Title: Reproducible diagnosis of Chronic Lymphocytic Leukemia by flow cytometry: an European Research Initiative on CLL (ERIC) & European Society for Clinical Cell Analysis (ESCCA) harmonisation project

Running title: ERIC/ESCCA consensus: reproducible CLL diagnosis by flow cytometry

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Abstract

The diagnostic criteria for CLL rely on morphology and immunophenotype. Current approaches have limitations affecting reproducibility and there is no consensus on the role of new markers. The aim of this project was to identify reproducible criteria and consensus on markers recommended for the diagnosis of CLL.

ERIC/ESCCA members classified 14 of 35 potential markers as “required” or “recommended” for CLL diagnosis, consensus being defined as >75% and >50% agreement, respectively. An approach to validate “required” markers using normal peripheral blood was developed.

Responses were received from 150 participants with a diagnostic workload >20 CLL cases per week in 23/150 (15%), 5-20 in 82/150 (55%) and <5 cases per week in 45/150 (30%). The consensus for “required” diagnostic markers included: CD19, CD5, CD20, CD23, Kappa and Lambda. “Recommended” markers potentially useful for differential diagnosis were: CD43, CD79b, CD81, CD200, CD10, and ROR1. Reproducible criteria for component reagents were assessed retrospectively in 14,643 cases from 13 different centres and showed >97% concordance with current approaches. A pilot study to validate staining quality was completed in eleven centres.

Markers considered as “required” for the diagnosis of CLL by the participants in this study (CD19, CD5, CD20, CD23, Kappa and Lambda) are consistent with current diagnostic criteria and practice. Importantly, a reproducible approach to validate and apply these markers in individual laboratories has been identified. Finally, a consensus “recommended” panel of markers to refine diagnosis in borderline cases (CD43, CD79b, CD81, CD200, CD10, ROR1) has been defined and will be prospectively evaluated.
Background

The WHO, IWCLL and NCCN diagnostic criteria for CLL is based on the morphology and immunophenotype of the neoplastic B-cells with co-expression of CD19, CD5, CD23, with weak CD20 and monoclonal surface immunoglobulin (sIg) expression 1-3. Although there are several recurrent molecular abnormalities present in CLL, none is specific for CLL 4 and therefore immunophenotyping still plays a central role in the diagnosis of CLL.

The current diagnostic criteria have some limitations affecting reproducibility, in particular relating to flexibility in the requirement for each marker to be present or absent as well as in the required expression level of each marker. The WHO definition states that CLL/SLL cells “usually coexpress CD5 and CD23” and that “using flow cytometry, the tumour cells express dim surface IgM/IgD, CD20, CD22, CD5, CD19, CD79a, CD23, CD43 and CD11c (weak). CD10 is negative and FMC7 and CD79b are usually negative or weakly expressed in typical CLL. It is also considered that “some cases may have an atypical immunophenotype (e.g. CD5- or CD23-, FMC7+ or CD11c+, strong sIg, or CD79b+)” 1. In turn, the current IWCLL guidelines also permit variation in markers expression levels: “CLL cells coexpress the T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23. The levels of surface immunoglobulin, CD20, and CD79b are characteristically low compared with those found on normal B cells. Each clone of leukemia cells is restricted to expression of either kappa or lambda immunoglobulin light chains. Variations of the intensity of expression of these markers may exist and do not prevent inclusion of a patient in clinical trials for CLL” 2.

Although some degree of flexibility is required to ensure that CLL diagnostic criteria are widely applicable, this can pose problems to the reproducibility of diagnostic criteria, particular if a scoring system that may permit absence of either CD5 or CD23 is employed 5,6. In addition, several markers such as CD200 7,8 and ROR1 9-12 may contribute to the diagnosis of CLL and related disorders. However, there is no consensus yet on how such markers should be incorporated into diagnostic algorithms. Moreover, although the addition of new markers to established diagnostic panels may improve diagnostic precision, this would require a systematic and well-designed approach.

The primary aim of this project was to achieve consensus on the minimum set of markers required for the diagnosis of CLL and develop a reproducible approach to validate and apply these markers in different laboratories. A secondary aim was to identify additional markers deserving prospective evaluation.
Methods

Identification of consensus on required and recommended marker panels
ERIC/ESCCA members were invited to participate in a survey to classify 35 potential flow cytometry markers as being “required”, “recommended”, “suggested”, “uninformative”, or “not sure” for the diagnosis of CLL. The full survey is shown in supplementary data. Results from respondents indicating that they did not work in a diagnostic laboratory or hospital clinic setting, or who indicated that their institution did not perform flow cytometry in the diagnosis or monitoring of CLL were excluded from analysis. The 35 markers were selected based on the inclusion in the diagnostic panels reported in the WHO classification \(^1\), IWCLL guidelines \(^2\), Euroflow B-cell panel \(^8\), or if a pubmed search for “differential diagnosis chronic lymphocytic leukemia CD” identified a reagent in publications by two or more different groups (Figure 1). Consensus for a marker to be required for CLL diagnosis needed >75% of participants indicating that the marker was required, while a marker was put forward to review if > 50% of participants considered the marker to be “recommended” or “required”. Positive and negative control populations in normal peripheral blood and the relative signals required for acceptable markers were derived from the previous ERIC project for optimising CLL MRD \(^{13}\) or defined through literature search and review by participants.

The definition of “weak” expression requires a threshold that is reproducibly lower than normal expression, i.e. lower than acceptable variation due to assay imprecision or due to changes associated with sample stability. The ICCS/ICSH guidelines for validation of cell-based fluorescence assays \(^{14}\) recommend assay imprecision ideally below 10% CV (although this may be higher in some settings) while specimen stability is determined by identifying the latest time point at which repeat testing of ≥5 samples shows up to 20% change from baseline. Therefore difference in fluorescence intensity of <20% may not be reliably determined, so weak expression was defined as a median fluorescence intensity at least 20% lower than the median expression level by normal peripheral blood B-cells. As laboratories use different reagents, instrumentation and procedures, each laboratory was requested to determine their own reference range to be used for definition of weak expression.

Consensus for the proposed diagnostic panel specification was reached by approval of all participating authors followed by presentation of the proposal at ERIC and ESCCA meetings and open consultation on the final document distributed to all ERIC and ESCCA members 3 months prior to submission.

Retrospective evaluation
Survey participants were requested to retrospectively assess the proposed criteria based on the required markers (table 1) by providing the number of: total B-LPD cases evaluated; CD5+ B-LPD cases; cases meeting the proposed criteria and diagnosed as CLL; cases not meeting the proposed criteria and diagnosed with another B-LPD, e.g. mantle cell lymphoma; cases not meeting the proposed criteria and diagnosed with CLL; cases not meeting the proposed criteria with insufficient material to make a final diagnosis or reported to be unclassifiable based on available data

Assessing reagent/instrument quality
A gating strategy to identify the expression levels of component markers on normal peripheral blood lymphocytes was developed (Figure 2). Participants providing retrospective evaluation were
requested to assess the gating strategy on ten cases in which the B-cells were polyclonal. The median fluorescence intensity for the relevant markers on defined positive and negative control populations were recorded by eleven different laboratories and returned for central analysis where the relative fluorescence intensity signal value was calculated. A borderline result was defined as 1-3/10 cases below the minimum acceptable relative signal target value. A suboptimal result was defined as more than 3/10 cases below the minimum relative signal target value.
Results and Discussion

Identifying consensus on the markers required or recommended for the diagnosis of CLL

ERIC/ESCCA members were invited to identify markers that are “required” or “recommended” for the diagnosis of CLL from a potential panel of 35 markers. Responses were received from 154 members of which 150/154 were involved in CLL diagnosis (100 were diagnostic laboratory staff, 14 were clinicians and 36 were involved in both the laboratory and clinical diagnostic process). Responses from individuals not involved in CLL diagnosis were excluded from further analysis. The diagnostic workload was more than 20 cases per week in 23/150 (15.3%), 5-20 in 82/150 (54.7%) and <5 cases per week in 45/150 (30%). The survey participant consensus was that the minimum diagnostic panel should include: CD19, CD5, CD20, CD23, Kappa and Lambda (i.e. “required”). Survey participants recommended that the following markers may also be of value CD22, CD38, CD45, FMC7, CD79b, CD10, CD43 and CD200. The complete list of markers and participant responses are shown in Figure 1.

The minimum required diagnostic panel was put forward for identification of component marker specification that could be used to assess reagent and laboratory quality (see below). The recommended marker panel was reviewed by the steering committee (AR, PH, MH, PG, EM) and it was proposed that the application of CD22, CD38, CD45 and FMC7 is left to the individual laboratory preference (i.e. “not recommended”) because of a variety of reasons. In detail: FMC7 is an epitope of CD20, the inclusion of both markers being redundant; similarly the level of CD22 expression is closely correlated with CD20; CD45 is used for identification of leukocyte subsets and provides a backbone to many gating strategies but is not essential to identify CLL cells; CD38 is heterogeneously expressed in CLL difficult to standardise and it is also difficult to identify control populations with stable expression levels; therefore it was proposed that the application of CD38 in diagnosis and prognosis is determined by individual laboratories.

The markers sIgM, CD81, CD103, CD49d, CD11c, IgD, IgG, and CD25 were recommended by 20-40% of participants and therefore their application is best determined by individual laboratories with the exception of CD81 which has been extensively validated in detection of MRD, therefore understanding the expression profile prior to treatment may be informative for differential diagnosis. ROR1 was not on the initial survey because at the time of preparation there was limited access to commercial reagents. ROR1 was initially identified by gene expression profiling studies as a CLL-specific marker and the role of protein expression in diagnosis and prognosis has been analyzed in several different studies and may be particularly informative in the discrimination between CLL and CD5+ post-germinal centre B-cell disorders. Based on all these considerations, the markers recommended for additional analysis were: CD43, CD79b, CD81, CD200, CD10 & ROR1.

For each marker in the required and recommended panels, a positive and negative control population that could be readily recognized in normal peripheral blood was identified, with the exception of ROR1 for which B-progenitors in the bone marrow are the only normal positive control. A minimum and recommended relative signal was also determined either by consensus or by using data from the CLL MRD project which identified the relative signal on positive vs. negative control populations required to achieve optimal separation of CLL cells from normal B-cells. The required and recommended markers with relevant positive and control populations and expected relative signals are shown in Table 1. This information was distributed to all ERIC and ESCCA members for consultation in order to confirm consensus.
Retrospective application of the minimum required panel using the proposed specification

Survey participants involved in the diagnosis of CLL were requested to retrospectively assess the proposed criteria shown in Table 1, and 13/150 responded with the results shown in Table 2. The required criteria were assessed retrospectively in 14,643 cases referred for diagnosis of a potential B-LPD, of which 11,721 were diagnosed with a CD5+ B-LPD. Central laboratories for clinical trials identified cases which had been submitted for a CLL trial, i.e. considered to have a diagnosis of CLL by another centre, as “trial” cases (2427/11,721) while all other cases were classified as “primary referral” (9294/11,721).

The high proportion (7379/9294, 79%) of primary referral cases met the proposed criteria and obtained a diagnosis of CLL. A clear alternative diagnosis (e.g. mantle cell lymphoma) was made in 54% (1025/1915) of primary referral cases that did not meet the proposed criteria. For primary referrals not meeting the criteria and not having a clear alternative diagnosis, a final diagnosis of CLL was made using the diagnostic unit’s current practice in 2.7% of total CD5+ LPD cases (n=251, 28% of cases not meeting the proposed criteria); there was insufficient material or data on the final diagnosis was not available in 6.9% of total CD5+ LPD cases (n=639, 72% of the 890 cases not meeting the proposed criteria) of cases. For primary referral cases, there was concordance in 97.2% (9043/9294, comprising 7379 diagnosed with CLL, 1025 diagnosed with another non-CLL B-LPD and 639 non-diagnostic with both approaches) using the reproducible criteria compared to each laboratory’s current practice.

The vast majority (2267/2427, 93.4%) of trial referrals, i.e. cases previously considered to have a diagnosis of CLL at another centre, were confirmed to meet the proposed criteria by the referral centre (classed as true positive). There were 160 cases that did not meet the proposed CLL criteria, of which 54/160 had a clear alternative diagnosis and 93/160 were considered ineligible for the trial due to lack of sufficient diagnostic material or non-specific diagnosis (true negative n=147/2427, 6.1%). 13/160 were finally classified as CLL (false negative) and included in the relevant trial (false negative n=13/2427, 0.5%). Based on cases referred for entry into a clinical trial and using each centre’s current practice, the proposed criteria would have a negative predictive value of 92% with a positive predictive value, specificity and sensitivity for the diagnosis of CLL of greater than 99%.

Evaluation of a pilot study to assess reagent and instrument quality

The 13 participants providing retrospective data were invited to assess the proposed specifications for the 6 markers identified as “required” for diagnosis of which 11/13 responded. Using a simple gating strategy (Figure 2), participating centres evaluated ten cases with polyclonal B-cells. The details and performance characteristics for the individual markers used in the 11 different centres are shown in Table 3. Each centre used a different combination of reagents but the performance characteristics were optimal in 525/600 (88%) for individual reagents. Only 1/11 centres obtained optimal results (i.e. the relative signal was above the minimum relative fluorescence intensity of positive and negative control populations) for all 6 markers in all 10 cases. A further 3/11 centres had some borderline signals (defined as 1-3 cases with results below the relative signal target value), in most cases likely to reflect the samples rather than instrument/reagent quality (e.g. 2 cases with weak CD23 expression on the polyclonal B-cells at centre 4). Suboptimal signals (defined as more than 3 cases with results below the relative signal target value) were identified with respect to an individual marker in 7/11 centres. In one case this reflected a limitation of the proposed gating strategy for centres using a multiplex approach (CD20 at centre 3). Several centres had sub-optimal results for the CD5 reagent, which may indicate that this marker requires a more stringent
specification. The other sub-optimal results may be due to one or more of several factors (e.g., clone, fluorochrome, manufacturer, equipment, operating procedure) and do not necessarily relate to the reagent used. Optimising and standardising each component of the process can be labour-intensive and should be specifically addressed in order to improve the overall quality of CLL diagnosis, even when using the most “basic” markers. The relatively simple global approach developed by ERIC/ESCCA to assess the CLL diagnostic panel is applicable to a variety of reagent and instrument suppliers and can easily identify potential problems or confirm acceptable performance in individual laboratories. In addition, it can be utilized in the future as the basis for a more homogeneous and standardized diagnostic approach in CLL, allowing cross-centre comparison and reproducibility both in clinical trials as well as daily diagnostic procedures.

Summary

CLL is one of the most common diagnoses made by hematology-oncology laboratories. Flow cytometry plays a central role in diagnosis but differential diagnosis remains an issue in a small proportion of cases. Due to the lack of a pathognomonic molecular abnormality in CLL there is no a gold-standard for its diagnosis. Also, characteristic immunophenotypic features, such as weak expression of surface immunoglobulin and CD20, are difficult to define in a reproducible fashion, thus making it difficult to ensure consistent diagnosis across laboratories.

This study demonstrates clear consensus on the minimum set of markers required for the diagnosis of CLL: CD19, CD5, CD20, CD23, Kappa and Lambda. The identification of positive and negative control populations in normal peripheral blood, as well as uniform performance criteria facilitate the evaluation of the diagnostic quality and a reproducible diagnosis. The approach piloted in this study provides a comprehensive evaluation of the components of the diagnostic flow cytometry process including technical equipment and specific combinations and concentrations of reagents allowing a reproducibility and comparability among different laboratories. The results demonstrate that this goal has still to be consistently achieved, particularly with respect to the CD5 reagents.

A further component of this project was to identify a panel of reagents that may improve differential diagnosis. The identification of markers that could contribute to differential diagnosis is confounded not only by the lack of a diagnostic gold-standard, but also because new markers are often assessed along with others that may also contribute to the differential diagnosis. In this regards, it is recommended that in addition to the minimum panel, reference centres and those involved in research assess CD43, CD79b, CD81 (required also for subsequent disease monitoring and MRD assessment), as well as CD200, CD10 & ROR1 (useful for differential diagnosis of CLL vs. mantle cell lymphoma, and germinal-centre B-LPD vs. post-germin al centre B-LPD respectively)\textsuperscript{16,23,24}. This should provide a stable platform for evaluating the contribution of cellular markers to the diagnosis and prognosis in CLL.
Acknowledgements:
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Table 1: required and recommended markers for use in the diagnosis of CLL with reagent specification based on expression patterns in normal peripheral blood.

<table>
<thead>
<tr>
<th>Inclusion in Diagnostic Panel</th>
<th>Antigen</th>
<th>Expression in CLL (% pos vs. control)</th>
<th>Control Population in normal peripheral blood</th>
<th>Minimum relative fluorescence intensity of positive and negative control populations (preferred)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Required</td>
<td>CD19</td>
<td>Positive (&gt;95%)</td>
<td>CD20+ B-cells</td>
<td>≥10*</td>
</tr>
<tr>
<td></td>
<td>CD5</td>
<td>Positive (&gt;20%)</td>
<td>CD3+ T-cells</td>
<td>≥30 (≥65)</td>
</tr>
<tr>
<td></td>
<td>CD23</td>
<td>Positive (&gt;20%)</td>
<td>CD23+ B-cells</td>
<td>≥5*</td>
</tr>
<tr>
<td></td>
<td>CD20</td>
<td>Weak</td>
<td>CD19+ B-cells</td>
<td>≥10 (≥20)</td>
</tr>
<tr>
<td></td>
<td>Igκ, Igλ</td>
<td>Weak &amp; restricted</td>
<td>CD20+ B-cells</td>
<td>≥5*</td>
</tr>
<tr>
<td>Recommended</td>
<td>CD43</td>
<td>Positive (&gt;20%)</td>
<td>CD3+ T-cells</td>
<td>≥15 (≥40)</td>
</tr>
<tr>
<td></td>
<td>CD79b</td>
<td>Weak</td>
<td>CD20+ B-cells</td>
<td>≥15 (≥30)</td>
</tr>
<tr>
<td></td>
<td>CD81</td>
<td>Weak</td>
<td>CD3+ T-cells</td>
<td>≥12 (≥20)</td>
</tr>
<tr>
<td></td>
<td>CD200</td>
<td>Positive (&gt;20%)</td>
<td>CD19+ B-cells</td>
<td>≥5*</td>
</tr>
<tr>
<td></td>
<td>CD10</td>
<td>Negative (&lt;20%)</td>
<td>Granulocytes</td>
<td>≥10*</td>
</tr>
<tr>
<td></td>
<td>ROR1</td>
<td>Positive (&gt;20%)</td>
<td>B-progenitors</td>
<td>≥5*</td>
</tr>
</tbody>
</table>

Required = consensus from >75% of participants. Recommended = consensus from >50% of participants with the following exceptions determined by the steering committee and confirmed by further consensus: exclusion of FMC7 (epitope of CD20)\(^{15}\), CD38 & CD45 (used for prognostic information and gating orientation but not specifically required for diagnosis), and inclusion of ROR1 which is closely associated with CLL\(^{9,10}\) but diagnostic antibodies were not widely available at the time of the survey.

Definition of weak: median fluorescence intensity at least 20% (identified as the minimum measurable difference based on ICSH/ISLH guideline recommendations for acceptable variation due to assay imprecision and specimen stability\(^{14}\)) lower than the median expression level by normal peripheral blood B-cells. Each laboratory was requested to determine their own reference range.

* specifically validated otherwise consensus, defined as approval of all contributing authors with no disagreement on open consultation by ERIC/ESCCA members. Values refer to the relative signal on
positive vs. negative control populations required to achieve optimal separation of CLL cells from normal B-cells.
Table 2: Retrospective assessment of the proposed criteria for diagnosis of CLL

<table>
<thead>
<tr>
<th>Total CD5+ B-LPD diagnoses</th>
<th>Meeting the proposed criteria and diagnosed with CLL</th>
<th>Not meeting the proposed criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Other diagnosis, e.g. Mantle Cell Lymphoma</td>
<td>Requires MDT or trial-specific decision</td>
</tr>
<tr>
<td>Primary referral</td>
<td>9294</td>
<td>7379 (79.4%)</td>
</tr>
<tr>
<td>Trial</td>
<td>2427</td>
<td>2267 (93.4%)</td>
</tr>
</tbody>
</table>
Table 3: Assessment of the reagents and instrument set-up in different centres by evaluating the relative signal of required diagnostic markers on control samples.

The signal for each marker on the internal positive and negative controls was determined using a simple gating strategy applied to ten control cases (see figure 2). The table shows the median relative signal (range) for the cases above the clone and fluorochrome (supplier).

* indicates that the results were sub-optimal in 1-3 of the ten cases, typically reflecting issues with individual samples rather than instrument/reagent quality.

** with a shaded cell indicates that the results did not meet the specified criteria in >3/10 cases due to one or more of several factors such as clone, fluorochrome, manufacturer, equipment or operating procedures as well as factors related to the evaluation procedure such as a limitation in the proposed gating strategy with multiplex approaches (e.g. CD20 at centre 3).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>CD19</th>
<th>CD20</th>
<th>CD5</th>
<th>Kappa</th>
<th>Lambda</th>
<th>CD23</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥10</td>
<td>≥10</td>
<td>≥30</td>
<td>≥5</td>
<td>≥5</td>
<td>≥5</td>
</tr>
<tr>
<td>Centre 1</td>
<td>225 (123-479)</td>
<td>127 (51.9-183)</td>
<td>**56.3 (16.2-5892)</td>
<td>24.4 (12.6-87.6)</td>
<td>100 (44.8-302)</td>
<td>11 (7.4-17.9)</td>
</tr>
<tr>
<td></td>
<td>HD37 RPE-Cy5</td>
<td>L27 FITC</td>
<td>DK23 APC</td>
<td>Polyclonal FITC</td>
<td>Polyclonal PE</td>
<td>MMH6 FITC</td>
</tr>
<tr>
<td>Centre 2</td>
<td>5462 (4291-6393)</td>
<td>64.8 (36.6-103)</td>
<td>*41.1 (17.7-57.2)</td>
<td>**17.1 (4.9-37.6)</td>
<td>**2.9 (2.1-4.9)</td>
<td>**4 (3.1-6.8)</td>
</tr>
<tr>
<td></td>
<td>LT19 APC</td>
<td>2H7 APE-eF7B0</td>
<td>L17F12 V450</td>
<td>G20-193 APC-H7</td>
<td>1-155-2 APC</td>
<td>Tu1 FITC</td>
</tr>
<tr>
<td>Centre 3</td>
<td>2126 (85.1-14264)</td>
<td>J3-119 PE-Cy7</td>
<td>**5.4 (2.5-7.1)</td>
<td>**44.2 (2.8-102)</td>
<td>20.2 (7.1-55.5)</td>
<td>35.8 (8.4-116)</td>
</tr>
<tr>
<td></td>
<td>J3-119 PE-Cy7</td>
<td>L27 V450</td>
<td>L17F12 PerCP-Cy5.5</td>
<td>L27 FITC</td>
<td>Polyclonal PE</td>
<td>MMH6 FITC</td>
</tr>
<tr>
<td>Centre 4</td>
<td>17.9 (5.6-23.5)</td>
<td>175 (102-306)</td>
<td>237 (52.8-368)</td>
<td>35.6 (12.6-60)</td>
<td>430 (148-612)</td>
<td>*43.2 (0.8-1670)</td>
</tr>
<tr>
<td></td>
<td>SJ25C1 PerCP-Cy5.5</td>
<td>L27 FITC</td>
<td>L17F12 PE</td>
<td>TB28-2 FITC</td>
<td>1-155-2 PE</td>
<td>EBVCS-5 PE</td>
</tr>
<tr>
<td>Centre 5</td>
<td>16.5 (11.2-18.8)</td>
<td>24.6 (16.7-30.2)</td>
<td>*42.9 (15.1-56.7)</td>
<td>22.6 (10.3-65.1)</td>
<td>17.5 (10.3-24.2)</td>
<td>18.7 (8.6-31.7)</td>
</tr>
<tr>
<td>SJ25C1 PerCP-Cy5.5</td>
<td>L27 APC-H7</td>
<td>L17F12 PE-Cy7</td>
<td>G20-193 BV421</td>
<td>JDC-12 FITC</td>
<td>M-1233 BV421</td>
<td></td>
</tr>
<tr>
<td>Centre 6</td>
<td>56.8 (32.8-81.9)</td>
<td>2812 (398-5030)</td>
<td>*37.4 (24.105)</td>
<td>19.7 (11.4-65.6)</td>
<td>37.2 (24.4-105)</td>
<td>74.4 (13.6-31.7)</td>
</tr>
<tr>
<td>J3-119 PE-Cy7</td>
<td>J3-119 PE-Cy7</td>
<td>2H7 PacBlue</td>
<td>L17F12 PerCP-Cy5.5</td>
<td>L17F12 PerCP-Cy5.5</td>
<td>Polyclonal PE</td>
<td>MMH6 FITC</td>
</tr>
<tr>
<td>Centre 7</td>
<td>106 (89.9-175)</td>
<td>53.6 (41.2-67.4)</td>
<td>**26.2 (17-39)</td>
<td>22.1 (6.9-45.1)</td>
<td>149 (72.2-287)</td>
<td>16.9 (8.6-35)</td>
</tr>
<tr>
<td>SJ25C1 APC</td>
<td>53.6 (41.2-67.4)</td>
<td>L17F12 FITC</td>
<td>G20-193 BV421</td>
<td>JDC-12 FITC</td>
<td>M-1233 BV421</td>
<td></td>
</tr>
<tr>
<td>Centre 8</td>
<td>217 (130-234)</td>
<td>82 (51.2-123)</td>
<td>25.3 (10.7-80.1)</td>
<td>19.6 (7.4-74.8)</td>
<td>10.3 (5.8-14.1)</td>
<td>EBVCS-5 PE</td>
</tr>
<tr>
<td>J3-119 PE-Cy7</td>
<td>J3-119 PE-Cy7</td>
<td>2H7 Pacific Blue</td>
<td>BL1a APC</td>
<td>Polyclonal PE</td>
<td>9P25 FITC</td>
<td></td>
</tr>
<tr>
<td>Centre 9</td>
<td>*16.3 (5.5-130)</td>
<td>29.9 (18.3-58.7)</td>
<td>**5.4 (2.4-45.6)</td>
<td>*12.3 (4.2-29.7)</td>
<td>46.6 (6.5-75.5)</td>
<td>19.1 (9.8-48.4)</td>
</tr>
<tr>
<td>J3-119 PE-Cy7</td>
<td>J3-119 PE-Cy7</td>
<td>B-Ly1 FITC</td>
<td>BL1a PE</td>
<td>Polyclonal PE</td>
<td>9P25 FITC</td>
<td></td>
</tr>
<tr>
<td>Centre 10</td>
<td>31.6 (22.6-41.7)</td>
<td>82.1 (38.4-119)</td>
<td>**16.6 (3-31.5)</td>
<td>**6.1 (1.7-11.2)</td>
<td>18 (12.3-37)</td>
<td>9.1 (7.1-13.6)</td>
</tr>
<tr>
<td>J3-119 ECD (Coulter)</td>
<td>J3-119 ECD (Coulter)</td>
<td>B9E9 Pacific Blue</td>
<td>BL1a APC-AF750</td>
<td>Polyclonal PE</td>
<td>9P25 FITC</td>
<td></td>
</tr>
<tr>
<td>Centre 11</td>
<td>142 (20.2-10558)</td>
<td>99.5 (46.9-240)</td>
<td>**24.1 (9.7-45.8)</td>
<td>87.1 (33.3-3398)</td>
<td>160 (55.4-2821)</td>
<td>70.6 (31.3-290)</td>
</tr>
<tr>
<td>SJ25C1 APC</td>
<td>SJ25C1 APC</td>
<td>L27 FITC</td>
<td>L17F12 PerCP-Cy5.5</td>
<td>L27 FITC</td>
<td>1-155-2 PE</td>
<td>EBVCS-5 PE</td>
</tr>
</tbody>
</table>
Figure 1

The percentage of participants ranking each marker as required or recommended for evaluation in the diagnosis of CLL.
Figure 2

Simple gating strategy for defining positive and negative internal control populations to assess the relative signal on markers required for diagnosis according to the consensus criteria.

1. Mononuclear cell (MNC) gate
2. Identify T-cell and B-cell gates
3. Identify κ+λ- & κ-λ+ B-cell gates

Define CD23 pos/neg threshold using T-cell expression and identify CD23+ B-cells