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Methodological Comparison of Two Anti-ZAP-70 Antibodies.

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Abstract

Background—ZAP-70 expression is a stage independent prognostic marker in CLL. However, inter-laboratory variation is large and there is neither a consensus nor regulatory approved methodology.

Methods—Two anti-ZAP70 clones (1E7.2 and SBZAP) were compared in 45 untreated CLL patients. Nine different methods for ZAP-70 expression analysis were evaluated: M1, isotype control to determine negative; M2, internal residual T-cell to determine positive; M3, normal donor (ND) T-cell to determine positive; M4, internal T-cell/clone ratio; M5, ND residual T-cell/clone ratio; M6, clone/normal remaining B-cell ratio; M7, clone/ND B- cell ratio; M8, CLL- Z score; M9, modified CLL-Z score. A scoring system was designed integrating both 1E7.2 and SBZAP clones to assign ZAP-70 expression.

Results—The correlation coefficients for the four selected highest statistically significant methods were as follows (M1=0.71, M3=0.72, M7= 0.67, and M9=0.64). These four methods were used to generate a combined score. The two reagents showed agreement using the designed scoring system for 37/45 samples (82%) and 8/45 (18%) showed equivocal result with one of the two clones. Seven of the eight equivocal samples were resolved using the scoring system.

Conclusions—Four of the nine methods of analysis were compared for each reagent. The use of two independent ZAP-70 reagents increases analytical certitude and the scoring method aids in the resolution of equivocal results. The combined use of two reagents, four methods of analysis and a scoring method allowed for assignment of ZAP-70 expression in 44/45 samples (98%) tested and improved performance of this important prognostic assay.

Keywords

chronic lymphocytic leukemia; ZAP-70 score; Flow cytometry; 1E7.2/AF488; SBZAP/PE

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common hematolymphoid neoplasm of adults in Western countries (1). CLL has been adequately described by the World Health Organization classification (WHO) and the NCI working guidelines (2, 3) and a precursor state has been defined (4). One of the most intriguing features of the disease is its clinical heterogeneity with some patients progressing rapidly to early treatment, whereas others exhibit indolent disease never requiring treatment. To that end, several prognostic biomarkers have been developed to allow identification of low, intermediate and high-risk patients for the appropriate stratification for the care and treatment of the CLL patient (5, 6).

The mutational status of a patient's immunoglobulin heavy chain variable region (IGHV) gene has recently been identified as a robust indicator of disease outcome. Patients with mutated phenotype (M-IGHV) have a more indolent clinical course and a longer survival than those with the un-mutated phenotype (U-IGHV) (7, 8). Zeta chain-associated protein-70 (ZAP-70) expression in CLL correlates with U-IGHV and has been proposed as a surrogate biomarker for IGHV mutational status (9, 10). In addition, ZAP-70 has emerged as a powerful independent prognostic factor in CLL (11).

Among the available techniques for ZAP-70 detection, flow cytometry is optimal as it allows the simultaneous detection of ZAP-70 protein expression levels in CLL cells and residual normal lymphocyte subsets. However, the flow cytometric analysis of ZAP-70 suffers from difficulties in standardization and interpretation. Several factors contribute to reported inter-laboratory variations. These factors include the anti-ZAP-70 antibody clone and its fluorochrome conjugate, the fixation and permeabilization procedure, and gating strategies. Although ZAP-70 expression has been standardized in several laboratories, a single consensus method has not yet been validated. And no ZAP-70 assay has either CE marking and/or FDA clearance. This comparative methodological study was designed to optimize the staining for two different anti-ZAP-70 clones using the same fixation and permeabilization method for each reagent. Two clones, three fluorochromes, various methods of fixation and permeabilization, gating strategies and methods of reporting results were compared in order to define the variables involved in the ZAP-70 assay. Three questions were raised: 1) what is the best method for reporting ZAP-70 expression? 2) Does using more than one method of ZAP-70 reporting lead to more reliable results? 3) And does the use of two reagents result in a better assay for determining ZAP-70 expression?

Material and Method

Sample Specimens from Normal Donor and CLL patients

A preliminary study on fresh whole blood samples from 10 normal donors was conducted to determine the impact of different whole blood fixation and permeabilization techniques on the intensity of ZAP-70 staining. This study used heparin treated peripheral blood samples obtained from 45 newly diagnosed, untreated CLL patients (21 female and 24 males with 1.14:1 male: female ratio; median age 62.4 with range of 47–81. These patients were enrolled on NHLBI IRS approved clinical study registered with clinicaltrials.gov under identifier (NCT00923507), and under NCI study 97-C-0178 (clinicaltrial.gov, identifier: NCT00019370). The diagnosis of CLL was made on the basis of clinical examination, as well as morphological and immunological criteria according to established criteria of Hallek et al. (3). Anonymous normal donor blood samples were obtained from the NIH Department of Transfusion Medicine and used as controls.

Antibody Staining Panels for Normal Donors and Patient Samples

Table 1 is a summary of reagents used in this study. The ZAP-70 clone /conjugate were selected based primarily on the extensive experience of one laboratory with the 1E7.2 clone both with FITC and AF488 (12, 13). The second reagent was selected on the basis of that SBZAP clone had the highest known signal to noise ratio (14, 15). After an initial comparison of 1E7.2 AF 488 and 1E7.2 FITC, we selected 1E7.2 AF 488 as a reference reagent for further study (See Fig. 1).

Staining for Cell Surface Antigens

Surface staining was carried out using 100 µl of washed (Phosphate Buffer Saline × 2) whole blood, which was added to pre-wetted tubes containing the designated antibody (Table 1). The ND control tube, isotype control tube and the CLL tube were stained separately for each sample. The cells were stained for 30 minute at room temperature in the dark and then fixed with 4% formaldehyde for 10 minutes.

Intracellular ZAP-70 Staining

For each sample, intracellular ZAP-70 protein staining was performed. Three methods of permeabilization were evaluated: Triton X-100 (14), a saponin based method (13), and the commercial available Fix & Perm (16). After permeabilization by each method, the cells were stained with the manufacturer's recommended concentration of 1E7.2 AF 488, 1E7.2 FITC, and SBZAP-PE or corresponding isotype control for 30 min at room temperature.

Flow Cytometric Analysis

All analyses were performed on a FACSCanto II (Becton Dickinson, CA, USA) flow cytometer. FACSDiva software was used for acquisition. Cytometry setup and tracking beads (CST, BD) were used to initialize PMT settings. Unstained control cells as well as a single stained tubes for AF488, PE, PerCPCy5.5, PECy7, APC-Cy7, and eFluor 450 were prepared and used to set up flow cytometric compensation. In some experiments, rat anti-mouse kappa light chain Comp Beads (BD) were used to set the compensation and were stained according to the manufacturer's instruction. The number of events collected in these experiments varied from 500, 000 to 1 million events. FlowJo software (Tree Star, Ashland, OR) was used for data analysis and display.

Gating Strategies for Definition of ZAP-70 Expression Status

Nine different methods were used for ZAP-70 expression analysis. M1 is the isotype method of analysis ZAP-70 expression (17, 18) (Fig. 2, panel D). M2 this method utilizes the residual internal T-cell population to determine the "percent positive" (19) (Fig. 2, panel E). M3 this method depends on ND T-cells as a reference point for the determination of "percent positive" as described by Rassenti et al. (9) (Fig. 2, panel F). Different ratios using mean fluorescent intensity (MFI) were also determined. M4 is the MFI of ZAP-70 staining by patient T-cell/ CLL cell ratio as originally defined by Bakke et al., and Rossi et al. (20, 21). M5 is the MFI of ZAP-70 staining by ND T-cell/ CLL cell ratio. M6 is the MFI CLL cell/normal residual B-cell ratio (20). This value could not be calculated in some samples due to insufficient numbers of normal remaining B-cells. M7 is the MFI CLL cell/ND B-cell ratio as described by the GEIL group (22). M8 is the CLL- Z index = $(\text{CLL Clone MFI} - \text{B-cell MFI}) / (\text{T-cell MFI} - \text{B-cell MFI}) \times 100$ as originally reported by Shankey et al. (14). Also this value could not be calculated in some samples due to insufficient numbers of normal remaining B-cells in some CLL samples. M9 is a modified CLL-Z score using ND B-cell and ND T-cell $(\text{CLL MFI} - \text{ND B-cell MFI}) / (\text{ND T-cell MFI} - \text{ND B-cell MFI}) \times 100$. We used a cut-off 11% for the M1 approach; 20% for M2 and M3; a value of < 3.0 was considered positive for M4 and M5; a value of > 1.4 was considered positive for M6 and

M7; and a value of 0.3, 6.0, and 27.0 for negative, low positive, and high positive ZAP-70 expression as suggested by Shankey et al., for the M8 determination. Also we used an arbitrary cut-off of 20.0 to discriminate between positive and negative ZAP-70 cases for M9 method (See Figure 3 for the nine displayed scattered plots which were used to estimate the cut-off used in this study). Because ZAP-70 expression levels were very close to the cutoffs for some patients, we also defined a “borderline” value that were within 2% of each individual cut-off, for example, between 19.6 and 20.4 for a cutoff of 20.

Statistical Analysis and Scoring System for CLL ZAP-70 Expression

The Spearman rank correlation method with exact p value calculation was used to compare the two clones 1E7.2 AF 488 and SBZAP PE. This statistical method was also used to compare all nine methods of detecting ZAP-70 expression. A scoring system was designed from 0 to 4 to reduce ZAP-70 discordant results that were observed using one antibody clone type with a single method of analysis. Our proposed scoring system selected four out of the nine methods (M1, M3, M7, and M9) based on the preference of the percentile positive (9, 17, 18, 19) cell vs. ratio metric method (14, 20, 21, 22). We selected two percentile methods (M1, and M3) out of three that showed the highest correlation coefficient, as well as two ratio metric methods (M7, and M9) that also showed the highest correlation coefficient between the two antibody clones with a common reference. For a given specimen, each individual method of analysis was given a score of 1 point out of 4 if it was positive, and 0 point if it was negative. When summing up the results of all four methods the following possibilities could occur 0, 1, 2, 3, and 4 depending on the number of methods positive for ZAP-70 expression with each antibody type. We considered the cases that show no or one positive method (score 0 or 1) as a negative (N), the cases that showed positivity by two method (score 2) as equivocal (E), and those with three or four positive method (score 3 and 4) as a positive (P). This score was calculated for each clone separately and then integrating both clones together with the following possibilities: N/N, P/P, N/E, E/N, P/E, E/P, E/E for the 1E7.2/ SBZAP clones. See Table 2 for the summary of ZAP-70 scoring.

Results

Fixation and Permeabilization in Normal Donor ZAP-70 Expression

A preliminary study was conducted on 10 normal donors using three methods of permeabilization: Triton 100-X permeabilization (14), the optimized saponin method (13), and the commercially available Fix & Perm method (16). The sequence of analytic steps for identification of CD20+/CD5– B-cell and CD20–/CD5+ T-cell is presented in [Fig. 1, panel A and B]. The results of the ZAP-70 staining using these antibodies with the three methods of permeabilization are shown in [Fig. 1 panels C, D, and E]. The highest (brightest) levels of ZAP-70 fluorescence were achieved when the cells were permeabilized using the non commercial saponin based method with 1E7.2 AF488, 1E7.2 FITC, as well as with SBZAP-PE. There was no apparent difference in expression between 1E7.2 FITC and 1E7.2 AF488 regardless of the permeabilization method used, so the latter was chosen. These findings led us to select the saponin based method together with 1E7.2AF488 and SBZAP/PE for subsequent analysis of ZAP-70 expression in CLL cells.

Correlated CLL ZAP-70 Expression

The gating strategies for different methods of analysis of ZAP-70 in CLL are shown in (Fig. 2). ZAP-70 expression analysis for both 1E7.2 AF488 and SBZAP PE was performed with nine different methods (M1–M9) (See Table 1s, and 2s Supplementary data). Comparison of the two anti ZAP-70 clones across the nine methods of analysis was shown in (Fig. 3). The correlation coefficient of these methods were as follow: M1, 0.71; M2, 0.67; M3, 0.72; M4,

0.53; M5, 0.58; M6, 0.11; M7, 0.67; M8, 0.20; M9, 0.64. Of these nine methods of analysis, four were selected for detailed analysis (M1, M3, M7, and M9), and were used for further ZAP-70 scoring (See Statistical Analysis and Scoring System in the Material and Methods Section).

CLL ZAP-70 Expression

For the M1 analysis both clones showed a nearly identical pattern 22–23% positive, 73 % negative and 2–4% borderline values (within 2% of the cutoff). The M3 method in which the ZAP-70 evaluation is driven by the use of ND T-cell population to adjust the cursor for positivity, 21 (47%) and 18 (40%) cases were defined positive for AF488 and SBZAP respectively; 23 (51%) and 25 (56%) cases were defined as negative for AF488 and SBZAP respectively; and 1 (2%) and 2 (4%) cases show borderline results for AF488 and SBZAP respectively.

In the M7 method, ZAP-70 expression is evaluated by taking into account the MFI of the patients clone over the ND B-cell MFI with the optimal cut-off value of 1.4 as previously reported by GEIL group. This method revealed that 19 (42%) and 16 (36%) cases were defined positive for AF488 and SBZAP respectively; 26 (58%) and 28 (62%) cases were defined as negative for AF488 and SBZAP respectively; and 0 (0%) and 1 (2%) case show borderline results for AF488 and SBZAP respectively.

The M9 modified CLL Z-index is based on using ND T- and B-cells as an extension of CLL Z- score of Shankey et al. This method showed the greatest discrepancy between the two antibody clones with 11 positive for AF488 and 6 positive for SBZAP. The summary of ZAP-70 expression levels obtained by the four selected statistically significant methods was shown in (Table 3).

The ten borderline values (in the list above) are observed in 8 of the patients, when these 8 are dropped from the scoring system, the proportion of patients with equivocal results drops from 8/45 (18%) to 4/37 (11%). The proportions of patients with equivocal results (4/8 among patients with borderline values vs. 4/37 among patients without borderline values) are significantly different ($p=0.024$ by Fisher's exact test).

In summary, both clones showed a nearly identical pattern: 10 or 11 samples were positive with the M1 isotype analysis; this rose to 21 or 18 positive samples with the M3 (ND T cells for percent positive cells) for both clones and remained at 19 or 16 positive samples for M7 (ND B cells as a reference; and decreased to 11 or 6 positive samples for M9, the modified Z score. This latter analysis shows the greatest discrepancy between the two clones (Table 3).

CLL ZAP-70 Scoring System

ZAP-70 expression analysis was independently scored for each ZAP-70 reagent using the four methods of analysis M1, M3, M7, and M9 (Table 2). 31/45 (69%), 10/45 (22%), and 4/45 (9%) showed negative, positive, and equivocal score results respectively with the 1E7.2 AF 488 clone. On the other hand 31/45 (69%), 9/45 (20%), and 5/45 (11%) showed negative, positive, and equivocal score results respectively with SBZAP PE clone. Three groups were defined when both clones were integrated together (Table 4): Negative group: N/N 29/45 (64%) showed negative results with both 1E7.2/SBZAP clones with scores 0 or 1 for each clone; Equivocal group: 8/45 (18%): showed equivocal results with a score of 2 for one or both 1E7.2/SBZAP clones (P/E, E/P, E/N, N/E, E/E); Positive group: P/P 8/45 (18%) showed positive results with both 1E7.2/SBZAP clones with scores of 3 or 4 (See Table 4).

Equivocal results were seen in 8 out of 45 (18%) cases, where one clone showed equivocal result (score of 2) while the other was either negative (score of 0 or 1) or positive (score of 3

or 4): E/N (2 cases), N/E (2 cases), E/P (1 case), P/E (2 cases), and E/E (1 case). Further assessment of these eight equivocal samples integrating both clone together showed that 4 cases were considered negative with the combined ZAP-70 score (2 E/N cases, and 2 N/E cases). One of these N/E cases showed a combined score of two positive methods (M3 (23.3%), and M7 (2.14)) with the 1E7.2 clone and one positive method M3 (20.1% border line) for the SBZAP clone. The other E/N case showed a combined score of two positive methods (M7 (2.09), and M9 (20.3 border line)) with the 1E7.2 clone and one positive method M7 (1.4 borderline) for the SBZAP clone.

The reverse situation of N/E score was also seen in two cases. One N/E case showed M7 positivity (2.44) with the 1E7.2 and M7 (2.4), and M9 (20.3 border line) positivity with the SBZAP clone. The other N/E case showed M3 positivity (95.5%) with the 1E7.2 clone and M1 (11.1% borderline), and M3 (75.5%) positivity with the SBZAP clone.

On the other hand three cases were evaluated as positive with the combined ZAP-70 score. One case shows (E/P) (two positive methods M7 (2.39), and M9 (30.2) with the 1E7.2 clone, and three positive methods M3 (58.3%), M7 (2.02), and M9 (36.2) with the SBZAP clone. The other two cases showed a P/E score, one of the two cases showed M1 (12.7), M3 (46.6%), M7 (2.52), and M9 (26.7) positivity with the 1E7.2 clone and M1 (21.4), and M3 (34.4) positivity with the SBZAP clone. The second P/E case showed M1 (28.2), M3 (64.2), M7 (1.78), and M9 (37.6) positivity using the 1E7.2 clones, while the SBZAP clone showed M1 (16.4%), and M3 (45.6%) positivity only. Out of these eight cases, only one case had an equivocal combined (E/E) score with two positive methods M1 (18%), M3 (25.9%) with the 1E7.2 clone vs. M1 (12.8%), and M3 (21.7%) for the SBZAP clone.

In summary, in the four cases that were considered negative with the combined ZAP-70 score (2 E/N cases, and 2 N/E cases), both clones had the same positive method (M3 for two samples and M7 for two samples), but one clone had a second positive method, which was a borderline result in two cases. Of the three cases evaluated as positive with the combined ZAP-70 score, two were positive for all four methods with the 1E7.2 clone, but only positive for M1 and M3 with the SBZAP clone, while the third was positive for M7 and M9 by both clones and also for M3 with the SBZAP clone.

Discussion

In this study, 45 untreated CLL blood samples were analyzed for ZAP-70 expression. Two different monoclonal antibodies and two different fluorochromes were examined using an identical fixation and permeabilization method. Fresh whole blood samples were stained within 1–2 hours of venipuncture and analyzed immediately upon completion of the staining process. Nine methods of reporting ZAP-70 expression were compared. Four methods were selected namely M1, M3, M7, and M9. Both antibody clones showed a nearly identical patterns of concurrence when using the isotype negative control or normal donor T or B cells as external positive controls. ZAP-70 expression for each reagent was scored independently in this series of 45 samples. The results obtained with both reagents were then integrated together to confirm ZAP-70 expression and re-evaluate those cases showing equivocal results with one reagent only. Concordance was achieved using this scoring system in 37 of 45 (82%) samples (29 cases were negative showing N/N results, and 8 were positive showing P/P results).

Equivocal results were seen in 8 cases, where one clone showed equivocal result while the other was either negative or positive: E/N, N/E, E/P, P/E, and E/E. The equivocal results from the scoring system might be due to borderline values for each method of analysis. Further evaluation of these eight equivocal samples integrating both reagents together

revealed that seven out of the eight equivocal results (88%) could be scored as positive (3/7) or negative (4/7). One of the 8 cases remained equivocal with both antibody clones and testing would need to be repeated. If we look at these equivocal eight cases more closely, we find that one of these cases has two sub-clonal populations within the CLL clone (data not shown). When ZAP-70 expression was evaluated over the whole CLL clone, it scored as equivocal with SBZAP reagent and positive with 1E7.2. On the other hand if each clone was assessed separately for ZAP-70 we found that one sub clone was positive for ZAP-70 and the other was negative by both 1E7.2 and SBZAP clones.

Overall, these findings confirm the earlier report of Orchard et al. (17), regarding the use of isotopic control as well as the later detailed report by Zucchetto et al. (18). Although the M2 or internal T cell control of Crespo et al. (19) was not significant in our hands, the practice of Rassenti et al. (13), using ND T cells as a positive reference worked well. Also our results confirmed the recent report of the GEIL group on the advantage of adding ND B-cells, which is intuitively attractive (22). It is not surprising that the use of external donor T and B-cells improves the original Z-score of Shankey et al. (14). Also it is not possible to do the calculations for M6 and M8 in those samples that have no normal remaining B-cell and because of this; these two methods don't achieved statistical significance. This is a clear-cut argument to run a normal donor control with the CLL sample.

In summary, the M9 method may be too restricted in that it unduly reduces the number of positive samples compared to the M3 and M7 methods. Indeed, it would seem that the use of the isotype control and modified Z-score methods underestimate positive samples while the use of T and B cells overestimates the number of positive samples. And it is surprising that both methods of using T cell to clone ratios (internal and ND) did not reach statistical significance. This would suggest the need for micro bead standardization. Finally it is envisioned that a single combined tube containing both external ND and patient sample would reduce some of the complexity and variation of the ZAP-70 analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Gating strategy for T, NK cells in Normal Donor

A. left hand panel FSC-A vs. SSC-A lymphocyte gate in red. B. CD20 vs. CD5. B-cell, T-cell, and NK cell gates shown. C. Comparison of ZAP-70 antibody clones: Blue histograms are ZAP-70 negative normal B-cells (analysis gate through lymphocyte gate in A and B - Cell gate in B). Red histograms are ZAP-70 positive normal T-cells (analysis gate through lymphocyte gate in A and T- Cell gate in B). Saponin based method for permeabilization used. D. Comparison of commercially available Fix & Perm (red histograms) and Triton X-100 (blue histograms) permeabilization methods gated on ND T-cell in B. Anti- ZAP70 antibody used is labeled. E. Comparison of saponin (red histograms) and Triton X-100 (blue histograms) permeabilization methods gated on ND T-cell in B. Anti- ZAP70 antibody used is labeled.

histograms) permeabilization methods gated on ND T-cell in B. Anti- ZAP70 antibody used are labeled.

Figure 2. Gating Strategies for CLL Sample Analysis

A. FSC-A vs. SSC-A for CLL whole blood sample. Lymphocyte gate is in red. B. CD3 on X-axis vs. SSC-A on Y-axis. Gate R1 contains CD3+ T-cell. Gate R2 contains CD3- cells, B and NK). C. CD20 vs. CD5 gated on R2. Clone-analysis gate containing CLL cells. NR B-cell- analysis gate containing normal remaining B-cells. NK- analysis gate containing normal remaining NK cells. D. Example of setting the cursor for percent positivity with 1E7.2 AF488 based on the isotype control. E: Setting the cursor Percent positive determination using internal residual T-cells (in red) as a control. CLL cells in blue. F. Setting the cursor for percent positive determination using normal donor T-cell (in red) as a control. CLL cells in blue. The insert in figure E & F are the single parameter histogram showing the patient CLL clone (blue) vs. the T-cell control (red). Identical process was used for SBZAP/PE gating strategy analysis.

Figure 3.

The ZAP-70 expression values for 45 CLL patients are displayed in nine scattered plot. These scattered compare the two anti-ZAP-70 clones across the methods (M1–M9) of assessing ZAP-70 expression. **M1** ZAP-70 positive cells by isotype references; **M2** ZAP-70 positive cells by patient T-cell references; **M3** ZAP-70 positive cells by ND T-cell references; **M4** patient T-cell/clone ratio; **M5**: ND T-cell/clone ratio; **M6**: clone/NR B-cell ratio **M7**: clone/ND B-cell ratio, **M8**: CLL Z index, **M9**: Modified CLL Z-index.

Table 1

Antibody Panel for Surface Labeling in Conjunction with Intracellular ZAP-70 Staining.

Tube #	AF488/FITC	PE	Per CpCy5.5	PE Cy7	APC Cy7	Pacific Blue
Tube#1 ND	ZAP-70 *	-----	CD19	CD5	CD3	CD20 eFluor 450
Tube#2 ND	-----	Z A P-70 **	CD19	CD5	CD3	CD20 eFluor 450
Tube#3 ISO	ISO AF488	-----	CD19	CD5	CD3	CD20 eFluor 450
Tube#4 ISO	-----	ISO PE	CD19	CD5	CD3	CD20 eFluor 450
Tube#5 CLL	ZAP-70 *	-----	CD19	CD5	CD3	CD20 eFluor 450
Tube#6 CLL	-----	ZAP-70 **	CD19	CD5	CD3	CD20 eFluor 450

Note: ISO: Isotype control, ND: Normal Donor sample, CLL: Chronic lymphocytic Leukemia patient sample, ZAP-70

* 1E7.2 clone; ZAP-70

** SBZAP clone.

AF488: Alexa fluor 488; PE: phycoerythrin; PerCpCy 5.5: peridin chlorophyll protein-cyanin 5.5; PE Cy7: PE- cyanin 7; APC-Cy7: allophycocyanin-Cy7; and CD20-eFluor450. The unstained control tubes and a single stained tube for each fluorochrome were not included in the table. **Reagents:** The 1E7.2 clone and its isotype IgG1 control were obtained from Invitrogen (1E7.2 AF488 Camarillo, California) and from e-Biosciences (1E7.2 FITC, San Diego, CA). The SBZAP clone conjugated with phycoerythrin (PE) and its isotype control IgG1-PE were obtained from Beckman Coulter, Inc. (SBPE, Fullerton CA). Permeabilization reagents were the commercially available Fix and Perm (Invitrogen, catalogue # GAS004, Camarillo, CA), saponin, and Triton X-100 were purchased from (Sigma, St. Louis, MO). CD20-eFluor 450 was obtained from eBiosciences (San Diego, CA). CD19-PerCpCy 5.5, CD5 PECy7, and CD3 APC-Cy7 were obtained from BD Biosciences (San Diego, USA).

Table 2

ZAP -70 scoring method using both 1E7.2 & SBZAP clone

1E7.2/AF488							SBZAP/PE						Combined	
M1*	M3*	M7*	M9*	score	Result		M1*	M3*	M7*	M9*	score	Result		1E7.2/SBZAP
11	20.8	1.07	2.3	1	N		9.4	17.3	0.59	0	0	N		N/N
5.0	12.3	1.22	3.8	0	N		0.01	9.4	1.07	3.06	0	N		N/N
0.093	20.4	0.52	0	1	N		4.13	19.8	0.49	0	0	N		N/N
7.9	12.4	1.43	7.5	1	N		2.6	5.3	1.22	6.07	0	N		N/N
0.01	12.9	1.15	3.2	0	N		0.37	18.3	1.28	8.76	0	N		N/N
1.2	14	1.67	7.6	1	N		2.7	11.9	2.07	1.25	1	N		N/N
12.7	46.6	2.52	26.7	4	P		21.4	34.4	0.98	0	2	E		P/E
21.4	31.8	2.58	37.0	4	P		19.3	22.0	3.42	47.0	4	P		P/P
20.1	35.2	2.1	65.3	4	P		17.4	29.8	2.07	30.52	4	P		P/P
5.0	23.6	2.14	19.4	2	E		0.29	20.1	1.06	3.8	1	N		E/N
19.6	39.4	2.55	67.3	4	P		21.3	37.8	2.7	41.8	4	P		P/P
19.4	39.7	2.48	59.8	4	P		36.7	30.3	1.48	15.3	3	P		P/P
8.7	15.6	1.51	11.73	1	N		5.12	12.2	1.47	5.01	1	N		N/N
2.0	6.9	1.26	5.9	0	N		0.82	9.3	1.28	3.03	0	N		N/N
1.54	16.5	2.39	30.2	2	E		10.2	58.3	2.02	36.2	3	P		E/P
10.9	21.2	3.8	83.22	3	P		17.4	31.7	3.87	39.47	4	P		P/P
18.2	42.5	2.14	33.6	4	P		12.9	22.1	1.83	11.4	3	P		P/P
17.9	65.1	1.9	26.7	4	P		15.2	48.5	2.1	33.3	4	P		P/P
18.7	25.9	1.12	4.3	2	E		12.8	21.7	1.0	0.26	2	E		E/E
3.3	6.7	2.44	16.0	1	N		5.3	1.19	2.4	20.3	2	E		N/E
0.05	15.9	2.18	19.3	1	N		1.1	12.77	1.22	3.4	0	N		N/N
0.005	0.11	2.09	20.3	2	E		0.21	0.92	1.4	5.4	1	N		E/N
5.47	18.7	1.64	20.0	1	N		9.43	13.3	1.89	17.6	1	N		N/N
1.25	18.9	0.91	0	0	N		10.2	13.5	1.05	1.5	0	N		N/N
0.035	9.1	0.99	0	0	N		4.46	5.3	1.26	4.09	0	N		N/N
4.9	10.9	1.25	3.4	0	N		8.47	5.8	1.14	1.98	0	N		N/N

1E7.2/AF488						SBZAP/PE				Combined	
M1*	M3*	M7*	M9*	score	Result	M1*	M3*	M7*	M9*	score	Result
0.22	26.9	1.23	4.3	1	N	4.38	10.6	1.26	3.48	0	N
12.3	61.2	2.63	35.8	4	P	20.8	39.3	1.98	15.3	3	P
3.11	9.3	1.22	4.8	0	N	0.75	2.9	1.56	8.73	1	N
4.74	14.9	1.10	3.46	0	N	7.17	39.1	1.13	5.8	1	N
5.33	20.7	0.97	0	1	N	7.65	29.7	0.91	0	1	N
7.1	17.2	1.12	4.05	0	N	8.7	36.6	1.26	7.6	1	N
3.77	18.9	1.14	4.64	0	N	6.7	10.4	1.13	3.88	0	N
9.4	20.7	0.91	0	1	N	7.43	11.7	1.63	15.4	1	N
2.9	8.4	1.03	0.67	0	N	3.4	8.8	1.01	0.36	0	N
4.83	14.7	0.65	0	0	N	5.62	10.9	1.52	7.3	1	N
2.22	21.3	0.55	0	1	N	2.62	10.5	0.91	0	0	N
28.2	64.2	1.78	37.6	4	P	16.4	45.6	1.16	5.7	2	E
6.48	23.0	0.56	0	1	N	1.8	22.9	1.07	3.5	1	N
5.1	11.8	1.24	15.6	0	N	1.15	15.6	1.3	11.4	0	N
1.73	21.1	0.46	0	1	N	3.9	0.005	0.64	0	0	N
5.7	95.5	0.93	0	1	N	11.1	57.5	1.10	4.2	2	E
8.4	11.7	1.10	3.26	0	N	4.9	10.9	1.02	0.97	0	N
1.99	17.5	0.71	0	0	N	7.43	19	0.97	0	0	N
10.3	29.6	1.26	9.08	1	N	9.8	32.4	1.17	4.9	1	N

M1* the percentage of positive using an isotype control,

M3* the percentage of positive using normal donor T-cell as references,

M7* the clone/ND B-cell ratio, and

M9* the modified Z-score of Shankey using both ND T&B-cell.

Score system points for each method: 0 if negative, 1 if positive. A scoring system was evaluated as a number of positive tests obtained by 1E7.2 and SBZAP separately. A score of 0 or 1 considered as negative (N), 2 considered as equivocal (E), 3 or 4 considered as positive (P) using that clone. Combined 1E7.2/SBZAP: the CLL score of both 1E7.2 and SBZAP were integrated together N/N negative with both 1E7.2 /SBZAP; P/P: positive with both 1E7.2 /SBZAP; P/E positive with 1E7.2 and equivocal with SBZAP; N/E negative with 1E7.2 and equivocal with 1E7.2 and positive with SBZAP; E/N equivocal with 1E7.2 and negative with SBZAP; E/E equivocal with both 1E7.2 and SBZAP (See Supplementary Tables 1s, and 2s).

Table 3
Summary of ZAP-70 Expression Levels Obtained by the Four selected Methods of Analysis.

	1E7.2 AF488				SBZAP/PE				Correlation Coefficient	
	N	P	B	N	P	B	N	P	B	
M1 [*]	33	10	2	33	11	1				0.71
M3 [*]	23	21	1	25	18	2				0.72
M7 [*]	26	19	0	28	16	1				0.67
M9 [*]	32	11	2	38	6	1				0.64

N: negative result, P: positive result, B: borderline result.

^{*} the four selected high correlation coefficient methods.

Table 4

CLL combined scoring system for ZAP-70 expression.

Group	1E7.2 clone score	SBZAP/PE clone score	1E7.2 score /SBZAP score	Total Number
Negative	0–1	0–1	N/N	29
Equivocal	2	1	E/N	2
	2	3	E/P	1
	1	2	N/E	2
	4	2	P/E	2
	2	2	E/E	1
Positive	3–4	3–4	P/P	8

Negative group (N/N) : scored negative with both clone and showed score of 0 or 1 with each one. Equivocal group: showed equivocal results with one of the two clone with a score of 2. Positive group: (P/P): scored positive with both clone and showed score of 3 or 4 with each one.