

Measurement of ZAP-70 Expression in CLL Using An Optimized Flow Cytometric Assay for ZAP-70 Protein Levels in Whole Blood Samples

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Abstract

Crespo et al (NEJM 348: 1764, 2003) published the first paper utilizing flow cytometry to ZAP-70 protein plays a critical role in the initiation of T-cell signaling through the T-cell receptor, and also regulates B cell differentiation. Studies aimed at understanding the relationship of maturation to disease course in the B-cell form of Chronic Lymphocytic Leukemia (B-CLL) have demonstrated a correlation of disease progression with t(12) configuration (germ line vs mutated). Flow cytometry-based assays for ZAP-70 expression in CLL patient samples have been previously reported, and generally show poor separation of ZAP-70 levels in samples considered as positive from negative, making interpretation of these data difficult, and reducing the likely agreement between laboratories. Using a unique fixation and permeabilization technique in conjunction with an optimal ZAP-70-PE antibody conjugate, we have developed a whole blood assay for the measurement of ZAP-70 protein expression which results in a significantly increased S/N of 19.24 (normal blood T-cells to B-cells) compared to previously reported flow cytometric assays (S/N of 0 to 8). Based on these data, we have developed a 5 antibody/4 color whole blood assay (CD5-FITC, ZAP-70-PE, CD19-PCy5, CD3-CD56-PECy7) which includes surface labeling before fixation and ZAP-70 labeling after permeabilization. Critical assay details including pre and post staining stability, instrument standardization, gating strategies and data interpretation will be presented and discussed. Using this optimized assay, we have undertaken a multi-institutional study to determine the inter-laboratory variability of ZAP-70 measurements using normal donors, and an assessment of the ability of this assay to increase the separation of B-CLL ZAP-70 positive from negative populations. Finally, we have developed a method to index differential ZAP-70 expression using internal controls, which obviates the use of quadrant analysis techniques.

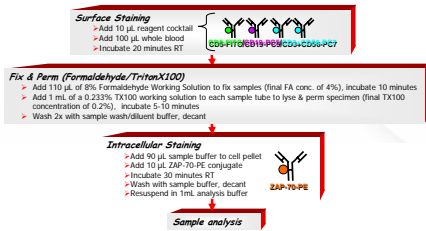
Materials

- ZAP-70 Antibodies evaluated (*clone-dry*)
 - SBZAP-PE – Beckman Coulter, Inc. Custom Design Services (CDS) Conjugate Surface Marker Antibodies - Custom Reagents by CDS, Beckman Coulter, Inc.
 - CD5(B1.1a)-FITC
 - CD5(NKH1)-PC7
 - CD19(J4.119)-PC5
 - CD19(J4.119)-APC
- Sample preparation Reagents – Optimized Fix/Perm Method (FATX100 Method)
 - 10% Formaldehyde (MeOH free, EM Grade), Polysciences, Inc. PN 04018
 - Dilute to 8% in PBS for assay working solution (8mL/10mL final volume)
 - 10% Triton X-100 Ampules, Pierce PN 28314
 - Dilute to 0.233% in PBS for assay working solution (233uL/10mL final volume) (warm to 37°C before use)
 - Wash & Diluent Buffer: PBS, 0.1mM EDTA, 2% BSA, 0.1% Na₂S₂O₈, pH 7.2
 - Analysis Buffer: PBS, 0.1% Paraformaldehyde (PFA), 0.1% Na₂S₂O₈, pH 7.2
 - Commercial Fix/Perm Reagents
 - IntraPrep™ Permeabilization Reagent – PN A07802, Beckman Coulter, Inc.
 - Fix & Perm™ Cell Permeabilization Reagents – PN G4S-003, Caltag Labs
 - BD™ Phosflow Reagents: LyseFix PNX5049 and Perm Buffer II PIN55052
- Instrument Set-up Reagents for the Cytomics FC500 Flow Cytometer
 - Flow-Set™ Fluorospheres – PN 6607007, Beckman Coulter, Inc.
 - PC7 (770/488) Setup Kit – PN 6607121, Beckman Coulter, Inc.
 - Flow-Check™ Fluorospheres – PN 6605389, Beckman Coulter, Inc.
 - Compensation Reagents – Beckman Coulter, Inc.
 - QuickCOMP 2 Kit (CD45-FITC/CD45-PE) – PN 177018
 - CD45-PC5 – PN IM2653, CD45-APC – PN IM2473 and CD45-PC7 – PN IM3548

Methods

- ZAP-70 AutoSetup Method Comparisons
 - AutoSetup
 - Setup was optimized for a 1 (488 nm Argon) and 2 laser (488 nm Argon and 633 nm HeNe laser) FC 500 flow cytometer using the appropriate set-up reagents.
- Sample Preparation Method Comparisons:
 - The ZAP-70 conjugate combined with the 5 surface marker antibody cocktail was evaluated for ZAP-70 expression on a normal donor comparing the optimized FATX100 method to three commercially available fix & perm reagent kits.
 - S/N was calculated based on MFI of the normal T-cell and B-cell populations.

Optimized FATX100 Assay Method Flow Chart (patent pending):



4-Color, 5 antibody ZAP-70 Assay Performance Studies

- Specimen Stability
 - Five normal donor bloods were collected and processed at three different time periods; within 2 hours, 24 hours, and 48 hours post-venipuncture. All specimens were stored at 22-28°C (RT) for the duration of the study.
 - S/N was calculated based on MFI of the normal T-cell and B-cell populations.
- Prepared Sample Stability
 - Prepared samples from the above Specimen Stability time points were stored at 4-8°C for 24 hours, brought to room temperature and rerun with the existing cytometer settings.
 - S/N was calculated based on MFI of the normal T-cell and B-cell populations.
- Representative Assay Results on Normal and Abnormal Specimens.
 - One normal donor and four B-cell CLL donor specimens were analyzed using the optimized assay.
 - ZAP-70 expression on the B cell, T cell and CLL populations were evaluated.

Results

4-Color, 5-Antibody ZAP-70 Assay Standardization and Analysis

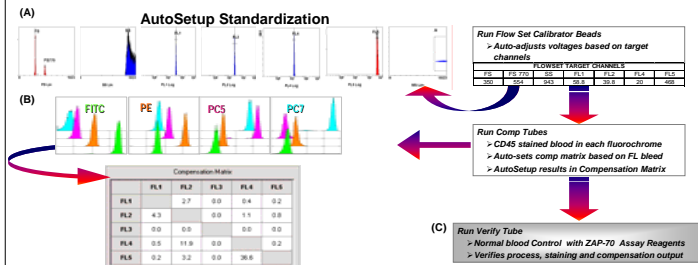


Figure 1: Instrument Set-Up. Target channels are set using Flow-Set calibrator beads (A). CD45 conjugates are then run for each fluorochrome to establish the compensation matrix (B). Finally, a normal blood specimen stained with complete assay reagents is run to verify the process, staining, and compensation matrix (C).

ZAP-70 Sample Preparation Comparisons

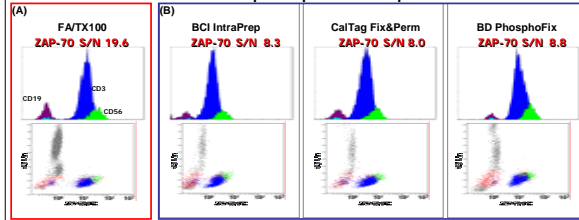


Figure 3: Comparison of the optimized FATX100 method (A) to three commercially available fix and perm kits (B) using the SBZAP-70-PE conjugate. The overlay plots of ZAP-70 signal within each gated lymphocyte subset (top panels) show negative expression in B cells, positive expression in T cells and highest expression in NK cells. The S/N for ZAP-70 expression is shown for each method using the MFI ratio for negative B cell to positive T cell populations.

ZAP-70 Method and Assay Performance

Analysis/Gating Approach (Normal Sample)

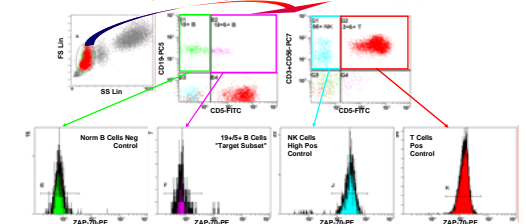


Figure 2: T, B, and NK cell lymphocytes are identified by the surface marker combinations in the two parameter histograms (top panel). ZAP-70 single parameter histograms (bottom panel) gated on the lymphocyte subsets give the Mean Fluorescence Intensity (MFI) for each subset and are used to calculate the MFI ratios of T/normal B and T/NK. These ratios are used as internal reference standards for evaluation of the ZAP-70 expression in the abnormal B cell (CD19+CD5+) population.

Stability of ZAP-70 Protein Expression in Normal Whole Blood

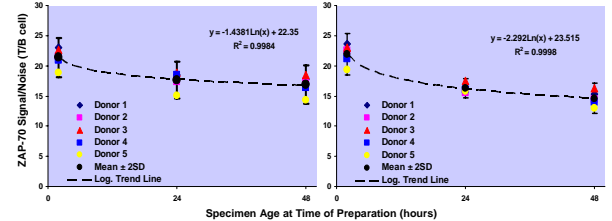


Figure 4: ZAP-70 protein stability in blood specimens through 48 hours post-venipuncture, analyzed immediately (Left Panel). A decrease of 17% (S/N) is observed within the first 24 hours, with an additional loss of 4% observed at 48 hours in unfixed specimens. ZAP-70 protein expression decreases logarithmically over time ($R^2=0.9984$). Fixed samples held at 4-8°C for 24 hours before analysis, using specimen prepared at 2, 24 and 48 hours (Right Panel) showed an additional loss of S/N ($R^2=0.998$).

ZAP-70 Assay Using CLL Samples

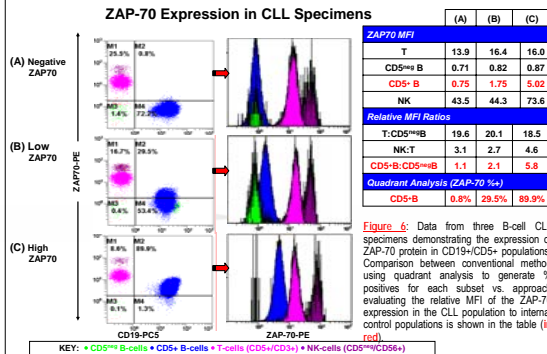


Figure 6: Data from three B-cell CLL specimens demonstrating the expression of ZAP-70 protein in CD19⁺CD5⁺ populations. Comparison between conventional method using quadrant analysis to generate % positives for each subset vs. approach evaluating the relative MFI of the ZAP-70 expression in the CLL population to internal control populations is shown in the table (in red).

Intra-Laboratory Results: ZAP-70 Expression in CLL Specimens

Sample	MFI Ratios		
	CLL/B-cells	CLL/T-cells	Z-Index ^a
Site A			
1	5.3	0.29	25.0
2	1.1	0.04	3.0
3	4.8	0.30	26.0
4	2.7	0.23	15.7
Site B			
1	1.1	0.05	0.3
2	2.1	0.11	6.0
3	5.8	0.31	27.0
Site C			
1	2.8	0.18	12.3
2	1.1	0.07	0.9
3	2.9	0.24	16.9
4	4.8	0.04	0
5	2.4	0.16	9.9
6	1.8	0.06	2.8
7	4.1	0.16	12.5
8	2.0	0.13	7.3
9	0.7	0.04	0
Site D			
1	1.2	0.08	11.8
2	7.1	0.48	44.5

Figure 7: (Left) Intra-laboratory variability of optimized whole blood assay for CLL samples. (Right) Inter-laboratory variability of CLL samples prepared and analyzed in four different laboratories, showing similar relative positions (and MFI values) for internal ZAP-70 negative and positive control populations. Table (above) summarizes the results of the analysis of eighteen different CLL samples analyzed in four different laboratories. Results in table are presented using three different techniques to index ZAP-70 levels in CLL population.

Conclusions

- The constitutive expression of ZAP-70 in T, NK and B cell populations (defined by the 4 surface markers) provides a method to measure ZAP-70 expression in the CLL population as a relative MFI ratio. This approach, including instrument standardization, enables equivalent results across laboratories and multiple instrument platforms.
- The optimized Formaldehyde/Triton X-100 method for whole blood fixation and permeabilization provides an approximate 2-fold increase in S/N compared to commercially available preparation reagents.
- ZAP-70 protein expression in normal T lymphocytes decreases logarithmically over time, with a mean loss of 17% in expression occurring within the first 24 hours. Additional loss occurs after sample processing.
- Preliminary results of the analysis of B-cell CLL specimens shows the robustness of using normal control ratios when comparing specimens and demonstrates varying levels of ZAP-70 expression for different CLL specimens.

Acknowledgements

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