods. Moreover, the use of the same method to stain cells from a patient at diagnosis and throughout the follow-up is recommended, to obtain more comparable cell parameters. Finally, LWSW, through the sample wash before the staining, prevents artifacts which may occur also in fresh samples and allows to acquire a higher number of useful events by reducing cell debris.

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CONFLICT OF INTEREST

There authors declare no conflicts of interest.

AUTHOR'S CONTRIBUTION

VEM, ES, and MG designed the study, analyzed, and interpreted the data; DO, MR, and SC collected data; BB, MB, and PO drafted and approved the final version of manuscript.

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