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

ZAP-70 protein plays a critical role in the initiation of T-cell signaling through the TCR, 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 IgV_H 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 18-24 (normal blood T-cells to B-cells) compared to previously reported flow cytometric assays (S/N of 3 to 8). Based on these data, we have developed a 5 antibody/4 color whole blood assay (CD5-FITC, ZAP-70-PE, CD19-PE-Cy5, 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-dye)*
- SBZAP-PE –Beckman Coulter, Inc. Custom Design Services (CDS) Conjugate
- 2F3.2-FITC – Upstate Cell Signaling Solutions, PN 16-210
- 136F12-Alexa Fluor 647 – Beckman Coulter, Inc. PN A24071
- 1E7.2-PE – Caltag Laboratories, PN MHZAP7004
- 29-FITC – BD Transduction Laboratories, PN 612588

Surface Marker Antibodies - Custom Reagents by CDS, Beckman Coulter, Inc.
- CD5(BL1a)-FITC
- CD3(UCHT1)-PC7
- CD19(J4.119)-PC5
- CD56(NKH1)-PC7

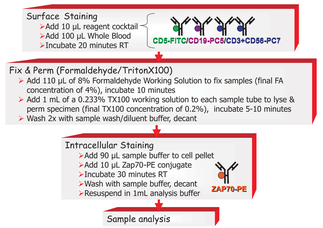
Sample preparation Reagents – Optimized Fix/Perm Method (FA/TX100 Method)
- 10% Formaldehyde (MeOH free, EM Grade), Polysciences, Inc. PN 04018
  - Dilute to 8% in PBS for assay working solution (8mL/10mL final volume)
- 10% Triton X-100 Ampules, Pierce PN 28314
  - Dilute to 0.233% in PBS for assay working solution (233uL/10mL final volume)
- Wash & Diluent Buffer: PBS, 0.1mM EDTA, 2% BSA, 0.1% NaN_3, pH 7.2
- Analysis Buffer: PBS, 0.1% Paraformaldehyde (PFA), 0.1% NaN_3, pH 7.2

Commercial Fix/Perm Reagents
- IntraPrep™ Permeabilization Reagent – PN A07802, Beckman Coulter, Inc.
- Fix & Perm™ Cell Permeabilization Reagents – PN GAS-003, Caltag Labs
- BD PhosFlow™ Reagents: Lyse/Fix PN558049 and Perm Buffer II PN558052

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 6605359, Beckman Coulter, Inc.
- Compensation Reagents – Beckman Coulter, Inc.
  - QuickCOMP 2 Kit (CD45-FITC/CD45-PE) – PN 177018
  - CD45-PC5 – PN IM2653
  - CD45-PC7 – PN Im3548

Methods

Optimized FA/TX100 Assay Method Flow Chart *(patent pending)*



ZAP-70 Antibody and Method Comparisons
- Antibody Comparisons
  - Five different ZAP70 antibody clones were evaluated to determine the comparative affinity and specificity of clone and conjugate.
  - Each antibody was stained with a fix & perm kit using a combination of formaldehyde and saponin, or the optimized FA/TX100 methodology.
  - Performance was assessed by determining the S/N of ZAP-70 detection in T cells.
- Sample Preparation Method Comparisons: The best ZAP-70 conjugate combined with the 5 surface marker antibody cocktail was evaluated for ZAP-70 expression on a normal donor comparing the optimized FA/TX100 method to three commercially available fix & perm reagent kits.

4-Color, 5 antibody ZAP-70 Assay Performance Studies
- Specimen Sample 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 22-28ºC (RT) for the duration of the study.
- Assay Results on Normal and Abnormal Specimens: Normal and B cell CLL donor specimens were analyzed using the optimized assay in three separate laboratories. ZAP-70 expression on the B cell and T cell populations were evaluated for assay reproducibility and performance.

Results

ZAP-70 Antibody and Method Comparisons

ZAP-70 Clone/Conjugate Comparisons

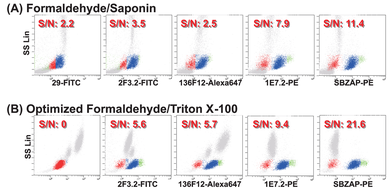


Figure 1: Comparative performance of five directly conjugated monoclonal antibodies to ZAP-70 in normal T lymphocytes. Both FA/Saponin (A) and the optimized FA/TX100 (B) fix & perm methods were evaluated to ensure that a particular epitope was not adversely affected by one method alone. All clones tested (except for clone 29-FITC) demonstrated an approximate 2-fold increase in ZAP-70 S/N with the optimized methodology. The SBZAP-PE clone showed highest S/N using either methodology.

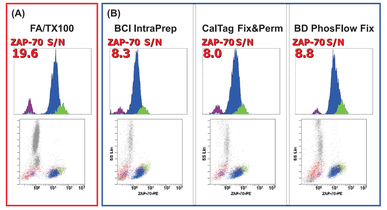


Figure 2: Comparison of the optimized FA/TX100 method (A) to three commercially available fix and perm kits (B) using the SBZAP-PE conjugate. The overlay plots of ZAP-70 signal within each gated lymphocyte subset (top panels) show negative expression in B cells, positive expression in T cells and highest 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.

Stability of ZAP-70 Protein Expression in Normal Whole Blood Specimens

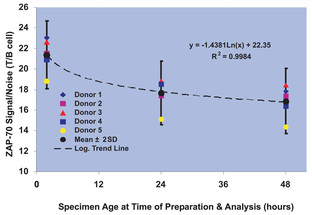


Figure 3: Stability of ZAP-70 protein in blood specimens stored at RT through 48 hours post-venipuncture. A mean decrease of 17% is observed within the first 24 hours, with an additional small loss (mean 4%) at 48 hours. The stability of the ZAP-70 protein expression correlates as a logarithmic function over time (R^2=0.9984 for the mean values) with an initial rapid rate of decrease that appears to level out after 24 hours.

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

Analysis Approach

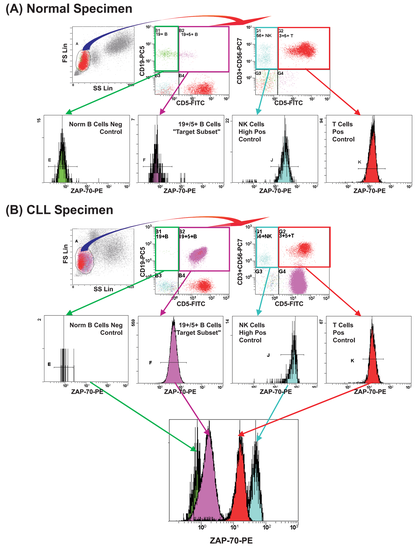
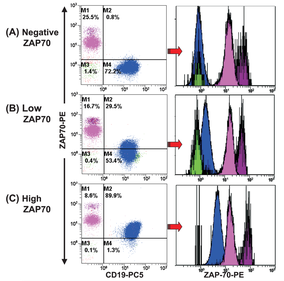


Figure 4 (A) & (B): T, B, and NK cell lymphocytes are identified by the surface marker combinations in the two parameter histograms (top panel). ZAP-70 single parameter histograms (middle panel) gated on the lymphocyte subsets give the Mean Fluorescence Intensity (MFI) for each subset and are used to 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. Overlay plot (bottom panel) demonstrates relative ZAP-70 expression in CLL population (CD19+/5+) compared to internal negative (CD19+/5- B-cells) and positive (CD3+ T-cells or CD56+ NK-cells) cell populations. In this case, the CLL demonstrates elevated ZAP-70 content compared to internal normal B-cell population (ZAP-70 negative).

ZAP-70 Expression Patterns in CLL Specimens



KEY: • CD5^{neg} B-cells • CD5+ B-cells • T-cells (CD5+/CD3+) • NK-cells (CD5^{neg}/CD56+)

	(A)	(B)	(C)
ZAP70 MFI			
T	13.9	16.4	16.0
CD5^{neg} B	0.71	0.82	0.87
CD5^{+} B	0.75	1.75	5.02
NK	43.5	44.3	73.6
Relative MFI Ratios			
T:CD5^{neg}B	19.6	20.1	18.5
NK:T	3.1	2.7	4.6
CD5^{+}B:CD5^{neg}B	1.1	2.1	5.8
Quadrant Analysis (ZAP-70 %+)			
CD5^{+}B	0.8%	29.5%	89.9%

Figure 5: 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. evaluation of MFI of ZAP70 expression in the CLL population relative to internal control populations is shown in the table (**in red**).

How reproducible is the assay within one laboratory?

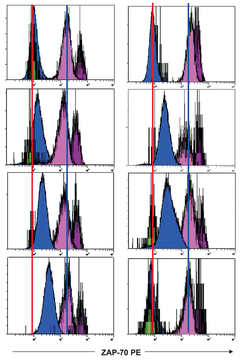


Figure 6: Intra-lab reproducibility; eight different B-cell CLL specimens were processed and run on three different days in one laboratory.

How reproducible is the assay between laboratories?

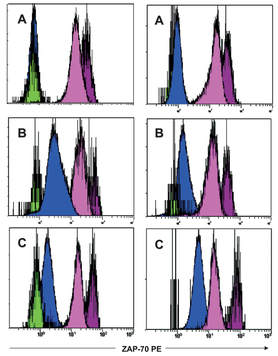


Figure 7: Inter-lab reproducibility; six different B-cell CLL specimens were processed and run in three different laboratories (A, B, C).

Conclusions

- **The performance characteristics of different antibody clones/conjugates demonstrate variability in the detection of ZAP protein expression in B, T, and NK cells.**
- **The optimized Formaldehyde/Triton X-100 method for whole blood fixation and permeabilization provides an approximate 2-fold increase in S/N for all conjugates tested except for clone 29.**
- **ZAP-70 protein expression in normal T lymphocytes degrades logarithmically over time, with a mean decrease of 17% in expression occurring within the first 24 hours.**
- **The constitutive levels of expression of ZAP-70 in T, NK and B cell populations (defined by the 4 surface markers) provides a method to quantify ZAP-70 expression in the CLL population as a relative MFI ratio. This approach allows standardization across laboratories and multiple instrument platforms.**

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