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Title: **Performing absolute neoplastic B-cell counts for peripheral blood samples in the context of a diagnosis of Monoclonal B-cell Lymphocytosis (MBL) vs Chronic B-cell Lymphoproliferative Disorders (CLL)**

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INTRODUCTION

Absolute neoplastic B-cell counts have gained importance in the distinction of monoclonal B-cell lymphocytosis (MBL) from chronic lymphocytic leukemia (CLL) and other chronic B-cell lymphoproliferative disorders as established by the new WHO Classification (Swerdlow WHO IARC 2017 Ref 12). Although the WHO classification establishes a threshold of greater than 5 x $10e^9$/L neoplastic B-cells to diagnose CLL, there are no guidelines or recommendations about the best laboratory method to accurately obtain this number. The goals of this module are to review the different approaches a flow cytometry laboratory can pursue to report absolute neoplastic B-cell counts and to discuss the variability that each approach may introduce to the final results.

HISTORY AND BACKGROUND

Historically, the criteria for diagnosing CLL included a blood cell absolute lymphocyte count of greater than 5 x $10e^9$/L coupled with the presence of clonal B-cells with a CLL phenotype by flow cytometry. These previous criteria were arbitrarily established from consensus input by clinical hematologists. In particular, the absolute lymphocyte count of 5 x $10e^9$/L was arbitrarily chosen as a value that reflected a presumably abnormal increased lymphocyte count independent of which hematology analyzer platform a laboratory may use. A patient without an increased absolute lymphocyte count would essentially not meet criteria for a diagnosis of CLL and would not need further studies. In patients with increased absolute lymphocyte count, flow cytometric studies established a qualitative assessment of the presence of B-cell clonality by surface light chain analysis and identification of a CLL phenotype. Flow cytometry did not play a significant role in the quantitation of disease for the diagnostic criteria.

Monoclonal B-cell Lymphocytosis (MBL) was established in the 2008 WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues (Swerdlow WHO IARC 2008 Ref 5) as researchers described the presence of small clonal B-cell proliferations with CLL-like immunophenotype in patients with CBC values within normal limits or, more specifically, those lacking lymphocytosis. These studies were population-based studies and only reported a relative percentage of B-cell clone size, without an absolute neoplastic B-cell count. Since these patients lacked lymphocytosis, they did not meet the criteria for a diagnosis of CLL. Of note, the term “monoclonal B-cell lymphocytosis” was selected to avoid the use of the term “leukemia” since these MBLs are considered benign findings in normal patients.

Concurrently, several researchers also studied and developed flow cytometric assays to identify and distinguish the presence of minimal amounts of neoplastic CLL B-cells separate from reactive polyclonal B-cells in the context of identifying minimal residual disease in treated patients. The performance of these assays was optimized to demonstrate the presence of a neoplastic phenotype vs. a normal B-cell phenotype. In the context of MRD testing, identification of an aberrant neoplastic B-cell phenotype by antigen expression was found to be a better approach compared with use of a surface light chain expression gating strategy. In the era of 5-color flow cytometry, these MRD assays often did not include CD45 in their design in order to add other markers that were more useful in the discrimination of neoplastic vs reactive B-cells. Although several studies have demonstrated this flow cytometry MRD approach to be sensitive and accurate particularly in identifying small populations, the more traditional approach using surface light chain analysis is still largely used particularly for initial diagnostic purposes. These MRD CLL assays are often used nonetheless now to quantify neoplastic B-cell counts although their initial purpose was the identification of minimal residual disease.

Once the description and definition of MBL was initially published, it was noted that some patients overlapped in meeting criteria for both MBL and CLL (Marti 2005 Ref 2). A consensus meeting by the CLL Working Group was held to redefine the diagnostic criteria, and a neoplastic B-cell count of 5 x $10e^9$/L was agreed upon to be used as the diagnostic threshold for CLL (Hallek 2008 Ref 3). This threshold was adopted by the WHO classification in 2017, which defines MBL as having "a monoclonal B-cell count <5x$10e^9$/L in the peripheral blood in subjects who have no associated lymphadenopathy, organomegaly, other extramedullary involvement, or any other feature of a B-cell lymphoproliferative disorder" (Swerdlow WHO IARC 2017 Ref 13). This definition of MBL is used for both cases with and without a CLL-like phenotype. Furthermore, the subtypes "low-count MBL" (<0.5x$10e^9$/L) and "high-count MBL" (≥0.5x$10e^9$/L) were defined. Small populations of clonal B-cells fitting the criteria for low-count MBL have been found in old but otherwise healthy individuals and seem to have little clinical consequence. These populations appear to exhibit clonal or oligoclonal immune responses and do not have the biological and genetic abnormalities of CLL. In contrast, cases of "high-count MBL" (≥0.5x$10e^9$/L) have similar biological and genetic abnormalities to CLL but with a neoplastic B-count below 5 x $10e^9$/L.

Studies have found no significant clinical or prognostic differences in patients with high-count MBL and early CLL (Rawstron 2010). A neoplastic B-cell count of 11 x $10e^9$/L, a relative decrease in the proportion of T-cells, and the presence of poor molecular markers have been found as more significant prognostic findings than the threshold of 5 x 10^9/L (Shanefelt 2008 Ref 4). Some have argued that there is no need for high accuracy in determining the exact neoplastic cell count and that a high variance assay in conjunction with the clinical picture may suffice. (Hanson 2009 Ref 6). Nonetheless, given the current diagnostic criteria, the distinction of these entities relies on accurate and reproducible absolute neoplastic count thresholds and validated laboratory methods are required for proper diagnosis.

THEORY

A flow cytometry analyzer can be used for both qualitative and quantitative assays. It has been described recently as "semi-quantitative" or "quasi-quantitative" in clinical flow labs. As abnormal populations are identified rather easily qualitatively with flow cytometry, and CBC analyzers provide accurate quantitative values one may infer that it may be equally as easy to join these two results and report absolute neoplastic B-cell numbers. However, instrument methods and pre-analytical, analytical, and post-analytical steps between flow cytometry and CBC hematology analyzers are significantly dissimilar which creates a high degree of variability. Simply applying percentages obtained by flow cytometry to CBC results may not be as accurate as it seems. Applying a percentage of neoplastic cells to

an absolute count and reporting that calculated result changes a test from *qualitative* to *quasi-quantitative*, and it must be validated as such.

TECHNICAL APPROACH AND CHALLENGES

This module attempts to identify the possible factors that must be taken into consideration when a lab plans to implement a quantitative flow cytometric assay. Such assays can be performed both as "dual platform", using both flow and hematology analyzers to obtain calculated neoplastic cell counts, or as "single-platform", using a flow cytometry analyzer that can directly obtain absolute cell counts. In this section, we will discuss the different pre-analytical, analytical and post-analytical considerations of a dual-platform assay, as these present more variables than single-platform.

Pre-Analytic Considerations and Variables

1. Anticoagulant: This choice (EDTA, Na Heparin or other) affects the stability of the sample being typically longer with Na heparin than with EDTA (Davis 2014 Ref 9).
2. Method used for determination of absolute numbers:
    a. Some labs prefer the single platform option in which the addition of beads provide the means of establishing absolute numbers
    b. For the Dual platform option, the preferred process would be to use the same sample tube for both the CBC and flow cytometry analysis.
3. Time: The time between collection and testing for both the CBC and flow cytometry assays should be as close as possible, but certain variables (weekend, holiday, transport etc.) may delay flow cytometry testing. The results of percentages of lymphocytes can significantly change in samples tested within 12 hours as compared to samples tested after 24 or 48 hours (Wood 2013 Ref 10).

Analytic Considerations

1. Sample Processing: The order of processing steps is important, e.g. lyse-wash, lyse-no wash, pre-wash, other. The number of washes that a sample undergoes can change the lymphocyte yield and its proportions. Use of automated sample preparation instruments may also be different than manual preps.
    a. *Lyse-no-wash* process is thought to have both higher sensitivity to detect small populations and higher accuracy, but it is unable to assess surface light chains expression. In contrast, the assessment of surface light chains requires a "good wash" approach. The use of extra washes for this purpose usually generates better results but this can significantly decrease the lymphocyte yield and the accuracy of the quantitative results. Please note that "washing" the sample also invalidates any attempt to perform a single platform absolute count, which as alluded to above, is the more accurate quantitative method (Rawstron 2013 Ref 8).
2. Antibody Selection: There is no industry standard for B-cell identification. All the variables related to antibody selection may be important, such as clone, fluorochrome, vendor, inclusion of CD45 and light chains, and testing protocols with single tubes vs multiple tubes. In established laboratories, minimal residual disease assay designs may have already been validated to best detect small neoplastic B-cell populations and distinguish them separately from normal reactive B-cells.
3. Gating Strategies: Each lab has its own preferred gating strategy for identifying lymphocytes, B-cells, and neoplastic B-cells. Variables include the hierarchic and gating strategies to identify the

proportions of these relevant subsets. The different gating strategy approaches that laboratories use is likely one cause of interlaboratory variability. (Hanson 2008 Ref 6)

4. Denominator: The denominator population should be chosen carefully. Neoplastic B-cell percentage can be calculated based on total events, cellular events excluding debris, or lymphocytes.
    a. Finding the percentage of neoplastic cells out of total events, or "cellular events", and then applying to the total WBC count is one method. Many laboratories have begun to exclude debris from their analysis and from their total denominator population. Using total events instead of "cellular events" as a denominator may erroneously skew results, as there are differences in the presence or absence of debris. However, using "cellular events" and excluding debris will need an additional gating strategy that may introduce additional variability. Of note, using the total cellular events as a denominator is in agreement with the Bethesda guidelines for reporting that recommends reporting the percentage / proportion of neoplastic cells of the sample (Wood et al 2007, Ref 14).
    b. Using the abnormal cells as a fraction of the gated lymphoid population and multiplying that by the absolute lymphocyte count obtained from the CBC is another method. This is similar to the method used for absolute CD4 counts particularly if it includes side scatter vs CD45 lymphoid gate strategy. Laboratories already have well established quality process for the quantitation of CD4 counts and may feel this is a more secure and accurate method. However, please note that the increased accuracy of the CD4 counts assays is predominantly due to the standardization of the different pre-analytical, analytical, and post analytical values mentioned in this module and having vendors manufacture IVD assays for this purpose rather than solely the use of the lymphoid population as the denominator.
5. Number of events acquired and sensitivity: A minimum number of events acquired should be defined to identify a population. If there are insufficient events to meet the set threshold, results should not be reported. This may be particularly significant if the laboratory is interested in detecting low-count MBL. Using 100 events as the minimum number of acquired events to define a population is safe and universally accepted in flow cytometry community. Laboratories with MRD experience may decide to use a lower number. Acquiring routinely 100,000 total events in peripheral blood samples is becoming a relatively routine practice. This definition of 100 events as the minimum number of required events and the acquisition setting of acquiring 100,000 events will theoretically create a sensitivity for the assay estimated at 0.1% (100 / 100,000 x 100). Validation studies for the Limit of Blank (LOB), Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ) should be considered.
6. Assays with more than one tube: It is a relatively common practice for a laboratory to run routinely more than one tube / aliquot. For example, the lab could run a tube with Kappa / Lambda and another tube with an MRD CLL approach without Kappa / Lambda. Both of these tubes can identify the neoplastic B-cells, but the proportion of neoplastic B-cells obtained from each will be different. It is recommended that the number of neoplastic B-cells is obtained from a single tube. Reporting an average number from two separate tubes is not recommended.

Post-Analytic Considerations

1. Reporting percentages only: Some laboratories may choose to report only a percentage of abnormal neoplastic B-cells relative to the total events or to the gated lymphocyte population and not report absolute counts. While this circumvents the issue of reporting absolute counts directly, the clinicians, needing an absolute count, logically take these percentages and perform their own calculations from CBC results. The lab then loses control of any carefully constructed

constraints, as these may be inappropriately applied, the wrong numbers selected for the calculations, or CBCs from different timeframes be used.

2. Reporting absolute cell counts: Some laboratories may choose to report absolute cell counts on diagnostic reports. To do so, the lab must have appropriately validated the assay as quasi-quantitative. According to the upcoming new CLSI H62 "Validation of Assays Performed by Flow Cytometry" document, quasi-quantitative assays must undergo validation for accuracy, precision, sensitivity, specificity, linearity, and stability; see below.
3. Pathologist reporting: Consistency of calculations is needed among pathologists performing post-analytic calculations.
4. Report disclaimers: Laboratories may include a disclaimer within their diagnostic reports, stating the relevant variable characteristics for their own assay. It is not uncommon for cell-based flow cytometry assays to have a high intra-assay variability (CV) of up to 30%. The variability in results can be further compounded by true biological variability in repeat samples.

Of note, a single platform assay will avoid all the pre-analytical and processing variables previously mentioned since this approach would essentially standardize the assay to a single tube assay and to the same sample collection. There are commonly available IVD single platform assays designed initially for absolute CD4 counts that now also include T-cells, B-cells, and NK-cells. The caveat for these assays is that they only identify B-cells as a whole and do not distinguish between reactive and neoplastic B-cells. However, it is not unreasonable to use these IVD assays for this purpose with the presumption that most if not all the B-cells present in the sample are neoplastic. A descriptive disclaimer should be noted in the report about the limitations of this method if this is the method that your laboratory chooses to use.

VALIDATION

Given the clinical need for determining absolute monoclonal B-cell counts and the fact that the flow cytometry laboratory is best suited for the job, laboratories must complete additional steps to ensure that the aforementioned considerations have been addressed and the results are accurate. The assay for neoplastic B-cell counts creates challenges when using a dual platform approach, with both validation of the qualitative flow cytometry assay to identify the neoplastic B-cells as well as the quantitative CBC analyzer values.

Reporting absolute values transforms the leukemia/lymphoma panel from a mostly qualitative one into a quasi-quantitative assay. According to the upcoming new CLSI H62 document, quasi-quantitative assays must undergo validation for 1) accuracy, 2) specificity and selectivity, 3) sensitivity, 4) precision, 5) linearity, and 6) stability. These extra validation studies can be performed in parallel with a new assay validation, but with a few extra steps, or they can be done later on as an add-on quasi-quantitative validation in a previously qualitative assay (Wood 2013 Ref 10). Please note that some of the nomenclature used for validation studies can be confusing. For example, the definitions and validation processes for "Sensitivity" and "Specificity" are not the same for qualitative and quantitative assays. Understanding these differences is important.

1. Accuracy: Accuracy is defined as the closeness in agreement between the average values obtained from a series of test results when compared to an accepted reference standard. Unfortunately, there is usually no accepted reference standard for flow cytometry assays. You will be essentially designing the reference standard for your own lab. A concordance table should be performed for accuracy if you are establishing a new qualitative assay with a comparison to your previous qualitative assay. The concordance table for accuracy will include a

qualitative specificity and sensitivity assessment of the assay. A quantitative comparison using a linearity R2 comparison could be considered as well using available IVD quantitative kits to count absolute T-cells and B-cells that are available from vendors particularly in the setting of monitoring CD4 counts. However, the caveat here is that you will likely need to do the comparison between all B-cells rather than neoplastic B-cells since there are no IVD assay kits available for neoplastic B-cells. In addition, this comparison will have some inherent additional variability due the comparison between a dual and single platform methods.

2. Specificity/Selectivity: Analytical specificity includes antigen selection, clones, titration, and evaluation of wash steps, gating strategy, and verification that antibodies work as expected. These will often have been already performed in your previous validation studies.
3. Sensitivity: This has also been described as the detection capability of the assay. The parameters that are investigated are the Limit of Blank (LOB), the Limit of Detection (LOD), and the Lower Limit of Quantification (LLOQ). The decision of the minimal number of events acquired and the laboratory definition minimum number of events to define a population should be made. Each of these parameters will usually need an experimental design study for their validation. Both the percentage of the neoplastic population detected, and its absolute value should be considered in the assessment of the experimental designs. Examples of experimental designs have been described in the ICCS Ask an Expert section under "How is sensitivity defined in rare event analysis?" and in references below.
4. Precision: Precision is the dispersion of replicate measurements under conditions of measurement. An intra-assay precision assessment to calculate the percentage of coefficient of variation (CV) should be done. Ideally this should be done with sample with values close to 5 x 10e9/L neoplastic B-cells. An additional lower value can also be considered. Precision studies for inter-assay, inter-operator, inter-instrument variables could be considered as needed for your own laboratory.
5. Linearity: The purpose of linearity verification is to determine that there is a linear relationship between the concentration of B-cells being measured and the result. This can be achieved by serial dilution studies.
6. Stability: Stability is the lack of variability in the measured value (neoplastic B-cells) relative to time and anticoagulant. The assessment of stability in this particular assay is complicated since dual platform method uses two separate samples. The time of draw to the performance assay can introduce significant variability in this assay. The laboratory should keep this in mind with regards to the acceptability criteria it establishes in its experimental design versus the practical aspects of collection and blood draws particularly if it receives samples from referral centers when transport delays are often unavoidable.

RECOMMENDATIONS

This module has described the background for the current clinical and laboratory requirements to have a flow cytometry assay to calculate absolute neoplastic B-cell counts. This was relatively arbitrarily established as a consensus decision to better define the patients populations rather than an actual real clinical prognostic difference.

1. Flow cytometrists should be aware of this issue and that the presence of some variability in your assay is acceptable.
2. Acknowledge in reports the presence of variability in this calculated absolute neoplastic B-cell count, particularly with the use of dual platform methods and the use of samples that may have a relatively prolonged time between the time of draw and testing.

3. In regard to technical recommendations, the authors have struggled to find specific recommendations to provide. As described in the validation section under "Accuracy" this assay has no reference standard for comparison and there are no published guidelines about how to best perform this assay. However, the authors still advise that a validation should be performed with a reproducible standard operating procedure for the calculation and reporting of neoplastic B-cell counts.
    a. The use of IVD single platforms kits that include absolute B-cell counts is not an unreasonable choice and it is the preferred choice by one of the authors; this choice is also used by at least one large international referral laboratory. This choice is able to produce more accurate quantitative counts but lacks any qualitative data (neoplastic versus reactive). The neoplastic nature of the B-cells would need to be obtained and confirmed by a separate qualitative assay as needed. Please note as well that the kit manufactures specifically state that their kit is "not intended for screening samples for the presence of leukemic cells or for use in phenotyping samples from leukemia patients".
    b. The use of leukemia / lymphoma panels with a dual platform method for the calculation of the neoplastic B-cells is also a reasonable choice that provides: 1. excellent qualitative data in recognizing the neoplastic B-cells, 2. acceptable quantitative data. and 3. values particularly when you have established a standard reproducible procedure. Of note, the authors recommend that calculations are done from a single tube assay and advise against the practice of averaging results between different tubes in a multi-tube assay.

4. Lastly, the authors would like to highlight some of the points mentioned in this module that will be helpful to obtain more accurate quantitative results using a dual platform method. These are: a) shorter time between time of collection and testing, b) fewer washes or even a lyse-no-wash method, c) identification of the neoplastic B-cell population separate from other events and debris with accurate gating and reproducible strategies, and, d) use of a larger number of marker antibodies in a multiparametric assay including the use of CD45 when possible is helpful.

CONCLUSION

The authors would like the readers to know that they understand very well (some by personal experience) that laboratories may have limitations in regards to having the resources and time to develop and validate a new assay in order to report absolute neoplastic B-cell counts. We hope that you find this module useful and that it provides you with the needed context and information needed for you to design and validate the assay that best fits your laboratory. The authors are also sharing some report examples of their assays done in their laboratories; please note that not all authors are currently performing and reporting neoplastic B-cell counts.

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APPENDIX / SAMPLE REPORTS

Some example reports and assay descriptions are provided below.

Example #1

This lab uses the following panels:

- "Triage panel" which also includes blast markers, B-cell and T-cell markers if there is no lymphocytosis (less than 5,000/cmm)
- A more targeted "CLL panel" is used if there is absolute lymphocytosis (>5,000/cmm)
    - A CLL comment is used if the absolute number of abnormal B-cell is greater than 5,000/cmm
    - An MBL comment is used if the absolute number of abnormal B-cell is less than 5,000/cmm

- The "CLL panel" and report are below:

TISSUE/SPECIMEN: 1. Peripheral Blood in EDTA (CLL vs reactive lymphocytosis)

DIAGNOSIS: IMMUNOPHENOTYPIC FINDINGS CONSISTENT WITH CHRONIC LYMPHOCYTIC LEUKEMIA

Comment: Flow cytometric analysis confirms the presence of a monoclonal B-cell population (approximately 66% of cells; absolute count of 14,236/uL) with the following phenotype: CD45+, CD5+ (dim), CD10-negative, CD19+, CD20+ (decreased), CD22+(decreased), CD23+, CD38-negative, CD200+, and with kappa (dim) surface light chain restriction. This phenotype is most consistent with CLL/SLL but may rarely be seen in other B-cell lymphoproliferative disorders. Clinical correlation is recommended for complete interpretation of these results.

Clinical History: Lymphocytosis

Flow Results: ***Immunophenotypic analysis*** is performed using antibodies to the following epitopes: CD45, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD22, CD23, CD26, CD38, CD200 as well as kappa and lambda surface light chain immunoglobulins. A peripheral blood smear was reviewed for adequacy.

An **abnormal mature lymphoid** population is identified (approximately 66% of the total peripheral blood cells and an absolute count of 14,236/uL) with the following phenotype:

B-cell markers:		**Other markers:**	
CD10 (Foll. center cell):	negative	**CD5 (pan T-cell, B-cell subset):**	**positive (dim)**
CD19 (pan B-cell):	**positive**	**CD45 (pan-Leukocytes):**	**positive**
CD20 (pan B-cell):	**positive (decreased)**	CD38:	negative
CD22 (pan B-cell):	**positive (decreased)**	**CD200 (CLL):**	**positive**
CD23 (pan B-cell):	**positive**		
Kappa light chains:	**positive (dim)**		
Lambda light chains:	negative		

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

- The triage panel and MBL report are below:

TISSUE/SPECIMEN: 1. Peripheral Blood in EDTA (CLL vs reactive lymphocytosis)

DIAGNOSIS: MONOCLONAL B-CELL POPULATION IDENTIFIED (See comment)

Comment: Flow cytometric evaluation demonstrates a monoclonal B-cell population with an absolute monoclonal B-cell count of 522/uL and a phenotype is typically seen in CLL/SLL. However, patients with CLL/SLL typically have absolute numbers of monoclonal B-cells greater than 5,000/uL.

Monoclonal B-cell lymphocytosis (MBL) is defined by a monoclonal B-cell count less than 5,000/uL in patients who have no associated lymphadenopathy, organomegaly, or other features of a B-cell lymphoproliferative disorder. Clinical correlation is recommended as some B-cell lymphomas may have low level peripheral blood involvement. The prevalence of monoclonal B-cell lymphocytosis increases with age. Patients who have monoclonal B-cells less than 500/ul (Low count MBL), generally have low risk of progression to CLL and do not necessarily require specific follow-up. Patients who have monoclonal B-cells greater than 500/ul (High count MBL) have a 1-2% progression per year to CLL requiring treatment therefore clinical follow-up is recommended in these patients.

Reference: Swerdlow SH, et al.: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, revised 4th edition. Lyon, International Agency for Research on Cancer, 2017.

Clinical History: Atypical lymphocytosis

Flow Results: ***Immunophenotypic analysis*** is performed using antibodies to the following epitopes: CD45, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD22, CD23, CD26, CD38, CD200 as well as kappa and lambda surface and cytoplasmic light chain immunoglobulins. A peripheral blood smear was reviewed for adequacy.

1. Approximately 68% of the total peripheral blood cells are lymphoid, comprising of:
    a. 80% CD3+ T-cells (CD4:CD8 ratio is 1:1)
    b. 12% CD19+ B-cells with a mixture of polyclonal B-cells and **monoclonal B-cells with the phenotype CD45+, CD5+ (dim), CD19+, CD20+ (dim), CD22+ (decreased), CD23+, CD38-negative, CD200+ and with cytoplasmic kappa light chain restriction (approximately 6% of the total peripheral blood cells, absolute count of 522/ul)**
    c. 8% CD3-negative/CD19-negative cells
2. Approximately 6% of the total peripheral blood cells are CD4+ monocytic cells.
3. Approximately 26% of the total peripheral blood cells are CD10+ granulocytic cells.

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCDS-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

- A triage panel and normal report are below.

TISSUE/SPECIMEN: 1. Peripheral blood in EDTA (leukemia/lymphoma evaluation)

DIAGNOSIS: **1. NO CIRCULATING BLASTS**
2. NO IMMUNOPHENOTYPIC EVIDENCE OF A LYMPHOPROLIFERATIVE DISORDER

Comment: There is no evidence of a monoclonal B-cell population. There is no evidence of aberrant T-cell antigen expression. No circulating blasts are identified by flow cytometry. Clinical correlation is recommended.

Clinical History: Leukocytosis

Flow Results: ***Immunophenotypic analysis*** is performed using antibodies to the following epitopes: CD45, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD13, CD14, CD16, CD19, CD20, CD22, CD23, CD26, CD33, CD34, CD38, CD56, CD117 as well as kappa and lambda surface light chain immunoglobulins. A smear was reviewed for adequacy.

1. Approximately 16% of the total peripheral blood cells are lymphoid, comprising of:
   a. 79% CD3+ T-cells (CD4:CD8 ratio is 1:1)
   b. 6% CD19+ polyclonal B-cells (kappa: lambda ratio is 1.6:1)
   c. 15% CD3-negative/CD56+ natural killer cells (approximately 2% of the total peripheral blood cells).
2. Approximately 7% of the total peripheral blood cells are CD33++/CD14++ monocytic cells.
3. Approximately 77% of the total peripheral blood cells are granulocytic cells with a mature pattern of CD11b/CD16 expression. A population of eosinophils (4%) is noted.
4. Less than 0.05% blasts are seen based on CD34 and CD117 assessment.

The markers used for this flow cytometric analysis are labeled as Analyte Specific Reagents (ASR) and are used for clinical purposes. The performance characteristics of these markers have been determined by DCD S-Flow Cytometry Laboratory. Their use has not been approved by the U.S. Food and Drug Administration; the FDA has determined that such approval is not necessary.

EXAMPLE #2

My laboratory does not routinely provide absolute neoplastic B-cell counts. If clinician requests absolute counts, an unofficial result is obtained by using a dual-platform calculation of hematology count with the flow % result. An example of one my reports is provided below.

Flow cytometric analysis has been performed using peripheral blood to characterize the lymphoid cell population using a CD45+/CD14-/low side scatter gating strategy. The lymphoid gate accounts for 9% of total events.

B-cells account for 8% of the lymphoid gate. They show polytypic expression of surface immunoglobulin light chains and do not exhibit any immunophenotypic abnormalities.

T-cells account for 79% of the lymphoid gate with the CD4-positive and CD8-positive cells in a 4.9:1 ratio.

Accounting for approx. 30% of T-cells (approx. 25% of the lymphoid gate and 2% of total events) is a population expressing CD45RA, variable CD8, CD56 and CD57. These cells express CD2, CD3, CD5, CD7 and are negative for TRBC1.

NK cells account for 12% of the lymphoid gate and show no immunophenotypic abnormalities.

EXAMPLE #3

We currently use broad panels for all of our bloods/bone marrows/nodes. We do not triage although we are currently working on it. Therefore all samples get worked up for B-cell LPDs with 19/5/k/l tube and 20/23/10/FMC7 tube, as well as others. Attaching our standard comments for samples with clonal CD5 populations. If obvious CLL, we delete the second half of the "hi MBL" comment. We do not report absolute numbers. We are trying to develop a plan to validate this as we transition to 10-color.

HIGH COUNT MBL VS CLL

The results of flow cytometry are those of involvement by a population of clonal CD5-positive B-cells comprising ***% of the lymphocytes. The immunophenotypic profile is: CD5+, CD19+, CD20+(dim), CD10-, CD23+, FMC7-, surface light chain ***. This immunophenotype is typical of chronic lymphocytic leukemia (CLL) but this diagnosis requires the number of clonal lymphocytes to be greater than

5,000/cmm. Should this threshold not be met, a diagnosis of “high-count” monoclonal B-cell lymphocytosis (MBL) would be appropriate in the absence of extramedullary disease. MBL is a non-neoplastic condition that may represent a precursor lesion to a B-cell lymphoproliferative neoplasm and has been detected in 5-10% of otherwise healthy individuals, with increasing incidence with age. High-count MBL carries a risk of progression to CLL requiring therapy of 1-2% per year (Strati et al. Blood 2015;126 :454-62). Should this patient progress above the CLL threshold, CLL FISH panel is recommended for prognostic information. Correlation of these findings with morphologic and clinical data is essential.

LOW COUNT MBL

The results of flow cytometry are those of involvement by a small population of clonal CD5-positive B-cells comprising {only 3-4}% of the lymphocytes. The immunophenotypic profile is: CD5+, CD19+, CD20+(dim), CD10-, CD23+, FMC7-, surface light chain ***. Given the patient’s low absolute lymphocyte count and the CLL-like immunophenotype, this likely represents “low-count” monoclonal B-cell lymphocytosis (MBL), in the absence of extramedullary disease. MBL is a non-neoplastic condition that may represent a precursor lesion to a B-cell lymphoproliferative neoplasm and has been detected in 5-10% of otherwise healthy individuals, with increasing incidence with age. Low-count MBL does not seem to progress and these patients do not need clinical follow-up according to the literature. Correlation of these findings with morphologic and clinical data is essential.

EXAMPLE #4

Below is an example of one our peripheral blood reports. Our laboratory uses a drop-in formatted “Synoptic report”. The same 10-color screen is used for all peripheral blood samples. A complete phenotype panel is not repeated in the samples if it has been done in the previous four years. Results for absolute neoplastic B-cell counts are taken form the screen tube for practical purposes since that is usually the only tube performed in all cases. A CLL example report that include the neoplastic B-cell count and the description of the screen with its antibodies is provided below.

FLOW REPORT

Synoptic Report

A: FLOW SYNOPTIC REPORT

CLINICAL INFORMATION

Type of test performed: Leukemia/Lymphoma screening

Clinical History: Recommended by hematology for B-cell lymphocytes

Peripheral Blood CBC and Manual Differential:

- Collection Date: August 27 2019
- Hbg: 148 g/L
- WBC: 14.71 x10(9)/L
- Platelet: 154 x10(9)/L
- % Lymph: 67.6
- % Monos: 7.1
- Absolute Lymphocyte Count: 9.94 x10(9)/L

Previous In-House flow cytometry testing: Yes

SAMPLE DESCRIPTION

Specimen Received: Peripheral blood

Anticoagulant and/or preservative used: Heparin lithium

Sample amount (estimated): 4 ml

Cell count of cell suspension obtained after initial processing: 8.22 x 10e9/L
Antibody panel(s) utilized:
Peripheral Blood Screen Tube: CD8+Lambda+CD16, CD4+Kappa, CD34+CD117, CD19, CD3+CD14, CD33, CD38, CD20, CD45, CD56

QUALITY OF THE FLOW CYTOMETRY DATA
Acquisition:

- Total number of events acquired in screen tube: 100,000
- % Debris: 4

TECHNICAL DATA ANALYSIS
Additional data:

- **Total mature B-cell lymphocyte:**
  - Kappa: 1 %
  - Lambda: 52 %

Detailed population phenotype: No
Population Type: B-cell lymphocytes
Size of the population: Population represents approximately 25 % of total events.
Calculated disease specific counts: The neoplastic clonal B-cell count with a CBC absolute lymphocyte count and the flow cytometry % of neoplastic clonal B-cells from the "Lymph Sum" is estimated to be 5.95 x 10e9/L

Flow Cytometry Differential
A sample screening panel was done with the above listed antibodies. A cell population differential plus debris based on flow cytometric parameters is provided as a description of the sample.

Specimen Name	################		
Population	#Events	%Parent	%Total
Cellular events	95899		95.9
Monocytes	3270		3.3
CD16 pos neutrophils	47806		47.8
Immature grans	200		0.2
Mature B-cells	25133		25.1
T-cells	12896		12.9
NK cells	3057		3.1
CD34+CD117	16		0
Sum of Immature Events	266		0.3
Lymph Sum	41066		41.1
Neoplastic B-cells	24579	59.9	
Debris	4101		4.1

Comment

There is a clonal B-cell population with a Chronic Lymphocytic Leukemia phenotype in ~ 25% of the sample.

A calculated neoplastic clonal B-cell count with a CBC absolute lymphocyte count and the flow cytometry % of neoplastic clonal B-cells from the "Lymph Sum" is estimated to be 5.95 x 10e9/L. Please

note that these results may vary. However, per this estimate patient meets diagnostic criteria for a Chronic Lymphocytic Leukemia.

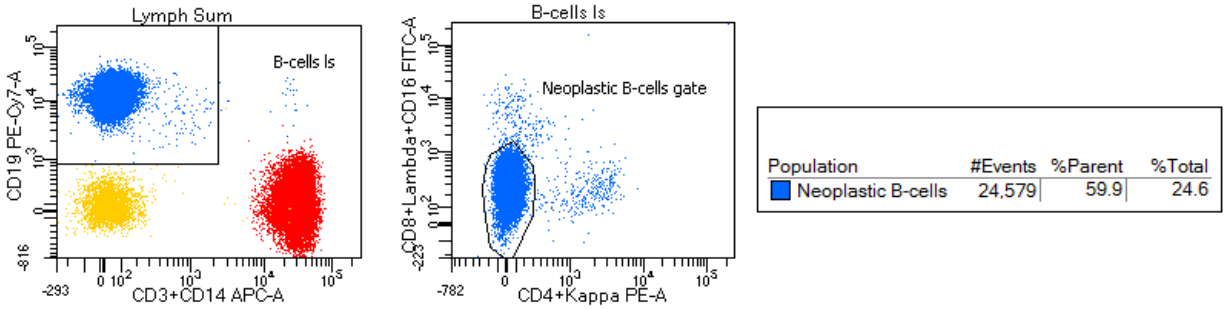
Some of the recent literature suggests that when the neoplastic B-cell counts are close to the diagnostic threshold between Chronic Lymphocytic Leukemia and Monoclonal B-cell Lymphocytosis of a B-cell count of 5 x 10e9/L that the possibility of both diagnoses be raised and considered in the context of the clinical information.

Flow Cytometry Report
Peripheral blood:

- Clonal B-cell population in ~ 25% of the sample.
- Findings are consistent with a Chronic Lymphocytic Leukemia.
- See comment.

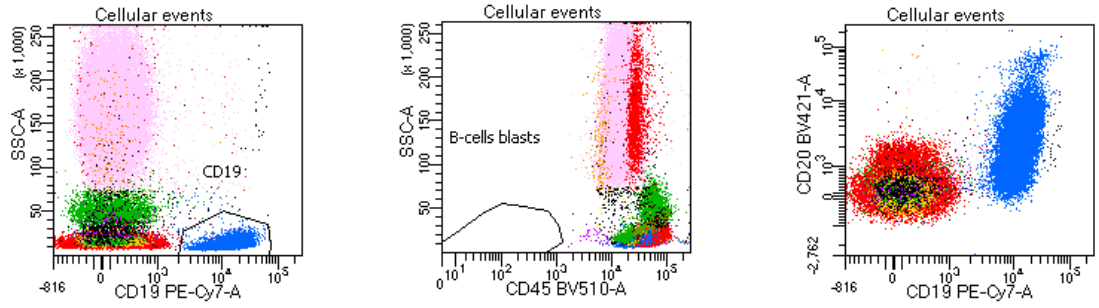
Images of our PDF

- Neoplastic B-cells

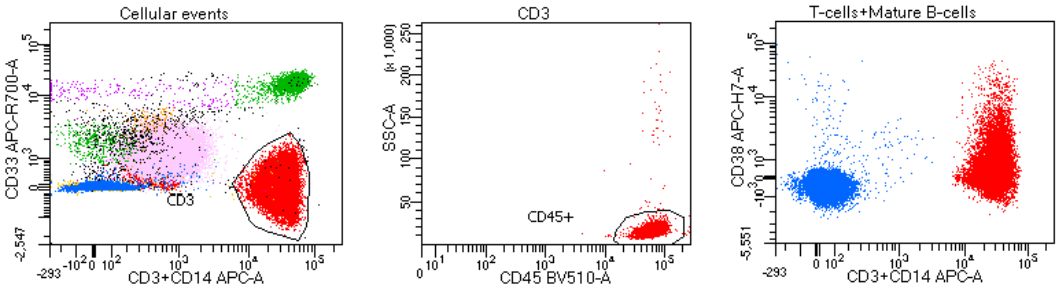


In peripheral blood samples we report an estimated neoplastic absolute B-cell count. This calculation is done in our laboratory with a dual platform method with a concurrent CBC absolute lymphocyte count and the percentage of neoplastic clonal B-cells taking out of the "Lymph Sum" as a denominator. There is Boolean gating strategy used to only include the B-cells out of the "Lymph Sum". The neoplastic clonal B-cell gate will be visually placed by the technologist to include the entire clonal B-cell population independently of its positive, dim or negative light chain expression. Do not calculate the B-cell neoplastic population using other gating strategies since the results may vary using different approaches.

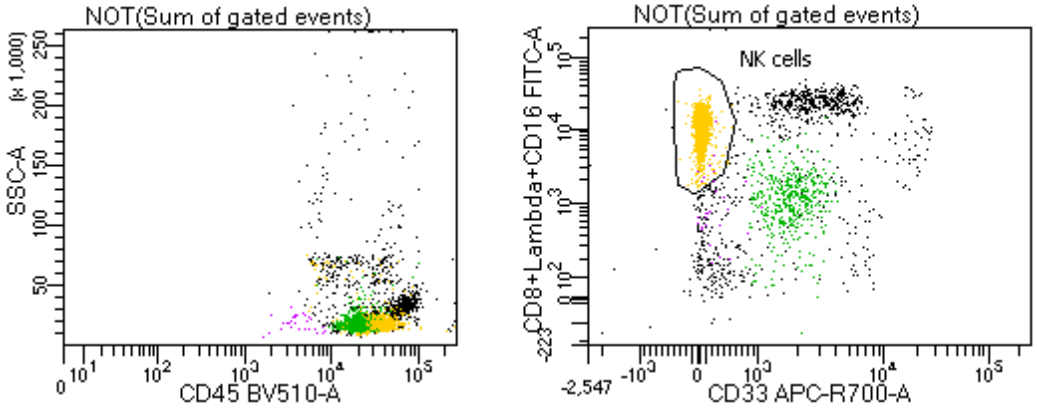
- B-cell gate



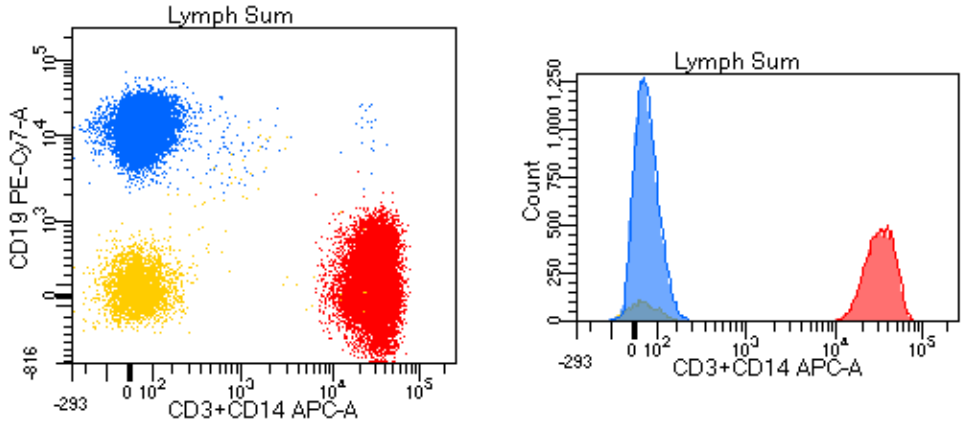
- T-cell gate



- NK-cell gate



- Lymphosum



Population	#Events	%Total
T-cells	12,896	12.9
Mature B-cells	25,133	25.1
NK cells	3,057	3.1
Lymph Sum	41,066	41.1