Research paper

Optimization and testing of dried antibody tube: The EuroFlow LST and PIDOT tubes as examples

Vincent H.J. van der Velden [a,*], Juan Flores-Montero [b], Martin Perez-Andres [b], Marta Martin-Ayuso [c], Oliver Crespo [d], Elena Blanco [b], Tomas Kalina [e], Jan Philippé [f], Carolien Bonroy [f], Maaike de Bie [a], Jeroen te Marvelde [a], Cristina Teodosio [b], Alba Corral Mateos [b], Veronika Kanderová [e], Mirjam van der Burg [a], Dennis Van Hoof [d], Jacques J.M. van Dongen [g], Alberto Orfao [b]

[a] Laboratory for Medical Immunology, Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
[b] Cancer Research Centre (IBMCC, USAL-CSIC), Institute for Biomedical Research of Salamanca (IBSAL) and Department of Medicine and Cytometry Service (NUCLEUS Research Support Platform), University of Salamanca (USAL), Salamanca, Spain
[c] Cytognos SL, Salamanca, Spain
[d] BD Biosciences, San Jose, USA
[e] Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Charles University (DPH/O), Prague, Czech Republic
[f] Department of Laboratory Medicine, University Hospital Ghent, Ghent, Belgium
[g] Department of Immunohematology and Blood Transfusion (IHB), Leiden University Medical Center (LUMC), Leiden, The Netherlands

ARTICLE INFO

ABSTRACT

Within EuroFlow, we recently developed screening tubes for hematological malignancies and immune deficiencies. Pipetting of antibodies for such 8-color 12-marker tubes however is time-consuming and prone to operational mistakes. We therefore evaluated dried formats of the lymphocytosis screening tube (LST) and of the primary immune deficiency orientation tube (PIDOT). Both tubes were evaluated on normal and/or on patient samples, comparing the mean fluorescence intensity of specific lymphocyte populations. Our data show that the dried tubes and liquid counterparts give highly comparable staining results, particularly when analyzed in multidimensional plots. In addition, the use of dried tubes may result in a reduced staining variability between different samples and thereby contributes to the generation of more robust data. Therefore, by using ready-to-use reagents in a dried single test tube format, the laboratory efficiency and quality will be improved.

1. Introduction

Current clinical immunophenotyping protocols generally include ≥ 8-color tubes, allowing precise characterization of normal, reactive and neoplastic leukocyte populations (van Dongen et al., 2012a; Theunissen et al., 2016; Berkowska et al., 2014). By combining multiple antibodies in the same fluorochrome position, the number of antibodies in single tubes may even be larger (Quijano et al., 2009). For example, in the EuroFlow Lymphocytosis Screening Tube (LST), both CD20 and CD4 are labeled with Pacific Blue (PacB), CD56 and Surface membrane (Sm) Immunoglobulin (Ig)Kappa are both conjugated with PE, CD8 and SmIgLambda are both conjugated with FITC, and CD19 and T-cell receptor (TCR)γδ are both conjugated with PC7, resulting in an 8-color tube with 12 markers (van Dongen et al., 2012a). Comparably, in the

EuroFlow orientation tube for primary immunodeficiencies (PIDOT) CD8 and SmIgD are both conjugated with FITC, CD16 and CD56 with PE, CD4 and SmIgM with PerCP-Cy5.5, and TCRγδ and CD19 with PC7, also resulting in an 8-color tube with 12 markers.

Pipetting of antibodies for such tubes is time-consuming and is prone to operational mistakes (1–4% of samples in our experience). In contrast, the use of dried tubes may result in a reduced staining variability between different samples due to prolonged stability of the dried reagent mixes, and thereby contributes to the generation of more robust data. Therefore, the laboratory efficiency and quality may be improved by using ready-to-use reagents in a dried single-test tube format (Hedley et al., 2015).

Within EuroFlow, the screening tubes and the diagnostic antibody panels were licensed to BD Biosciences and Cytognos, who aim to develop dried tubes for the various EuroFlow screening tubes as well as classification tubes. In this report, we describe the development and testing of the EuroFlow LST tube, which was released on the market in 2015 by BD Biosciences with the name of BD OneFlow™ LST (BD Biosciences, 2016), and the EuroFlow PIDOT tube, to be released by Cytognos SL.

* Corresponding author at: Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.
E-mail address: v.h.j.vandervelden@erasmusmc.nl (V.H.J. van der Velden).

2. Materials and methods

2.1. Dried tubes

Dried BD LST tubes were prepared by BD Biosciences (San Jose, CA, USA) (Chan et al., 2016) whereas the dried format of the PIDOT tube was manufactured by Cytognos SL (Salamanca, Spain). For the dried BD OneFlow™ LST tube, the reagents were dried in BD's proprietary buffer formulation to preserve the activity of the antibodies and extend the life and stability of the reagents.

2.2. Samples

The BD™ LST prototype tubes were evaluated on peripheral blood (PB) and bone marrow (BM) samples from normal donors, defined as individuals without any known clonal hematological disorder and with no evidence of such disorders in the tested sample (lot #1 (2198715): n = 14 PB; lot #2 (130228): n = 13 PB and 5 BM). The final lot (lot #3; 130716) was first evaluated on normal PB (n = 15) and subsequently tested on PB (n = 7) and BM samples (n = 6) of patients with mature B-cell malignancies. Lots were tested and data collected in two EuroFlow centers (Erasmus MC, Rotterdam, The Netherlands, and University of Salamanca, Salamanca, Spain). The final lot was subsequently clinically evaluated in a side-by-side comparison study between the EuroFlow liquid reagents and the BD OneFlow™ LST tube, performed at three external clinical sites. A total of 123 PB specimens, 53 BM specimens, and 31 fresh lymph node (LN) specimens were enrolled in this study.

The EuroFlow PIDOT dried tube was evaluated on PB samples from 25 normal donors, defined as individuals with no past history of immunological or hematological disorders and with no evidence of such disorders in the tested sample. Data were collected in four EuroFlow centers (Charles University, Prague, Czech Republic; Erasmus MC, Rotterdam, The Netherlands; Ghent University, Ghent, Belgium; University of Salamanca, Salamanca, Spain).

All samples were collected according to the guidelines of the Medical Ethical Committee of the University of Salamanca and Erasmus MC (MEC-2007-234).

2.3. Staining protocols

All samples were stained in parallel with the EuroFlow liquid LST reagent home-made cocktail or the PIDOT combination ("liquid" format) containing the reference reagents, and the BD dried LST tube or the Cytognos dried PIDOT tube (see Table 1 (BD OneFlow™ LST) and Table 2 (PIDOT)). Of note, whereas the EuroFlow liquid LST reagent cocktail contained the Pacific Blue (PacB) and Pacific Orange (PO)

fluorochromes, the dried LST tube contained the equivalent BD Horizon™ Violet (HV)-450 and the HV-500-C fluorochromes instead. For the PIDOT tube, the CD27-Brilliant violet (BV)421 and CD45RA-BV510 were not included in the dried tube, and were dropped-in when staining with the dried tube. All samples were stained with LST according to the EuroFlow Standard Operating Procedure (SOP) (Kalina et al., 2012). Briefly, PB or BM samples were first washed 2 or 3 times (see Results) using washing buffer (phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.09% sodium azide (NaN3)) and subsequently stained with the antibodies for 15 or 30 min (see Results) at room temperature in the dark. All stainings were performed in a final volume of 100 µl: 50 µl washed BM or PB and 50 µl of antibodies for the EuroFlow tubes and 50 µl washed BM or PB and 50 µl washing buffer for the dried BD LST tube. For the PIDOT tubes, samples were stained according to the EuroFlow bulk lyse SOP (for detailed protocol, see www.EuroFlow.org) (Theunissen et al., 2016; Flores-Montero et al., 2017). Briefly, 50 ml of ammonium chloride solution was mixed with 2 ml of PB, and incubated for 15 min at room temperature (RT) to lyse non-nucleated red cells. Then, nucleated cells were washed with washing buffer and counted in a hematological analyzer. In each case, 2×10^6 nucleated cells were subsequently stained in a final volume of 100 µl (30 min at RT); afterward, 2 ml of BD FACS™ Lysing Solution (BD Biosciences) diluted 1/10 (vol/vol) in distilled water was added and cells incubated for another 10 min (RT), washed and resuspended in washing buffer (final volume of 500 µl).

2.4. Instrument settings and data acquisition

Instrument settings and data acquisition were performed following the specific EuroFlow SOP (Kalina et al., 2012) using BD™ Cytometer Setup and Tracking (CS&T) beads (BD Biosciences) and Rainbow beads (Spherotech, Lake Forest, IL, USA). Specific compensation settings for each fluorochrome conjugate were used, whenever necessary. A minimum of 50.000 total events (LST) or 1.000.000 total events (PIDOT) were acquired on a BD FACSCanto™ II flow cytometer (BD Biosciences). Appropriate instrument performance and laboratory procedures were confirmed by results obtained in the EuroFlow quality assessment rounds (Kalina et al., 2015). For the LST clinical validation study, the BD OneFlow™ LST tube was acquired on a BD FACSCanto™ II flow cytometer set-up using the BD OneFlow Set-up beads, BD FC Beads for compensation, and the BD OneFlow™ LST reagents (BD Biosciences, 2016).

2.5. Data analysis

Data were analyzed using Infinicyt software (van Dongen et al., 2012b). For evaluation of normal PB/BM samples, mature lymphocytes and their major cell subsets were used as reference populations; the reference populations and the corresponding gating strategy are described

Table 1
LST tube: EuroFlow reference reagents and reagents in the dried BD LST tube.

	EuroFlow reference reagent					Tested reagent	
	MoAb	Manufacturer	Clone	Catalogue ref.	Staining volume (µL/test)	BD LST dried format	
						MoAb	Clone
1	CD8-FITC	Cytognos	UCH-T4	CYT-SLPC-50	20	CD8-FITC	SK1
2	Igλ-FITC	Cytognos	Polyclonal			Igλ-FITC	Monoclonal
3	CD56-PE	Cytognos	C5.9			CD56-PE	MY31
4	Igκ-PE	Cytognos	Polyclonal			Igκ-PE	Monoclonal
5	CD5-PerCPCy5.5	BD	L17F12	341109	15	CD5-PerCPCy5.5	L17F12
6	CD19-PC7	Beckman Coulter	J3-119	IM3628	5	CD19-PC7	SJ25C1
7	TCRγδ-PC7	BD	11F2	649806	3	TCRγδ-PC7	11F2
8	CD3-APC	BD	SK7	345767	3	CD3-APC	SK7
9	CD38-APCH7	BD	HB7	646786	3	CD38-APC	HB7
10	CD20-PacB	BioLegend	2H7	302328	1	CD20-HV450	L27
11	CD4-PacB	BioLegend	RPA-T4	300521	2	CD4-HV450	SK3
12	CD45-PacO	Invitrogen	HI30	MHCD4530	5	CD45-HV500-C	2D1

BD: BD Biosciences.

Table 2
PIDOT tube: EuroFlow reference reagents and reagents in the dried format PIDOT tube.

	EuroFlow reference reagent					Tested reagent	
	MoAb	Manufacturer	Clone	Catalogue ref.	Staining volume (µL/test)	PIDOT dried tube MoAb	Clone
1	CD8-FITC	BD	SK1	345271	5	CD8-FITC	UCHT-4
2	IgD-FITC	Biolegend	IA6-2	348205	1.25	IgD-FITC	IADB6
3	CD16-PE	BD	3G8	555407	5	CD16-PE	3G8
4	CD56-PE	Cytognos	C5.9	CYT-56PE	5	CD56-PE	C5.9
5	CD4-PerCPCy5.5	BDB	SK3	332772	7	CD4-PerCPCy5.5	RPA-T4
6	IgM-PerCPCy5.5	Biolegend	MHM-88	314511	2	IgM-PerCPCy5.5	MHM-88
7	TCRγδ-PC7	BD	11F2	549806	5	TCRγδ-PC7	TCR-1
8	CD19-PC7	Beckman Coulter	J3-119	IM3628	1	CD19-PC7	19–1
9	CD3-APC	BD	SK7	345767	2.5	CD3-APC	UCHT-1
10	CD45-APCH7	BD	2D1	641417	2	CD45-APCH7	HI30
11	CD27-BV421[a]	BD	M-T271	562513	1		
12	CD45RA-BV510[b]	BD	HI100	563031	2.5		

BD: BD Biosciences.
[a] In one laboratory CD27 BV421 from BioLegend (O323, catalogue reference 302,824; 1 µl/test) was used; this is an official EuroFlow-approved alternative antibody.
[b] In one laboratory CD45 BV510 from BioLegend (HI100, catalogue reference 304,142; 2.5 µl/test) was used; this is an official EuroFlow-approved alternative antibody.

in Table 3 (LST) and Table 4 (PIDOT). For each marker and its corresponding positive and negative reference population, the mean fluorescence intensity (MFI) and coefficient of variation (CV) was recorded for both the liquid format and the dried format reagent mixes. The difference was calculated by the formula (MFI dried tube-MFI liquid format)/(MFI liquid format) ∗ 100%. Since the expression of markers on abnormal B-cells varied between different patients, it was not appropriate to use the average values of all abnormal populations. Therefore, for the patient samples only the samples being positive for a specific marker (MFI > 1000) were taken into account for the evaluation. For the LST clinical validation study, samples were identified as being "Follow-up needed" or "No Follow-up needed" using the two systems, and the level of agreement was determined.

2.6. Acceptance criteria

MFI values of the normal reference populations obtained using the dried tubes were compared with the MFI values of the reference populations obtained using the liquid tubes. If the percentage difference (see formula above) was >30% (either up or down), the difference was considered to be significant; if the difference was <30%, the two reagents were considered equivalent.

3. Results

3.1. LST tube

3.1.1. Evaluation of BD™ LST prototype #1 on normal samples

As shown in Table 5, when compared to the reference (liquid) reagent cocktail, the majority of reagents tested in the dried format

(prototype 1) performed within the acceptance criteria of the evaluation: Sm IgLambda-FITC; CD8-FITC; CD56-PE; SmIgKappa-PE; CD5-PerCPCy5.5; CD3-APC; CD38-APCH7; CD20-HV450; CD4-HV450 and CD45-HV500C. Nevertheless, some of these reagents (CD8-FITC, CD4-HV450) showed a tendency to display a lower expression when compared with the reference reagents (Table 5). CD19-PC7 (−31%) and anti-TCRγδ-PC7 (+32%) showed larger differences, outside the acceptance criteria. Background staining was generally somewhat higher in the dried tube (Table 5), but this had no impact on data analysis and the results obtained. Furthermore, significant differences were found for SmIgKappa and, to a lesser extent, SmIgLambda staining between the two EuroFlow testing centers (data not shown), probably related to slight differences in the washing steps.

3.1.2. Evaluation of BD™ LST prototype #2 on normal samples

Based on the variation observed between the dried and liquid reagent cocktails with the first prototype, the incubation time was increased to 30 min; this did not result in clearly higher fluorescence intensities but appeared to be more robust (plateau phase of staining) than shorter incubation time (data not shown). In addition, three pre-washes were introduced to reduce the stain variability for Ig light chains. Furthermore, while prototype #1 was the EuroFlow original recipe just in dry format, in prototype #2 all antibodies were set to a stable titer. As shown in Table 5, the majority of reagents tested in the dried format (prototype 2) performed within the acceptance limits of the evaluation. In contrast, the CD56-PE and SmIgKappa-PE markers showed higher differences, outside the 30% arbitrary acceptance criteria established. Finally, a higher background staining was observed using the dried tube for both FITC and PC7 (Table 5), but this was no longer detected for the other fluorochromes.

Table 3
Positive and negative reference lymphocyte populations used for the various markers and their gating strategy for the LST tube.

Target marker	Positive reference population	Gating strategy	Negative reference population
CD8	CD8$^+$ T-cells	CD8^{hi}/CD45^{hi}/SSC^{lo}	CD4$^+$ T-cells
IgLambda	Igλ$^+$ B-cells	CD20$^+$/CD19$^+$/Igκ$^-$/CD3$^-$	CD4$^+$ T-cells
IgKappa	Igκ$^+$ B-cells	CD20$^+$/CD19$^+$/Igλ$^-$/CD3$^-$	CD4$^+$ T-cells
CD56	NK-cells	CD3$^-$/CD19$^-$/CD20$^-$/CD45^{hi}/SSC^{lo}/CD56$^+$	CD4$^+$/CD56$^-$ T-cells
CD5	CD4$^+$ T-cells	CD4$^+$/CD45^{hi}/SSC^{lo}	B-cells
CD19	B-cells	CD20^{hi}/CD45^{hi}/SSC^{lo}/CD3$^-$	NK-cells
TCRγδ	TCRγδ$^+$ T-cells	TCRγδ$^+$/CD45^{hi}/SSC^{lo}/CD3$^{very\ high}$	NK-cells
CD3	CD4$^+$ T-cells	CD4$^+$/CD45^{hi}/SSC^{lo}	B-cells
CD20	B-cells	CD19$^+$/CD45^{hi}/SSC^{lo}/Igκ$^+$ or Igλ$^+$/CD3$^-$	NK-cells
CD4	CD4$^+$ T-cells	CD4$^+$/CD45^{hi}/SSC^{lo}	NK-cells
CD38	NK-cells	CD3$^-$/CD19$^-$/CD20$^-$/CD45^{hi}/SSC^{lo}/CD56$^+$	NA

NA: not applicable; LST: lymphocytosis screening tube.

Table 4
Positive and negative reference lymphocyte populations for the various markers and their gating strategy for the PIDOT.

Target marker	Positive reference population	Gating strategy	Negative reference population
CD8	CD8$^+$ T-cells	CD3$^+$/CD4$^-$/CD8$^+$/CD19$^-$/CD45^{hi}/TCRγδ$^-$/FSC^{lo}/SSC^{lo}	CD4 + T-cells
SmIgD	preGC B-cells	CD3$^-$/CD19$^+$/CD16 + CD56$^-$/CD27$^-$/CD45^{hi}/smIgM$^+$/smIgD$^+$/FSC^{lo}/SSC^{lo}	Switched memory. B-cells
CD16 + CD56	NK-cells	CD3$^-$/CD19$^-$/CD16 + CD56^{hi}/CD45^{hi}/FSC^{lo}/SSC^{lo}	CD4 + T-cells
CD4	CD4$^+$ T-cells	CD3$^+$/CD4$^+$/CD8$^-$/CD19$^-$/CD45^{hi}/TCRγδ$^-$/FSC^{lo}/SSC^{lo}	CD8 + T-cells
SmIgM	Unswitched memory B-cells	CD3$^-$/CD19$^+$/CD16 + CD56$^-$/CD27$^-$/CD45^{hi}/smIgM^{hi}/smIgD$^+$/FSC^{lo}/SSC^{lo}	Switched memory B-cells
TCRγδ	TCRγδ$^+$ T-cells	CD3$^+$/CD19$^-$/CD45^{hi}/TCRγδ$^+$/FSC^{lo}/SSC^{lo}	NK-cells
CD19	B-cells	CD3$^-$/CD19$^+$/CD16 + CD56$^-$/CD45^{hi}/FSC^{lo}/SSC^{lo}	NK-cells
CD3	T-cells	CD3$^+$/CD19$^-$/CD45^{hi}/FSC^{lo}/SSC^{lo}	NK-cells

NA: not applicable; GC: germinal center; PIDOT: primary immunodeficiency orientation tube.

3.1.3. Evaluation of BD™ LST prototype #3 on normal samples

Prototype #3 had reduced concentrations of TCRγδ-PC7 and SmIgLambda-FITC to decrease non-specific staining. As shown in Table 5, the majority of reagents tested in the dried format (prototype 3) on 15 normal PB samples performed within the acceptance limits of the evaluation. CD19-PC7 (−41%) and SmIgLambda-FITC (−62%) showed larger differences, failing to fulfill the acceptance criteria. A representative example is shown in Fig. 1. Presenting the data of both the liquid EuroFlow tube and the dried tube in an automated population separator (APS) plot showed however highly overlapping populations, indicating that the variations seen for some individual markers did not significantly impact the identification of the corresponding cell populations via multidimensional analysis (Fig. 2). Since the CD19-PC7 MFI value could not be further improved (technical limit of conjugation) and since B-cells could still easily be identified, CD19-PC7 was accepted in its current format. Although the SmIgLambda staining was less than the original EuroFlow liquid staining, the SmIgKappa and SmIgLambda positive populations still could well be separated and therefore the lower SmIgLambda intensity of the dried tube was not considered to negatively impact the analysis. Therefore, prototype 3

was considered to be acceptable and further evaluated on patient samples.

3.1.4. Evaluation of BD™ LST prototype #3 on patient samples

Prototype #3 was subsequently evaluated on PB (n = 7) and BM samples (n = 6) of patients with mature B- or T-cell malignancies. In general, the patterns observed for the abnormal T- and B-cell populations reflected the patterns observed in the normal populations (Table 6 and Fig. 3). Similarly to the liquid format, the dried format allowed the identification of all normal and abnormal B and T-cell populations present in each individual sample.

3.1.4.1. Reproducibility of the intensity of staining on normal samples using the BD OneFlow™ LST tube.
The reproducibility of the Euroflow LST staining among different samples was evaluated for the EuroFlow liquid reagents LST tube and the dried BD LST tube. The CV values for both types of tests were comparable for most markers, except for TCRγδ and SmIgKappa that were both more robust using the dried BD OneFlow™ LST tube (Table 7).

Table 5
Results of the comparison between the LST liquid reagents and the three dried BD LST prototypes on normal peripheral blood samples[a].

Antibody	Negative reference population			Positive reference population		
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3
SmIgLambda–FITC	116%	205%	24%	6%	6%	–62%
SmIgKappa–PE	174%	–35%	36%	–5%	–69%	–12%
CD8–FITC	116%	205%	24%	–25%	–4%	–12%
CD56–PE	174%	–35%	36%	–17%	–51%	–19%
CD5–PerCP–Cy5.5	60%	–4%	–81%	–17%	21%	11%
CD19–PC7	389%	172%	9%	–31%	–25%	–41%
TCRγδ–PC7	389%	172%	9%	32%	15%	–7%
CD3–APC	153%	–11%	–28%	21%	–1%	–8%
CD38–APCH7	NA	NA	NA	0%	–17%	–6%
CD4–PacB/HV450	–73%	–1%	–104%	–23%	–22%	–29%
CD20–PacB/HV450	–73%	–1%	–104%	–15%	4%	–5%
CD45–PO/HV500	NA	NA	NA	8%	–24%	–20%

    [a] For each sample, the difference was calculated as follows: (MFI dried tube-MFI liquid format)/(MFI liquid format) ∗ 100%. Data presented reflect the mean difference of the analyzed samples (lot #1: n = 14; lot #2: n = 18; lot #3: n = 15). NA = not analyzed. Red numbers indicate those percentages that are outside the acceptance criteria for the positive reference population; orange numbers indicate those percentages that are outside the (arbitrary) acceptance criteria for the negative reference population of <30%.
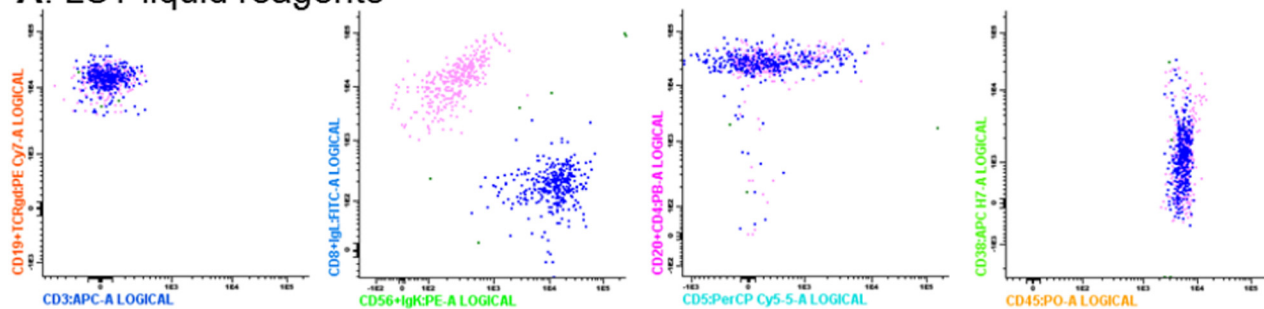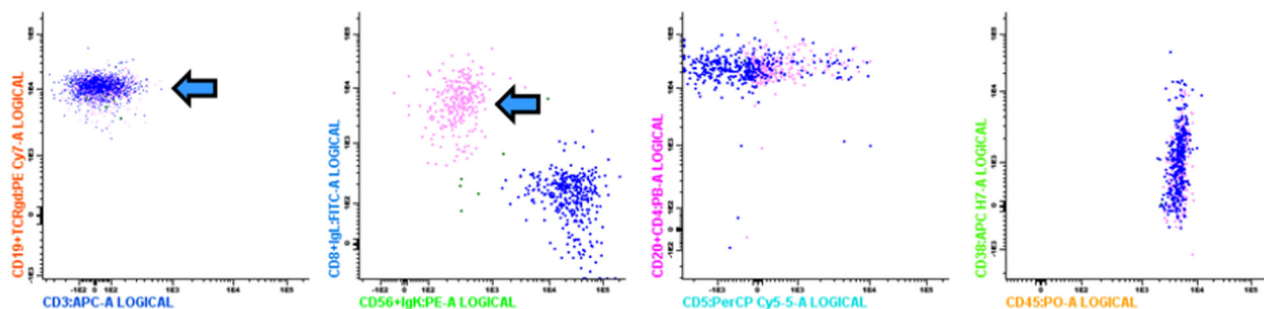
Fig. 1. Representative example of a PB sample stained with the EuroFlow LST tube liquid reagents (top row) and the same sample stained with the dried LST tube (prototype 3; bottom row). Only B-cells are shown (blue: SmIgKappa+; purple: SmIgLambda+). The arrows indicate the two markers with staining intensities over 30% different, between the two tubes. For data analysis the Infinicyt software (Cytognos) was used.

3.1.4.2. Clinical validation of the BD OneFlow™ LST tube. The BD OneFlow™ LST tube was finally clinically validated by comparing the results with those obtained using the EuroFlow liquid reagents in a total of 207 specimens. Using both methods, abnormal mature B-cells were identified in 81 samples, abnormal T-cells in 35 samples, abnormal NK-cells in 6 samples, and other abnormalities in 9 samples (e.g. plasma cell disorders); resulting in a total of 131 samples that needed follow-up. Seventy-six samples were defined as normal by both methods. Therefore, agreement between both methods was obtained in 100% of cases. Evaluation of the percentages of the major lymphocyte populations showed

equivalency of the BD OneFlow™ LST to the EuroFlow liquid reagents system (BD Biosciences, 2016).

3.2. PIDOT tube

3.2.1. Evaluation of the PIDOT dried tube on normal samples

As shown in Table 8, the majority of the reagents in the dried PIDOT format tested on 25 normal samples performed within the acceptance limits of the evaluation. A representative example of the staining obtained by the liquid tube and dried tube is shown in Fig. 4. Slightly
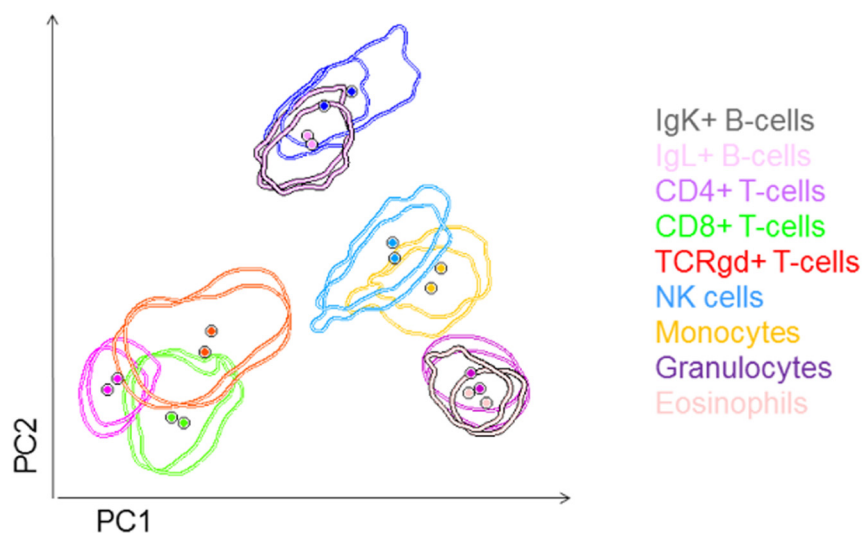

Fig. 2. Multidimensional evaluation of the EuroFlow LST tube liquid reagents and the dried LST tube (prototype 3) stained on the same PB sample, using visualization in an APS plot. The median and 2SD contours of the various populations are shown. For data analysis the Infinicyt software (Cytognos) was used. PC: principal component.

Table 6

Results of the comparison between the liquid LST reagents and the dried BD LST prototype #3 on peripheral blood or bone marrow samples from patients with a mature lymphoid neoplasm.

Reagent	n[a]	Average difference on positive cases[b]
IgLambda-FITC	4	−48%
CD8-FITC	2	−12%
IgKappa-PE	7	+8%
CD56-PE	–	–
CD5-PerCPCy5.5	8	+11%
CD19-PC7	13	−45%
TCRγδ-PC7	–	–
CD3-APC	2	+2%
CD38-APCH7	2	+9%
CD4-PacB/HV450	–	–
CD20-PacB/HV450	13	−8%
CD45-PO/HV500c	13	−10%

[a] Please note that not all markers could be assessed in every case, the number of cases evaluated for each marker depending on the cell populations identified in sufficient numbers in the sample.

[b] Average of the difference between dried and liquid LST format calculated for each individual marker.

Table 7

Reproducibility of staining intensity on normal samples using the LST liquid tube and dried BD LST tube.

| Marker	Population	Coefficient of variation of MFI values	
		Liquid LST reagents	Dried LST tube
CD8-FITC	CD8+/CD4- T cells	17%	17%
CD56-PE	NK cells	30%	32%
SmIgLambda-FITC	Mature IgLambda B cells	27%	26%
SmIgKappa-PE	Mature IgKappa B cells	81%	24%
CD5-PerCP Cy5.5	CD4+ T cells	13%	14%
CD19-PE Cy7	Mature B cells	22%	22%
TCRγδ-PE Cy7	CD4-/CD8- or dim/TCRγδ+	49%	37%
CD3-APC	CD4+ T cells	12%	11%
CD38-APC H7	NK cells	73%	72%
CD20-HV450	Mature B cells	16%	18%
CD4-HV450	CD8-/CD4+ T cells	10%	11%
CD45-HV500	CD4+ T cells	7%	7%

Results expressed as coefficient of variation (CV) of the mean fluorescence intensity (MFI) of the specified marker for 15 peripheral blood samples stained with the liquid and the dried LST reagents.

higher differences were observed for CD4-PerCP-Cy5.5 (35%) and TCRγδ-PC7 (40%), thus failing to fulfill the acceptance criteria. However, presenting the data of both the EuroFlow reagents PIDOT tube and the dried PIDOT tube visualized in an APS plot showed highly overlapping populations, indicating that the variations seen for some individual markers did not significantly impact the identification of the distinctly stained cell populations in a multidimensional analysis (Fig. 5). Moreover, when analyzing the reproducibility of the staining among the different samples (Table 9), it was observed that the CD4 MFI was more

similar between samples using the dried PIDOT tube. Because of this, CD4-PerCP-Cy5.5 was accepted in its current format.

3.2.2. Reproducibility of the intensity of staining on normal samples using the dried PIDOT tube

A higher reproducibility of the EuroFlow PIDOT staining among different samples was observed using the dried PIDOT tube. All tested reagents showed a lower CV when using the dried PIDOT tube as compared to the EuroFlow PIDOT tube reagents, evaluated in parallel
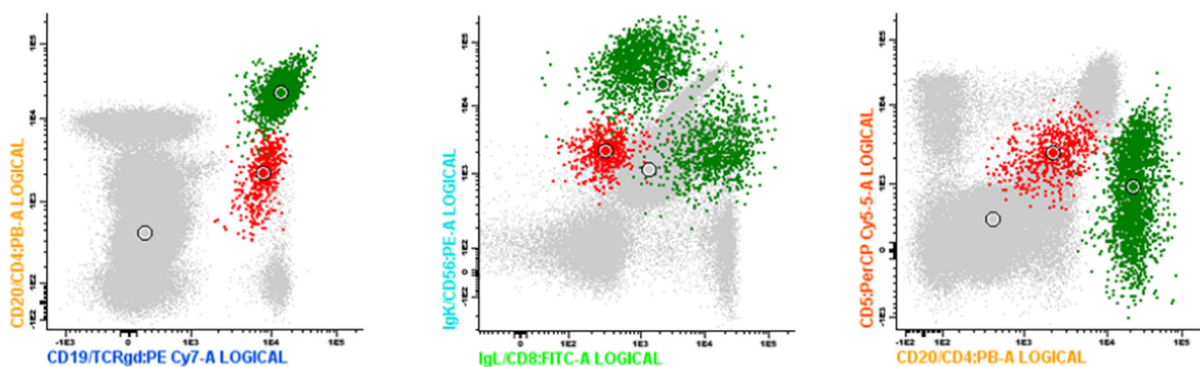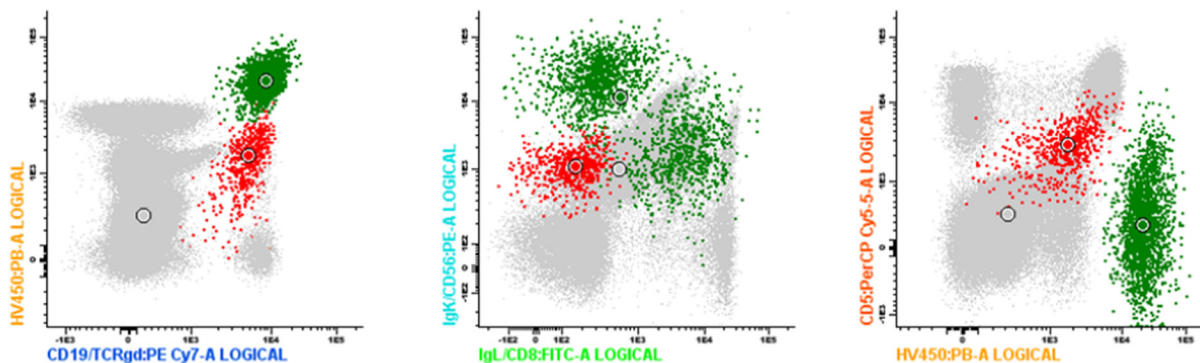

Fig. 3. Representative example of a PB sample stained in parallel with the EuroFlow liquid reagents (A) and the dried LST tube (prototype 3; B). In addition to the normal B-cells (shown in green), an abnormal B-cell population (shown in red) was observed. This monoclonal B-cell lymphocytosis population comprised 0.4% of the leukocytes (19% of the B-cells) and could easily be identified in both the EuroFlow liquid and dried LST tubes. For data analysis the Infinicyt software (Cytognos) was used.

Table 8
Results of the comparison between the Euroflow PIDOT liquid reagents and the dried tube on normal blood samples[a].

Marker	Negative reference population	Positive reference population
CD8	−36%	10%
SmIgD	−17%	−4%
CD16+CD56	2%	3%
CD4	18%	35%
SmIgM	6%	6%
TCRγδ	17%	40%
CD19	17%	4%
CD3	95%	21%
CD45	NA	0%

[a] For each sample, the difference was calculated as follows: (MFI dried tube-MFI liquid format)/(MFI liquid format) ∗ 100%. Data presented reflect the mean difference of the analyzed samples (lot #1: n = 14; lot #2: n = 18; lot #3: n = 15). NA: not analyzed. Red numbers indicate those percentages that are outside the acceptance (arbitrary) criteria of <30% for the positive reference population; orange numbers indicate those percentages that are outside the (arbitrary) acceptance criteria of <30% for the negative reference population.

in the same set of PB samples from 25 healthy controls (Table 9). The highest difference in the CV between the dried and the liquid format was observed for CD4-PerCP-Cy5.5 (10% vs. 48%; Table 9), meanwhile SmIgM and SmIgD showed very small differences in the reproducibility of the staining (CV of 21% versus 23% and 19% versus 20%, respectively). In this regard it should be noted that the CV obtained for distinct reagents tested in their corresponding target population, among different samples, was most commonly 10%–20% for the dried PIDOT tube and 20%–40% for the liquid format, except for the expression of

CD16 + CD56 in NK-cells that showed a CV > 100% with both formats of the EuroFlow PIDOT tube (Table 9).

4. Discussion

Here we report the performance of the dried format of two antibody cocktail combinations: the EuroFlow LST tube and EuroFlow PIDOT tube. Both tubes are screening tubes, the LST for patients with a lymphocytosis and the PIDOT for patients suspected of a lymphoid immune deficiency. Since both tubes contain 12 different antibodies, pipetting of all antibodies is time-consuming and is prone to operational mistakes. Operational mistakes, such as accidental omission of one reagent is an error that can be difficult to uncover in order to repeat the staining procedure and repetition increases the cost. Therefore, the laboratory efficiency and quality may be improved by the use of ready-to-use reagents in a dried single-test tube format (Hedley et al., 2015). Our data show that the developed dried tubes (BD™ OneFlow LST and PIDOT from Cytognos) give comparable data as the liquid counterparts. Of note, some makers showed a difference in MFI compared with the respective liquid formats of over 30%, which was arbitrarily defined beforehand as the acceptance range. However, these differences were accepted for several reasons. First, although the SmIgLambda staining was less bright than the original EuroFlow liquid staining, the SmIgKappa and SmIgLambda positive populations still could well be separated and therefore the lower SmIgLambda intensity of the dried tube was not considered to negatively impact the analysis. Second, for most markers the difference was minimal and just over 30% (CD4: 35%, TCRgd: 40%; CD19: ~45%). Finally, once evaluated in a
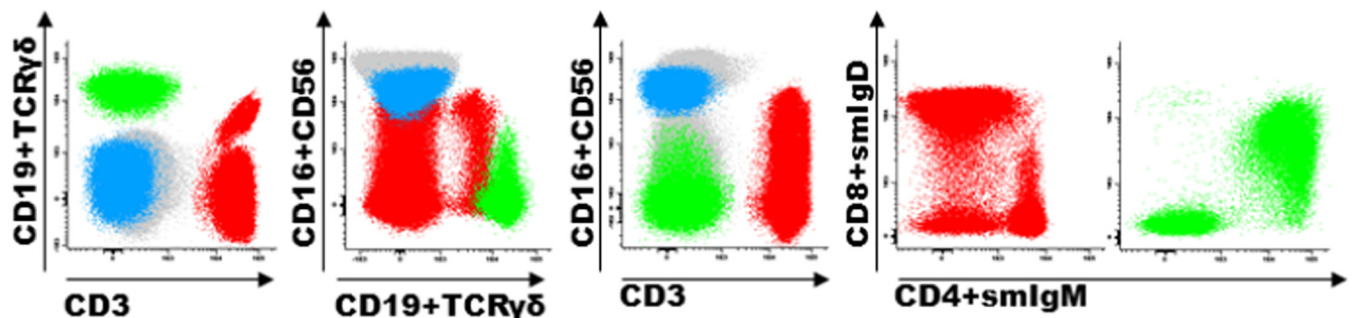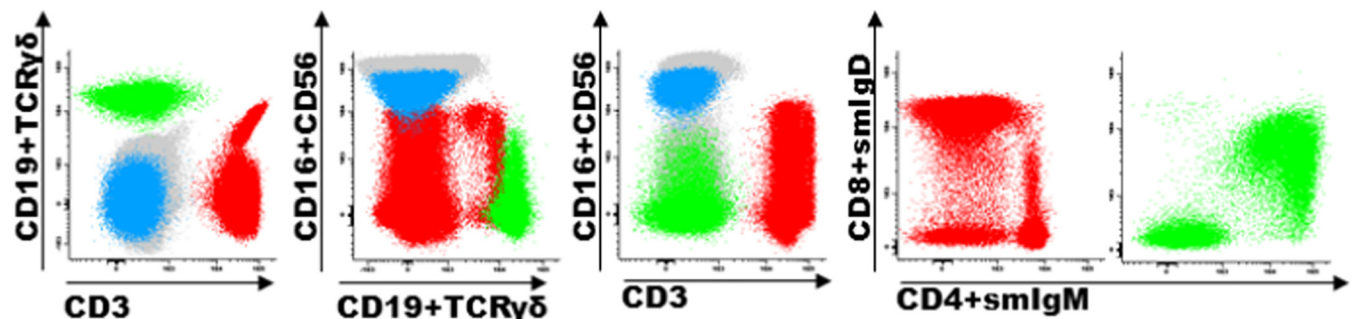

Fig. 4. Representative example of a PB sample stained with the EuroFlow liquid reagent (top row) vs dried PIDOT tube (lower row). T-cells (red), B-cells (green) and NK-cells (blue) are shown. For data analysis the Infinicyt (Cytognos) software was used.
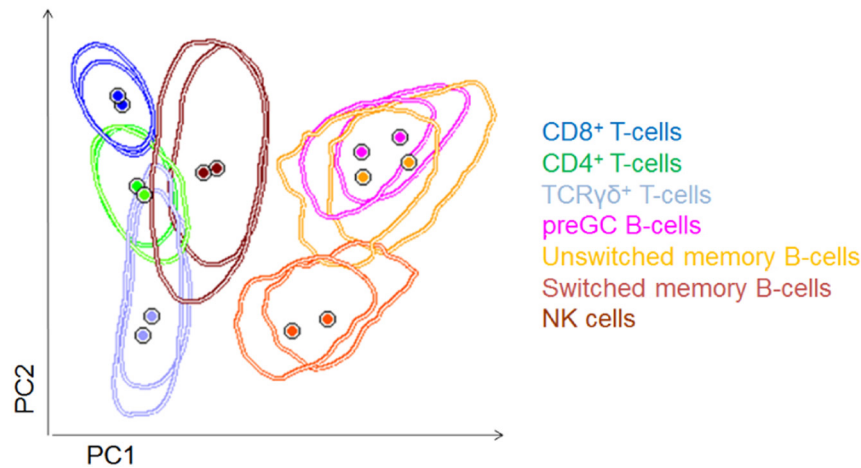
Fig. 5. Comparison of stainings for distinct target populations with the EuroFlow PIDOT liquid reagents tube versus the dried tube in a 12-dimensional space. The quality of the separation of the subsets and clustering of events is represented by the distance among the circles. Each circle represents a single population stained with the EuroFlow PIDOT tube liquid reagents or the dried PIDOT tube and reflects the median fluorescence expression for all parameters. Lines represent the homogeneity of the subpopulations in the 12-dimensional space, expressed as 2SD of the events from each target population represented. For data analysis the Infinicyt software (Cytognos) was used. PC: principal component.

multidimensional approach (APS plots), these differences appeared not to affect the separation of the various populations. Therefore, while the liquid cocktails and the dried reagent formats do not provide exactly identical data, both give comparable results when looked in multidimensional analysis. It is therefore anticipated that results obtained with both tube formats (liquid and dried) can be used in automated gating and classification software tools that are currently being developed by EuroFlow. (Pedreira et al., 2013).

The use of dried tubes is not only time-saving and less prone to operational mistakes, it also significantly reduces the time spent in antibody inventory management (ordering of reagents and acceptance testing of antibodies (one single tube versus 12 individual antibodies), including all the corresponding registrations). At Erasmus MC, the BD OneFlow™ LST tube has been used for diagnostic care since its introduction in 2015, with almost 2000 evaluations being performed yearly. Similarly, both the BD OneFlow™ LST tube and the PIDOT dried tube have been introduced in routine diagnostic screening at USAL, with optimal performance.

Finally, the use of dried tubes may result in a reduced staining variability between different samples and thereby contribute to more robust data. Although biological factors might contribute to differences in the markers' intensity of expression among healthy controls, some molecules have shown to be relatively stable among healthy controls. Accordingly, previous studies have shown that the CV of CD3, CD4, CD5, CD8, CD19, CD20, and CD45 expression on normal lymphocytes

are between 7% and 33%, within a single laboratory and using the same instrument (Bikoue et al., 1996). In line with this, the EuroFlow quality assessment program has shown that CVs below 30% are reached for CD19, CD45, CD4, CD8 and CD3 evaluated in different healthy controls from different laboratories, when instrument setup is strictly standardized (e.g. photomultiplier tube (PMT) voltages and fluorescence compensation values) and the EuroFlow staining protocols are applied) (Kalina et al., 2015). However, it is not clear whether variability is mostly dependent on normal biological factors among individuals or if there are other technical variations that might be affecting the reproducibility (e.g. pipetting differences). The results present in this study suggest that CV values <20% can be reached for most markers evaluated in healthy controls when using dried tubes, even among samples from different centers. Next to the stable markers reported above, we observed that SmIg staining (SmIgM, SmIgD, SmIgKappa, SmIgLambda) also showed a CV <30% among different healthy donors, despite the extensive surface Ig staining protocol with more than three washing steps. In contrast, a higher biological variation was observed for CD16 + CD56 staining of NK-cells, TCRγδ staining on T-cells, and CD38 expression on B-cells. This is likely related to differential expression of these markers by different cell subsets or their dependence on the activation status, and the affinity of the antibody clone used (Lima et al., 2001; Perez-Andres et al., 2010).

In conclusion, dried reagent tubes with staining characteristics comparable to the liquid counterpart have been developed and may improve both laboratory efficiency and the quality of immunophenotypic data. Dried tubes may also contribute to increased staining reproducibility (generally resulting in CV values below 20%), which will increase the capacity of flow cytometry to better detect small changes in the expression levels of individual markers and immunophenotypic profiles.

Table 9
Reproducibility of the staining using the Euroflow PIDOT liquid reagents and the dried tube on normal blood samples.

Marker	Population	Coefficient of variation of MFI values	
		Liquid PIDOT reagents	PIDOT dried tube
CD8	CD8⁺ T-cells	26%	18%
smIgD	preGC B-cells	20%	19%
CD16 + CD56	NK cells	122%	103%
CD4	CD4⁺ T-cells	48%	10%
smIgM	Unswitched memory B-cells	23%	21%
TCRγδ	TCRγδ⁺ T-cells	22%	16%
CD19	B-cells	21%	18%
CD3	T-cells	30%	19%
CD45	Lymphocytes	40%	14%

Results expressed as coefficient of variation (CV) of the mean fluorescence intensity (MFI) of the specified marker for 25 peripheral blood samples stained in parallel with the liquid and the dried PIDOT reagents. GC: germinal center.

References

BD Biosciences, 2016. BD OneFlow™ LST.2016 [cited 2017 14-march]; Available from:. https://www.bdbiosciences.com/documents/BD-OneFlow-LST-Manual.pdf December.

Berkowska, M.A., Heeringa, J.J., Hajdarbegovic, E., van der Burg, M., Thio, H.B., van Hagen, P.M., et al., 2014. Human IgE(+) B cells are derived from T cell-dependent and T cell-independent pathways. J. Allergy Clin. Immunol. 134 (3), 688–697 Sep. (e686).

Bikoue, A., George, F., Poncelet, P., Mutin, M., Janossy, G., Sampol, J., 1996. Quantitative analysis of leukocyte membrane antigen expression: normal adult values. Cytometry 26 (2), 137–147 (Jun 15).

Chan, R.C., Kotner, J.S., Chuang, C.M., Gaur, A., 2016. Stabilization of pre-optimized multi-color antibody cocktails for flow cytometry applications. Cytometry B Clin. Cytom. (Mar 22).

van Dongen, J.J., Lhermitte, L., Bottcher, S., Almeida, J., van der Velden, V.H., Flores-Montero, J., et al., 2012a. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 26 (9), 1908–1975 (May 3).

van Dongen, J.J., Lhermitte, L., Bottcher, S., Almeida, J., van der Velden, V.H., Flores-Montero, J., et al., 2012b. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 26 (9), 1908–1975 (Sep).

Flores-Montero, J., Flores, L.S., Paiva, B., Puig, N., Garcia-Sanchez, O., Bottcher, S., et al., 2017. Next generation flow (NGF) for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. Leukemia 20 (Jan).

Hedley, B.D., Keeney, M., Popma, J., Chin-Yee, I., 2015. Novel lymphocyte screening tube using dried monoclonal antibody reagents. Cytometry B Clin. Cytom. 88 (6), 361–370 (Nov–Dec).

Kalina, T., Flores-Montero, J., van der Velden, V.H., Martin-Ayuso, M., Bottcher, S., Ritgen, M., et al., 2012. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. Leukemia 26 (9), 1986–2010 (Sep.).

Kalina, T., Flores-Montero, J., Lecrevisse, Q., Pedreira, C.E., van der Velden, V.H., Novakova, M., et al., 2015. Quality assessment program for EuroFlow protocols: summary results of four-year (2010–2013) quality assurance rounds. Cytometry A 87 (2), 145–156 (Feb).

Lima, M., Teixeira, M.A., Queiros, M.L., Leite, M., Santos, A.H., Justica, B., et al., 2001. Immunophenotypic characterization of normal blood CD56+lo versus CD56+hi NK-cell subsets and its impact on the understanding of their tissue distribution and functional properties. Blood Cells Mol. Dis. 27 (4), 731–743 (Jul–Aug).

Pedreira, C.E., Costa, E.S., Lecrevisse, Q., van Dongen, J.J., Orfao, A., EuroFlow, C., 2013. Overview of clinical flow cytometry data analysis: recent advances and future challenges. Trends Biotechnol. 31 (7), 415–425 (Jul).

Perez-Andres, M., Paiva, B., Nieto, W.G., Caraux, A., Schmitz, A., Almeida, J., et al., 2010. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. Cytometry B Clin. Cytom. 78 (Suppl. 1), S47–S60.

Quijano, S., Lopez, A., Manuel Sancho, J., Panizo, C., Deben, G., Castilla, C., et al., 2009. Identification of leptomeningeal disease in aggressive B-cell non-Hodgkin's lymphoma: improved sensitivity of flow cytometry. J. Clin. Oncol. 27 (9), 1462–1469 (Mar 20).

Theunissen, P., Mejstrikova, E., Sedek, L., van der Sluijs-Gelling, A.J., Gaipa, G., Bartels, M., et al., 2016. Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood (Nov 30).