A Comparison of the BD IntraSure™ Kit to Other BD Fixation and Permeabilization Reagents and Invitrogen's FIX & PERM® Kit for the Detection of Intracellular Agents

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Contents

Abstract

Flow cytometry is commonly used to characterize cell surface antigens and can also be used to study intracellular antigens. The quality of intracellular staining depends on the ability of the fixation and permeabilization reagent to sufficiently permeabilize the cell (or other) membrane so that antibodies can traffic and bind to the target of interest. These reagents also have to maintain the cell integrity and the antigenic epitopes that a particular antibody recognizes and to which it binds.

There are many fixation and permeabilization reagents available that can be used to prepare cells for intracellular staining. Some are specific to a particular application or antigen(s) and can present workflow challenges when the expression of multiple intracellular antigens, which require different fixation and permeabilization procedures, is required from a single specimen.

BD

The BD IntraSure™ Kit (BD IntraSure) is a reagent kit designed to fix and permeabilize cells prior to intracellular immunofluorescence staining with monoclonal antibodies with minimal effect on existing cell surface staining and cell recovery. This reagent kit has been shown to be suitable for the detection of several different intracellular antigens.[1]

The objective of the preliminary research study outlined in this application note was to compare the effectiveness of the previously optimized fixation and permeabilization procedures using BD FACS™ lysing solution (FL) alone (2 hr FL) or in combination with BD FACS™ permeabilizing solution 2 (FP2) (10 min FL + FP2) with BD IntraSure and Fix & Perm® Cell Permeabilization Reagents (Invitrogen) (InvFP) for the detection of a variety of commonly used intracellular antigens. The effect of these procedures on scatter properties and leucocyte recovery was also compared.

Preliminary data presented in this study shows that all of these procedures were very similar with regard to scatter, discrimination of lymphocyte, monocyte, and granulocyte populations, and leucocyte recovery.

The previously optimized fixation and permeabilization procedures, 10 min FL + FP2 or 2 hr FL, were optimal for the detection of the intracellular antigens tested, resulting in clear discrimination of the cell populations of interest on a consistent basis. InvFP is suitable for 9 out of 11 of the intracellular antigens tested. InvFP is not suitable for the detection of Bcl-2 and is suboptimal for the detection of ZAP-70. In contrast, BD IntraSure can be used for the detection of 10 out of the 11 intracellular antigens tested and is not optimal for the detection of cytoplasmic (cyt) IgM.

Introduction

Flow cytometry is commonly used to characterize cell surface antigens. As this technology advances, more researchers are also using flow cytometry to study intracellular antigens to understand how cells communicate and respond to their environment. There are many different types of intracellular antigens that can be subdivided according to their physical location within the cell, such as the cytoplasm, inside vesicles, vesicle membranes, nuclear membrane, or inside the nucleus. The quality of intracellular staining depends on the ability of the fixation and permeabilization reagent to sufficiently permeabilize the cell (or other) membrane so that antibodies can traffick and bind to the target of interest. These reagents also have to maintain the cell integrity and the antigenic epitopes to which a particular antibody recognizes and to which it binds.

There are many fixation and permeabilization reagents available that can be used to prepare cells for intracellular staining. BD Biosciences offers a selection of products. Some are specific for a particular application, for example, BD Phosflow™ products for the detection of intracellular phosphoproteins, or BD FastImmune™ products for intracellular cytokine detection. Others have a more generic application, for example, BD Cytofix/Cytoperm™, BD FACS lysing solution (FL), and BD FACS permeabilizing solution 2 (FP2).

The BD IntraSure Kit is a reagent kit designed to fix and permeabilize cells prior to intracellular immunofluorescence staining with monoclonal antibodies with minimal effect on existing cell surface staining and cell recovery. Preliminary data has shown that this reagent is suitable for the detection of a variety of intracellular antigens for research applications.[1]

- The BD Oncomark™ product CD3/Anti-myeloperoxidase/CD79a can be used to determine the lineage of leukemic blasts in research applications. Cytoplasmic CD3 (cytCD3) identifies cells in the T-lymphoid lineage, anti-myeloperoxidase (MPO) identifies cells in the myeloid lineage, and cytoplasmic CD79a (cytCD79a) identifies cells in the B-lymphoid lineage.[2]
- B-cell lymphoma 2 (Bcl-2) is an intracellular (mitochondrial) protein that regulates apoptosis, and overexpression of this protein is known to result in a resistance to cell death and is often seen in neoplastic cells. Anti–Bcl-2 is often used in research studies of apoptosis, B-cell malignancies, chemotherapeutics, and follicular t(14;18) chromosomal translocations.[3]
- Zeta-chain-associated protein kinase 70 (ZAP-70) is a protein kinase in the Syk family of protein tyrosine kinases and plays a crucial role in T-cell signal transduction and thymocyte development. ZAP-70 is normally expressed in T and natural killer (NK) cells and is also known to be expressed in some chronic lymphocytic leukemias (CLL).[4]
- Terminal deoxynucleotidyl-transferase (TdT) is a 60-kDa polymerase present in the cell nucleus that catalyzes the template-independent addition of nucleotides to single-stranded DNA. This molecule has been reported to be involved in the regulation or translocation of both DNA and gene rearrangements during normal T- and B-cell development. It is present in the nuclei of immature T and B lymphocytes found in the normal thymus and bone marrow. Some neoplastic cells of acute lymphoblastic lymphoma/leukemia have been shown to have high TdT activity.[5]
- Ki-67 is a nuclear cell proliferation-associated antigen expressed in all active stages of the cell cycle. Its expression can be used to study cell proliferation.
- Cytoplasmic immunoglobulin expression (cytIgκ, cytIgλ, and cytIgM) occurs in B lymphocytes as well as in a wide variety of cancer cells, epithelial cells, and neurons.[6]

Preliminary research data presented in this application note compares the recovery and scatter properties of leucocytes and flow cytometric analysis of bulk lysed normal human whole blood (bulk lysed WB) treated with a variety of fixation and permeabilization reagents. The data also compares the effect of the procedures used on the ability to detect a variety of commonly used surface and intracellular antigens. Comparisons are made between the effectiveness of previously optimized research procedures using FL alone (2 hr FL) or in combination with FP2 (10 min FL + FP2), BD IntraSure, and Fix & Perm Cell Permeabilization Reagents (InvFP).

Objective

The objective of this application note is to compare the effectiveness of the previously optimized fixation and permeabilization research procedures of 2 hr FL or 10 min FL + FP2 with BD IntraSure and InvFP on bulk lysed WB with respect to:

- Scatter properties
- Comparison of lymphocyte, granulocyte, and monocyte populations (based on scatter)
- Total leucocyte recovery
- Detection of the following intracellular antigens: CD3, MPO, CD79a; Bcl-2 and ZAP-70; TdT; Ki-67; cytIgκ and cytIgλ; cytIgM.

Methods

Antibodies

Product Description	Fluorochrome	Vendor	Cat. No.
Anti-Bcl-2	FITC	BD Biosciences	340575
Anti-Human Ki-67 set	FITC	BD Biosciences	556026
Anti-Kappa (Ig Light chain)	APC	BD Biosciences	341088
Anti-Lambda (Ig Light chain)	PE	BD Biosciences	642919
Anti-TdT	FITC	BD Biosciences	347194
Anti-ZAP-70 (1E7.2)	PE	BD Biosciences	344635
BD Oncomark CD3/MPO/CD79a	FITC, PE, PerCP-Cy™5.5	BD Biosciences	340961
CD5 (IgG_{2a})	PerCP-Cy5.5	BD Biosciences	341089
CD19	PE	BD Biosciences	349209
	BD Horizon™ V450	BD Biosciences	560353
CD20	FITC	BD Biosciences	347673
	PerCP	BD Biosciences	347674
CD38	V450	BD Biosciences	646851
CD45	APC-H7	BD Biosciences	641399
Mouse Anti-Human IgM	PE	BD Biosciences	555783
Mouse IgG_1 fluorescence control	APC	BD Biosciences	340442
	APC-H7	BD Biosciences	641401
	FITC	BD Biosciences	349041
	PE	BD Biosciences	349043
	PerCP	BD Biosciences	349044
	PerCP-Cy5.5	BD Biosciences	347202
Mouse IgG_1 κ isotype control	PE	BD Biosciences	555749
	V450	BD Biosciences	560373
Mouse IgG_{2a} κ isotype control	PerCP-Cy5.5	BD Biosciences	552577

Note All antibodies listed are For Research Use Only. Not for use in diagnostic or therapeutic procedures.

Reagents and materials

Product Description	Vendor	Cat. No.
BD Cytofix™ fixation buffer	BD Biosciences	554655
BD Cytometer Setup and Tracking (CS&T) Bead Kit	BD Biosciences	641319
BD FACS lysing solution	BD Biosciences	349202
BD FACS permeabilizing solution 2	BD Biosciences	340973
Falcon® 12 x 75-mm round-bottom tubes	Corning	352058
BD IntraSure™ kit	BD Biosciences	641776
BD Pharmingen™ stain buffer (BSA)	BD Biosciences	554657
BD Pharm Lyse™ lysing buffer (10X concentrate)	BD Biosciences	555899
BD Trucount™ tubes	BD Biosciences	340334
BD™ CompBead particle set	BD Biosciences	552843
Fix & Perm® cell permeabilization reagents	Invitrogen	GAS-004
RPMI 1640 medium (Mediatech)	VWR	45000-396
Trypan blue (Mediatech)	VWR	45000-717

Specimens

Blood specimens were collected from normal healthy volunteers who consented to participate in an Institutional Review Board approved protocol.

Instruments

Samples were prepared using a BD FACS™ Sample Prep Assistant (SPA) II (software version 3.0.1).

Data from stained cells was acquired using a BD FACSCanto™ II system. The flow cytometer was set up using the BD Cytometer Setup and Tracking (CS&T) function, application settings were applied, and then compensation performed using BD CompBead particles prior to each experiment. BD FACSDiva™ version 6.1.3 software was used for data acquisition and analysis.

Methods

Isolation of Leucocytes (bulk lysis)

1. Blood specimens were collected into BD Vacutainer® EDTA tubes and 4–5 mL transferred into 50-mL conical tubes.
2. BD Pharm Lyse buffer was diluted 1:10 in DI water and added to each 50-mL tube containing the blood, filling the tube to the top.
3. The blood/buffer mixture was incubated for 15 minutes on a rocker, then centrifuged (400*g*, 5 min, 4˚C).
4. The supernatant was discarded, the cell pellet resuspended in 50 mL of stain buffer, and the cell suspension centrifuged (400*g*, 5 min, 4˚C).
5. The supernatant was discarded and the cell suspension resuspended in half the volume of original blood (2X concentration) in stain buffer.

Specimens processed in this way are subsequently referred to as bulk lysed whole blood (bulk lysed WB).

Surface staining for determination of cell recoveries

1. Five microliters of venous blood was collected from six independent donors, and bulk lysis performed as outlined previously.
2. Fifty-microliter samples were added to 12 x 75-mm tubes and 5 µL of CD45 APC-H7 was added to each tube using the SPA II.
3. Samples were incubated for 15 minutes and then fixed and permeabilized with each fix and perm combination as outlined in Table 1.
4. Mock staining for the intracellular markers was performed by adding an equivalent volume of stain buffer, and the procedure was completed as outlined in Table 1.
5. Once the procedure was completed, samples were transferred to BD Trucount tubes, and the absolute number of $CD45^+$ leucocytes present in each tube determined as outlined in the TDS.[1]

Procedure Details	2 hr FL	10 min FL + FP2	IntraSure	InvFP
Tube and markers	CD45 control, CD3/CD79a/MPO, cytIgκ/cytIgλ	CD45 control, Bcl-2/ZAP-70, TdT, Ki-67, cytIgM	All	All
Sample	Bulk lysed WB	• Bulk lysed WB for Bcl-2/ ZAP-70 and cytIgM • Bulk lysed WB + REH for TdT and Ki-67	• Bulk lysed WB for CD45 control, CD3/CD79a/MPO, Bcl-2/ZAP-70, cytIgκ/ cytIgλ and cytIgM • Bulk lysed WB + REH for CD45 control, TdT and Ki-67	• Bulk lysed WB for CD45 control, CD3/CD79a/MPO, Bcl-2/ZAP-70, cytIgκ/ cytIgλ and cytIgM • Bulk lysed WB + REH for CD45 control, TdT and Ki-67
Surface staining	Surface markers (15 min, SPA II)	• Surface markers (15 min, SPA II) • Also unlabelled IgM at 1:100 for cytIgM	Surface markers (15 min, SPA II)	Surface markers (15 min, SPA II)
Permeabilization and lysis	1. 2 mL FL, 2 hr 2. Centrifuge (500*g*, 5 min, RT) 3. Aspirate supernatants 4. 2 mL stain buffer 5. Centrifuge (500*g*, 5 min, RT) 6. Aspirate supernatants	1. 2mL FL, 10 min 2. Centrifuge (500*g*, 5 min, RT) 3. Aspirate supernatants 4. 2 mL stain buffer 5. Centrifuge (500*g*, 5 min, RT) 6. 1 mL FP2, 10 min 7. 2 mL stain buffer 8. Centrifuge (500*g*, 5 min, RT) 9. Aspirate supernatants	1. 100 µL IntraSure Reagent A, 5 min 2. 2 mL FL, 10 min 3. Centrifuge (850*g*, 5 min, RT) 4. Decant supernatants	1. 100 µL InvFP reagent A, 5 min 2. 2 mL stain buffer 3. Centrifuge (350*g*, 5 min, RT) 4. Aspirate supernatants
Intracellular staining	1. Intracellular markers (30 min, SPA II) 2. 2 mL stain buffer 3. Centrifuge (500*g*, 5 min, RT) 4. Aspirate supernatants	1. Intracellular markers (30 min, SPA II) 2. 2 mL stain buffer 3. Centrifuge (500*g*, 5 min, RT) 4. Aspirate supernatants	1. 50 µL IntraSure reagent B 2. Intracellular markers (15 min, SPA II) 3. 2 mL stain buffer 4. Centrifuge (850*g*, 5 min, RT) 5. Decant supernatants	1. 100 µL InvFP reagent B 2. Intracellular markers (20 min, SPA II) 3. 2 mL stain buffer 4. Centrifuge (350*g*, 5 min, RT) 5. Aspirate supernatants
Final fix	0.5 mL 1% PFA (BD Cytofix 1:4 in DPBS)	0.5 mL 1% PFA (BD Cytofix 1:4 in DPBS)	0.5 mL 1% PFA (BD Cytofix 1:4 in DPBS)	0.5 mL 1% PFA (BD Cytofix 1:4 in DPBS)

Table 1. Summary of different fixation and permeabilization procedures

Abbreviations: PFA – Paraformaldehyde; RT – room temperature; DPBS – Dulbecco's phosphate buffered saline

Note: All of these methods differ (by varying degrees) from those methods provided in the individual antibody or fixation and permeabilization buffer TDS to aid implementation of the study.[1-5, 8-11] Optimization and/or direct comparison of each method was compared to that provided in the TDS and was performed to ensure that the changes to the procedure were not deleterious to the outcome (data not shown).

Preparation of bulk lysed WB spiked with REH cells (bulk lysed WB + REH)

1. REH cells were cultured in RPMI 1640 medium + 10% FBS and passaged every 3–4 days.
2. Cells were harvested from dividing cultures and washed in stain buffer (400*g*, 5 min, RT).
3. The supernatant was discarded and the cell pellet resuspended in a small amount of stain buffer.
4. The viable cell number was determined using trypan blue exclusion, and the cell suspension made up to a concentration of 5 x 10^7 REH cells/mL.
5. One hundred microliters of REH was added to 900 µL of bulk lysed WB to achieve a final REH concentration of 5 x 10^6 REH cells/mL.

Surface and intracellular staining of samples

1. Five to 10 mL of venous blood was collected from six independent donors, and bulk lysis performed as outlined previously.
2. An aliquot of the bulk lysed WB was collected and spiked with REH cells as outlined previously.
3. Bulk lysed WB or bulk lysed WB + REH was used for the detection of different intracellular antigens:
   i. Bulk lysed WB was used for the detection of CD3/MPO/CD79a, Bcl-2/ZAP-70, cytIgλ/cytIgκ, and cytIgM, and the accompanying CD45 and fluorescence minus one (FMO) controls.
   ii. Bulk lysed WB + REH was used for the detection of TdT and Ki-67 and the accompanying CD45 and FMO controls.
4. One hundred microliters of sample (bulk lysed WB or bulk lysed WB +REH) was added to 12 x 75-mm tubes, and the appropriate volume of surface antibody added to each tube, according to each individual antibody TDS, using the SPA II.[12-17] A summary of the surface antibodies used to stain each tube is shown in Table 2.

   A complete panel of tubes containing a CD45 control, FMO control, and Test for each intracellular antigen was prepared for each fixation and permeabilization procedure tested as outlined in Table 1 (Sample).
5. Samples were incubated for 15 minutes and then processed with each fixation and permeabilization procedure as outlined in Table 1 (Permeabilization and lysis).
   i. Samples were processed using either the previously optimized 2 hr FL or 10 min FL + FP2 procedures (data not shown) as appropriate.
   ii. All samples were processed using InvFP and BD IntraSure.
6. The appropriate volume of intracellular detection antibody was added to each tube, according to each individual antibody TDS, using the SPA II.[2-5, 18-20]
7. Samples were incubated for the time specified, and the procedure completed as outlined in Table 1.
8. Fixed samples were stored overnight in the refrigerator and acquisition and analysis performed within 24 hours.

Staining panel for surface and intracellular antigens

The staining panel shown in Table 2 was used to stain samples for surface and intracellular antigens (shown in bold) using the volume of antibody recommended in the individual antibody TDS.[2-5, 12-18] FMO control tubes were prepared for each tube by substituting the intracellular antigen with an isotype-matched (by Ig subtype and concentration) antibody.

Tube name	PerCP-Cy5.5	PE	FITC	APC-H7	APC	BD Horizon V450
CD45 control	Isotype	Isotype	Isotype	CD45	Isotype	Isotype
CD3/MPO/CD79a	**CD79a**	**MPO**	CD3	CD45	–	–
Bcl-2/ZAP-70	CD5	**ZAP-70**	**Bcl-2**	CD45	–	CD38
TdT	CD20	CD19	**TdT**	CD45	–	–
Ki-67	CD20	CD19	**Ki-67**	CD45	–	–
CytIgκ/cytIgλ	CD5	**Igλ**	CD20	CD45	**Anti-Igκ**	CD19
CD3/MPO/CD79a	CD5	**IgM**	CD20	CD45	–	–

Table 2. Staining panel

Determination of cell recoveries

1. SSC parameters were set so as to maximize the visualization of the different cell populations in a CD45 vs SSC plot.
2. Ten thousand total events were acquired and the absolute number of CD45⁺ leucocyte events determined as outlined in the BD Trucount TDS.[7]
3. The relative percentages of lymphocytes, monocytes, and granulocytes were determined by gating on these individual populations using a CD45 vs SSC dot plot.

Expression of surface and intracellular antigens

1. SSC parameters were set using a CD45 vs SSC plot to accommodate all sample types without changing parameters.
2. Samples were automatically loaded and data automatically acquired on the BD FACSCanto II with the CD45⁺ stopping gate set to ensure the acquisition of at least 2,500 of the relevant intracellular events (based upon previous experience).
3. Data was analyzed using the gating strategies outlined in Figures 4–9 using the CD45⁺ and FMO controls to help with gating.

Results

Comparison of scatter properties

SSC parameters were set to maximize the visualization of the different cell populations in a CD45 vs SSC plot. Data is presented in Figure 1.

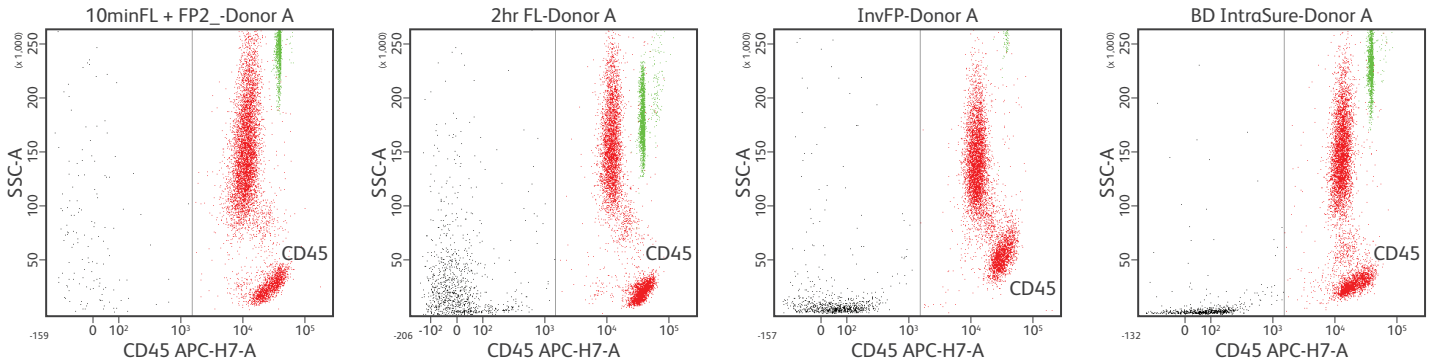

Figure 1. CD45 vs SSC plots of bulk lysed WB
BD Trucount beads are shown in green.

Absolute CD45 counts

The absolute count of CD45⁺ leucocytes remaining in each tube after each fixation and permeabilization procedure was determined using six independent donors. Data is presented in Figure 2. Horizontal lines represent the mean and 95% confidence interval (CI). Each donor is represented by a different color.

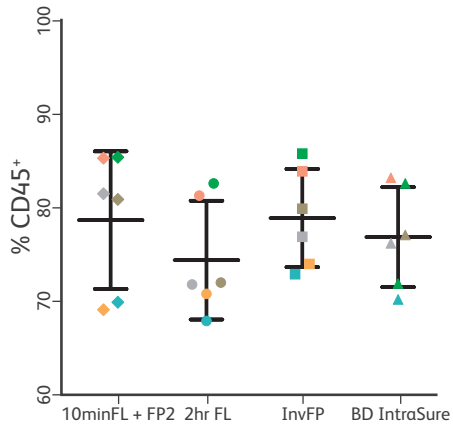

Figure 2. CD45⁺ leucocytes

Lymphocyte, monocyte, and granulocyte percentages

The percentage of CD45⁺ events in the lymphocyte, granulocyte, and monocyte gates was determined using six independent donors. Data is presented in Figure 3. Horizontal lines represent the mean and 95% CI. Each donor is represented by a different color.

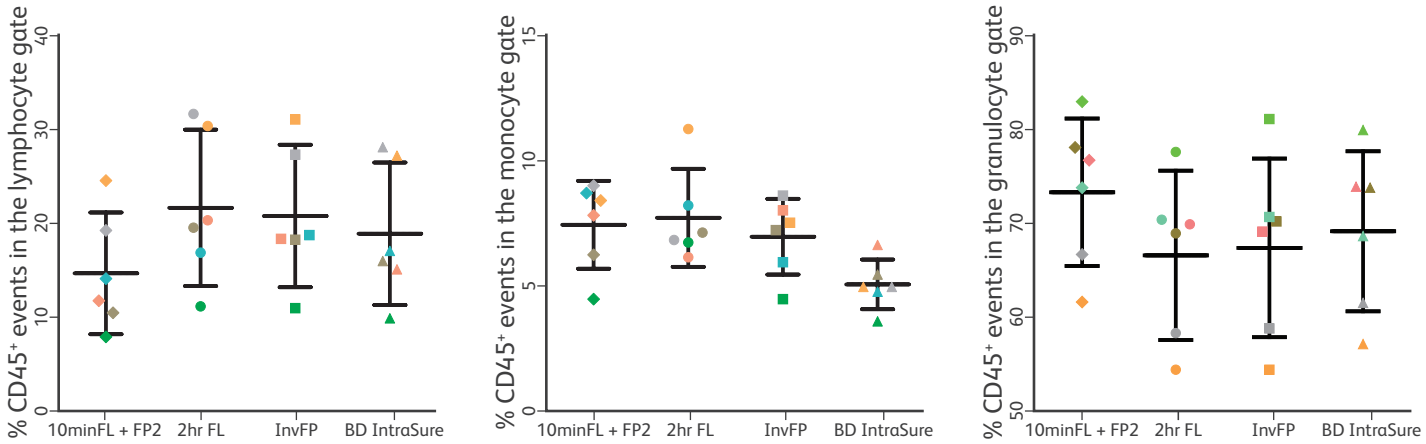

Figure 3. Percentages of lymphocytes, monocytes, and granulocytes

Detection of intracellular antigens

Representative dot plots of an individual donor and summary graphs summarizing the data from multiple donors are presented in Figures 4–9. Horizontal lines represent the mean and 95% CI. Each donor is represented by a different color in the summary graphs. Table 3 summarizes the research procedures and sample types used. Table 4 shows the numerical data for each summary graph.

Intracellular antigen(s)	Sample Type	Fix and permeabilization procedures used				Data presented in
		2 hr FL	10 min FL + FP2	InvFP	IntraSure	
CD3, CD79a and MPO	Bulk lysed WB	✓	–	✓	✓	Figure 4
Bcl-2, ZAP-70	Bulk lysed WB	–	✓	✓	✓	Figure 5
TdT	Bulk lysed WB + REH	–	✓	✓	✓	Figure 6
Ki-67	Bulk lysed WB + REH	–	✓	✓	✓	Figure 7
Igκ, Igλ	Bulk lysed WB	✓	–	✓	✓	Figure 8
IgM	Bulk lysed WB	–	✓	✓	✓	Figure 9

Table 3. Summary of research procedures used

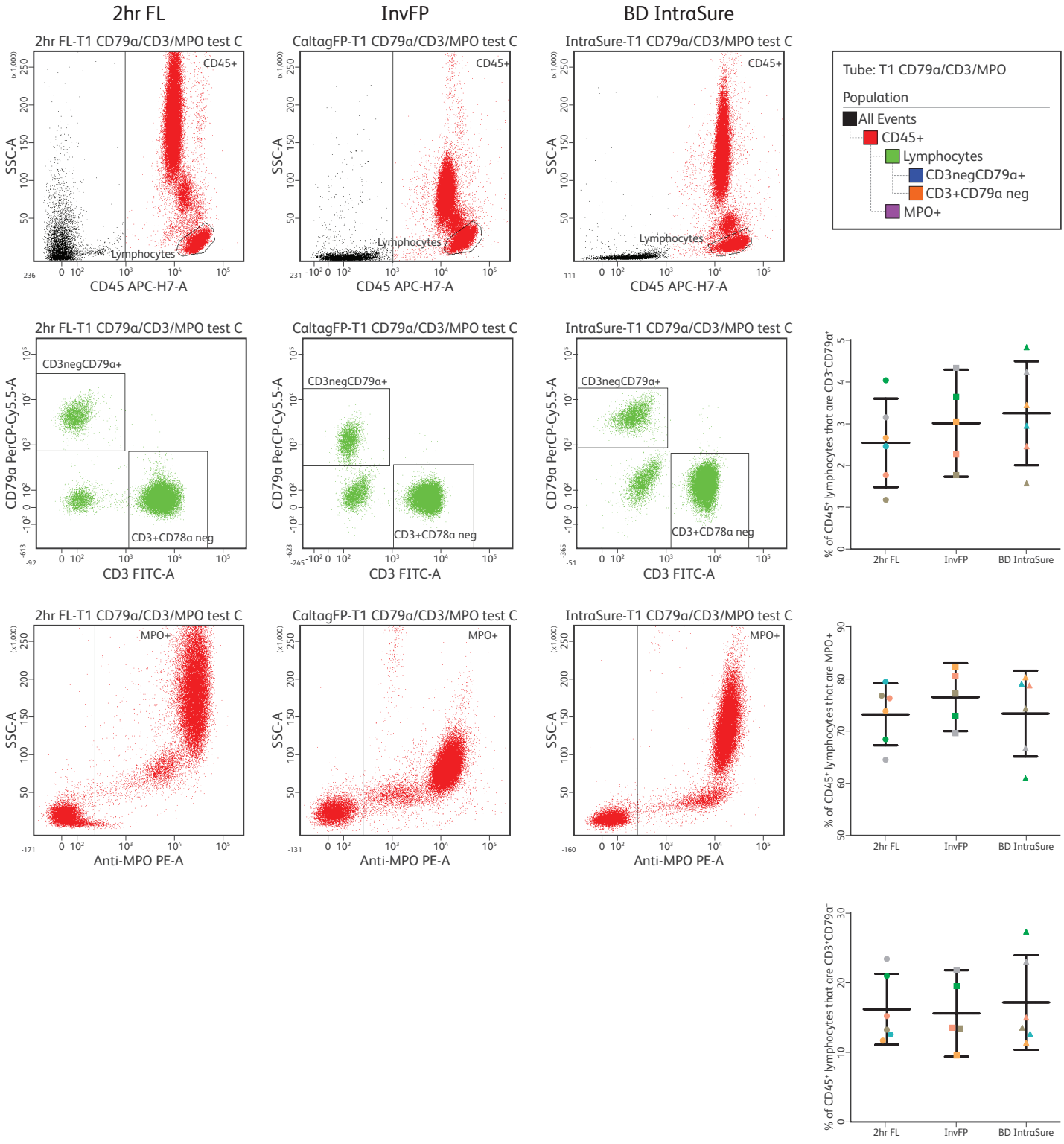

Figure 4. Intracellular CD79a, MPO, and CD3 staining

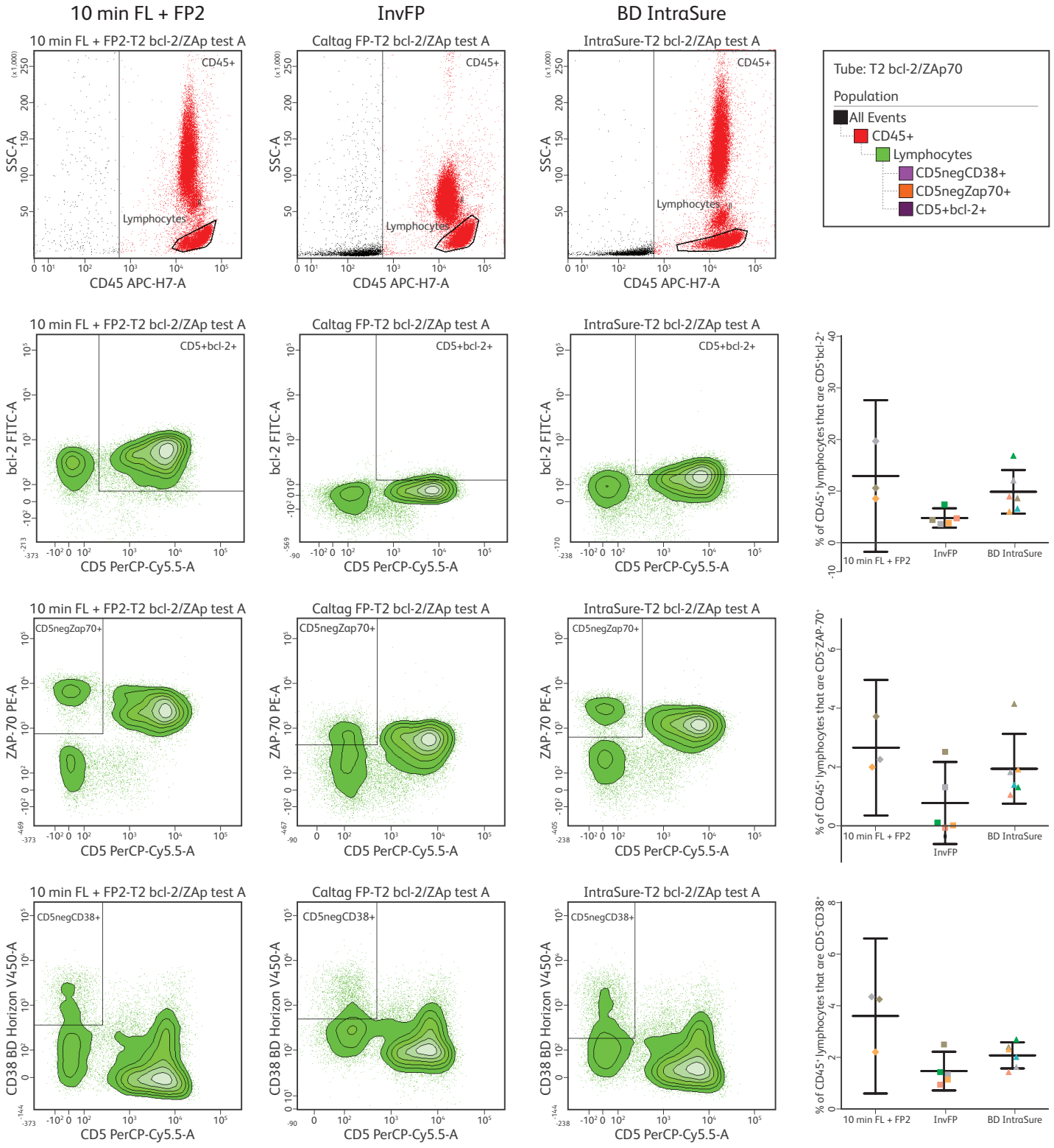

Figure 5. Intracellular Bcl-2 and ZAP-70 and surface CD38 staining

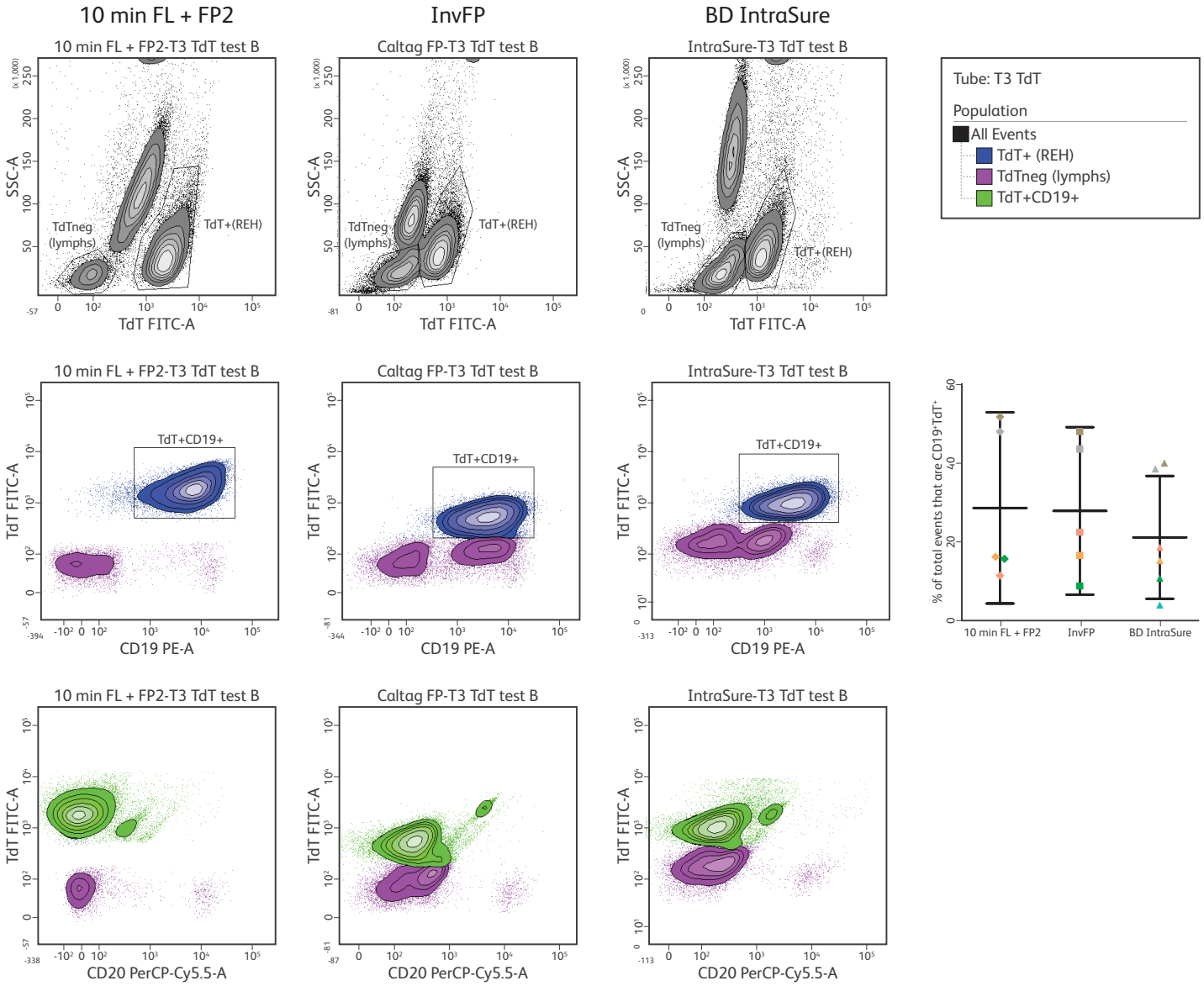

Figure 6. Intracellular TdT staining

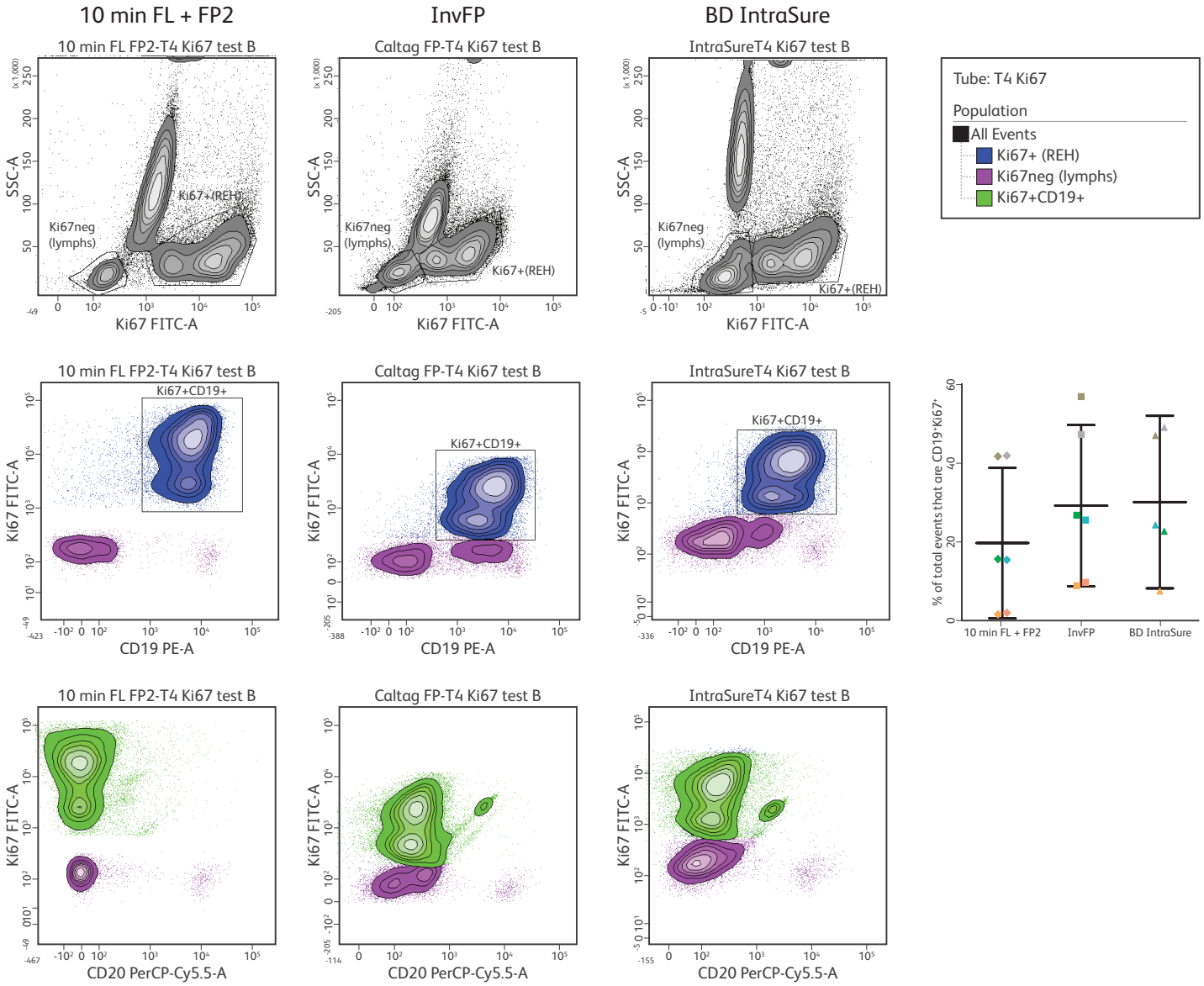

Figure 7. Ki-67 staining

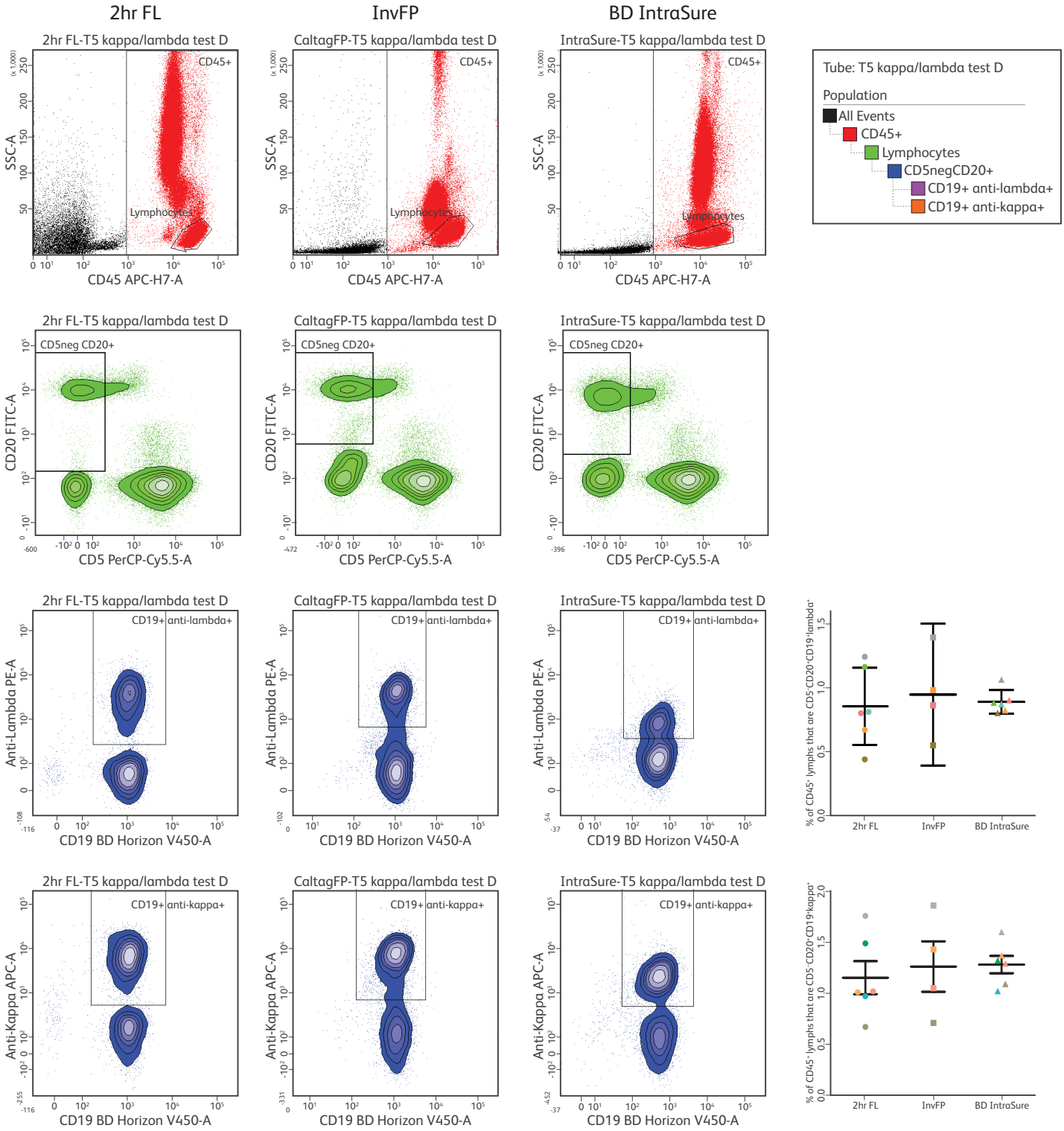

Figure 8. CytIgκ and cytIgλ staining

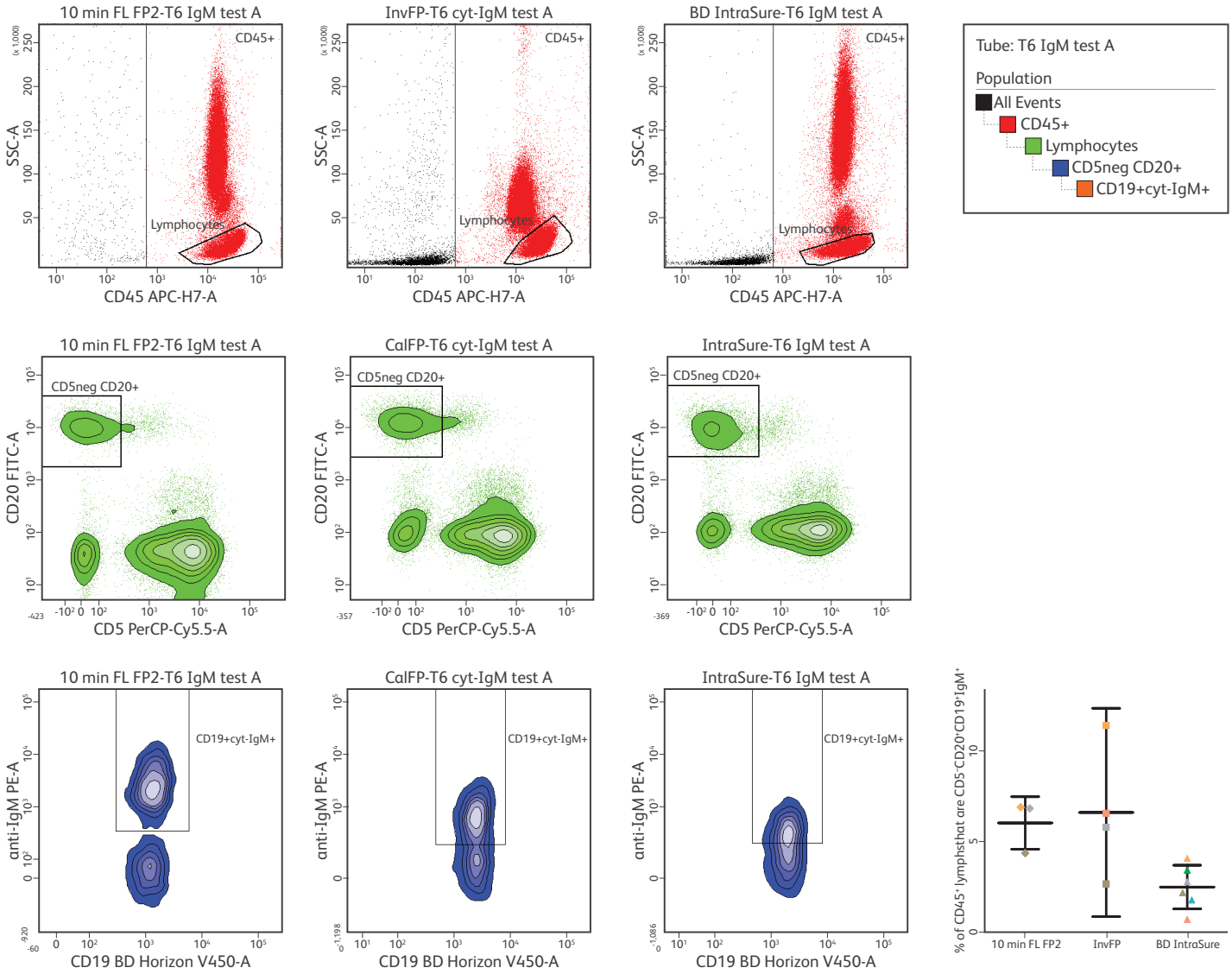

Figure 9. CytIgM staining

Phenotype	10 min FL + FP2			2 hr FL			InvFP			BD IntraSure		
	Mean	95% CI	n	Mean	95% CI	n	Mean	95% CI	n	Mean	95% CI	n
%CD45$^+$ lymphs that are CD3$^-$CD79a$^+$	NA			2.58	1.51–3.66	6	3.06	1.76–4.36	5	3.30	2.04–4.56	6
%CD45$^+$ lymphs that are CD3$^+$CD79a$^+$	NA			16.70	12.10–24.10	6	16.08	9.90–22.5	5	17.70	11.80–28.10	6
%CD45$^+$ lymphs that are MPO$^+$	NA			73.67	67.62–79.71	6	77.02	70.39–83.65	5	73.82	65.43–82.20	6
%CD45$^+$ lymphs that are CD5$^-$CD20$^+$Bcl-2$^+$	2.87	-0.64–3.38	3	NA			0.06	0.02–0.14	5	1.28	0.38–2.18	6
%CD45$^+$ lymphs that are CD5$^-$CD20$^+$CD38$^+$	2.85	1.25–4.45	3	NA			1.95	1.21–2.68	5	2.58	1.73–3.43	6
%CD45$^+$ lymphs that are CD5$^-$CD20$^+$ZAP-70$^+$	3.23	0.05–6.41	3	NA			1.11	-0.40–2.62	5	2.65	1.27–4.03	6
%CD45$^+$ lymphs that are CD19$^+$CD20$^-$TdT$^+$	23.12	0.53–45.71	3	NA			15.36	-0.36–31.0	5	14.98	-0.58–30.55	6
%CD45$^+$ lymphs that are CD19$^+$CD20$^-$Ki-67$^+$	22.73	4.89–40.60	3	v			22.58	3.74–41.43	6	21.50	4.37–38.63	5
%CD45$^+$ lymphs that are CD5$^-$CD20$^+$cytIgλ$^+$	NA			0.85	0.54–1.17	6	0.95	0.39–1.50	4	0.89	0.79–0.99	6
%CD45$^+$ lymphs that are CD5$^-$CD20$^+$cytIgκ$^+$	NA			1.15	0.74–1.57	6	1.26	0.48–2.05	4	1.28	1.06–1.50	6
%CD45$^+$ lymphs that are CD5$^-$CD20$^+$CD19$^+$cytIgM$^+$	5.93	2.40–9.46	3	NA			6.50	0.85–12.15	4	2.45	1.21–3.70	6

Table 4. Data summary table

Discussion

Comparison of scatter properties

All four procedures enabled the visualization of the three main leucocyte populations (lymphocytes, monocytes, and granulocytes) based on scatter, although individual adjustment of the SSC parameter was required to achieve good population separation (Figure 1). When the same SSC setting was used to view samples treated with different fixation and permeabilization reagents, samples treated with InvFP exhibited lower SSC properties compared to the other reagents (Figures 4–9).

A comparison of the relative percentages of $CD45^+$ events that were in the lymphocyte, monocyte, or granulocyte gate demonstrated consistency across all six donors (Figure 3).

Recovery

A comparison of the recovery of $CD45^+$ leucocytes after treatment with all four procedures was determined using BD Trucount beads. The data presented in Figure 2 shows similar recoveries with all fixation and permeabilization procedures.

Comparison of dot plots for intracellular antigens

Figures 4–9 show representative data comparing the appearance of dot plots after treatment of the samples with the different fixation and permeabilization procedures. Data compared within a particular figure is from a single donor. A single SSC parameter setting was used for all samples regardless of fixation and permeabilization procedure to facilitate the automatic acquisition of data.

2hr FL vs 10 min FL + FP2

During the course of setting up procedures for this preliminary study, some optimization was performed to streamline techniques, since there was some variation in procedures recommended in individual reagent TDSs or a procedure was not available for the detection of the antigen in its intracellular form.[1-5, 18-20] Consequently, in this preliminary research study, it was determined that a 2 hr FL treatment was appropriate for the simultaneous detection of intracellular CD3, CD79a, and MPO, cytIgκ and cytIgλ, and a 10 min FL + FP2 treatment was appropriate for the simultaneous detection of intracellular Bcl-2 and ZAP-70, and TdT, Ki-67, and cytIgM (data not shown).

Intracellular CD3, MPO, and CD79a, cytIgκ and cytIgλ expression in bulk lysed WB

As shown in Figures 4 and 8, intracellular CD79a, CD3, and MPO, and cytIgκ and cytIgλ expressing populations were clearly distinguished as single populations after sample treatment using 2 hr FL, InvFP, or BD IntraSure fixation and permeabilization procedures. This preliminary research study shows that these procedures can be used to detect these intracellular antigens and resulted in similar cell percentages for the phenotypes tested.

Intracellular Bcl-2 and ZAP-70 and cytIgM expression in bulk lysed WB

As shown in Figures 5 and 9, this preliminary research study shows that there were some differences in the detection of intracellular Bcl-2, ZAP-70, and cytIgM depending upon the fixation and permeabilization procedure used (10 min FL + FP2, InvFP, or BD IntraSure). Intracellular $Bcl\text{-}2^+$ and $ZAP\text{-}70^+$ events were clearly identified and resulted in similar cell percentages using 10 min FL + FP2 and BD IntraSure. Bcl-2 was not detected when InvFP was used to treat the cells. Intracellular $ZAP\text{-}70^+$ events could be distinguished only after careful gating compared to the FMO control, and the cell percentages were lower when compared to 10 min FL + FP2 or BD IntraSure, but were still within range of the 95% CI. $CytIgM^+$ events were clearly distinguishable as a distinct population only when cells were treated with 10 min FL + FP2 and required careful reference to FMO controls when InvFP or BD IntraSure was used to treat these cells. Cell percentages were extremely variable when InvFP was used to treat cells and diminished when BD IntraSure was used compared to 10 min FL + FP2.

Intracellular TdT and Ki-67 expression in bulk lysed WB + REH

As shown in Figures 6 and 7, this preliminary research study shows that 10 min FL + FP2 treatment resulted in excellent resolution of the TdT^+ and $Ki\text{-}67^+$ populations. InvFP and BD IntraSure were satisfactory but required more care in gating the appropriate positive population. There was a large variation in the percentage of TdT^+ and $Ki\text{-}67^+$ events measured with all fixation and permeabilization procedures, which might be due to differences in the proliferative characteristics of REH cells harvested on different days for individual experiments. Cell percentages were similar for all fixation and permeabilization procedures.

Conclusions

The objective of the preliminary research study outlined in this application note was to compare the effectiveness of two previously optimized fixation and permeabilization procedures (2 hr FL or 10 min FL + FP2) with BD IntraSure and InvFP for the detection of a variety of commonly used intracellular antigens and the effect of these procedures on scatter properties and leucocyte recovery.

Data presented in this research study shows that all of these procedures were very similar with regard to scatter, discrimination of lymphocyte, monocyte, and granulocyte populations, and leucocyte recovery.

The previously optimized procedures (10 min FL + FP2 or 2 hr FL) are optimal for the detection of particular intracellular antigens, resulting in clear discrimination of the cell populations of interest on a consistent basis. Since each procedure is optimal for different intracellular antigens, these procedures can present workflow challenges because different samples derived from a single specimen require different handling and preparation techniques. InvFP and BD IntraSure have the advantage that one fixation and permeabilization procedure can be used to prepare samples for the detection of multiple intracellular antigens from a single specimen. The preliminary research data presented in this application note demonstrates that InvFP is suitable for 9 out of 11 of the intracellular antigens tested. InvFP is not suitable for the detection of intracellular Bcl-2 and is suboptimal for the detection of ZAP-70. In contrast, BD IntraSure can be used for the detection of 10 out of the 11 intracellular antigens tested and is not optimal for the detection of cytIgM.

Customers should validate the procedures outlined in this preliminary research study in their own laboratory.

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