Sponsored and reviewed by ICCS Quality and Standards Committee

Title: Identifying appropriate reagents to assess CD5 expression

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OUTLINE:

The relative signal (CD5 median fluorescence intensity on T-cells vs. polyclonal B-cells) was used as a basic measure of CD5 reagent quality. The relative normal T:B-cell CD5 signal was calculated in 25 cases with optimal vs. sub-optimal discrimination of CLL cells from normal B-cells. A target relative signal on normal T-cells vs. normal B-cells of ≥30 was identified as a threshold to achieve optimal separation of CLL cells from normal B-cells. This target was subsequently evaluated by ten centers using a series of 100 control cases and was met in 61% of cases. There are several commercial reagents available which routinely achieve a median fluorescence intensity on T-cells of ≥30 relative to polyclonal B-cells in the same sample. Suboptimal signals may reflect laboratory processes and/or reagent quality. CD5 reagents that do not achieve this target need to be independently validated for the specific diagnostic assay.

PROCEDURE/PROCESS:

1] **Experimental identification of an appropriate relative signal for CD5.**
The procedure to identify the optimal CD5 relative signal is derived from the European Research Initiative on CLL study to harmonize minimal residual disease monitoring in CLL (1)

a. Leukocytes from patients with CLL (cases with >99.5% of the B-cells having a typical CLL phenotype, n=5) were labelled with CD19 PE-Cy7 and CD3 APC-H7 and from control samples (referred for investigation of a suspected lymphoproliferative disorder but shown to have normal T/B/NK-cell subsets, n=5) were labelled with CD19 PerCP-Cy5.5 and CD3 APC-H7. After washing, cells from the CLL samples were diluted into the control cells so that CLL cells represented approximately 5-15% of total leucocytes. This procedure allows the cells being used to evaluate CD5 expression (normal T-cells, normal B-cells, and CLL cells) to be identified without using CD5 for gating purposes.
b. To simulate the impact of varying relative signal, the mixtures of normal and neoplastic cells were then incubated with CD5 at varying concentrations (neat to 1:243, serial 1:3 dilutions), washed and acquired.
c. Normal B-cells were gated using CD19 PerCP-Cy5.5 expression vs. side scatter, CLL cells were gated using CD19 PE-Cy7 vs. side scatter and T-cells were gated using CD3 APC-H7 vs. side scatter. A histogram region was set to encapsulate 99% of CLL cells (excluding 1.0% of cells with the weakest CD5 fluorescence) and the percentage of normal B-cells falling within the region was calculated at each antibody dilution in all of the cases, with the histogram region on the control B-cells re-calculated for each antibody dilution in each case. The increase in percentage of normal B-cells overlapping with CLL cells was calculated for each antibody concentration, with an increase in expression overlap by more than 5.0% being defined as inadequate separation.
d. Relative signals were calculated for each antibody concentration as median fluorescence intensity from the positive control T-cell population divided by median fluorescence intensity for the negative control B-cell population (Fig 1).

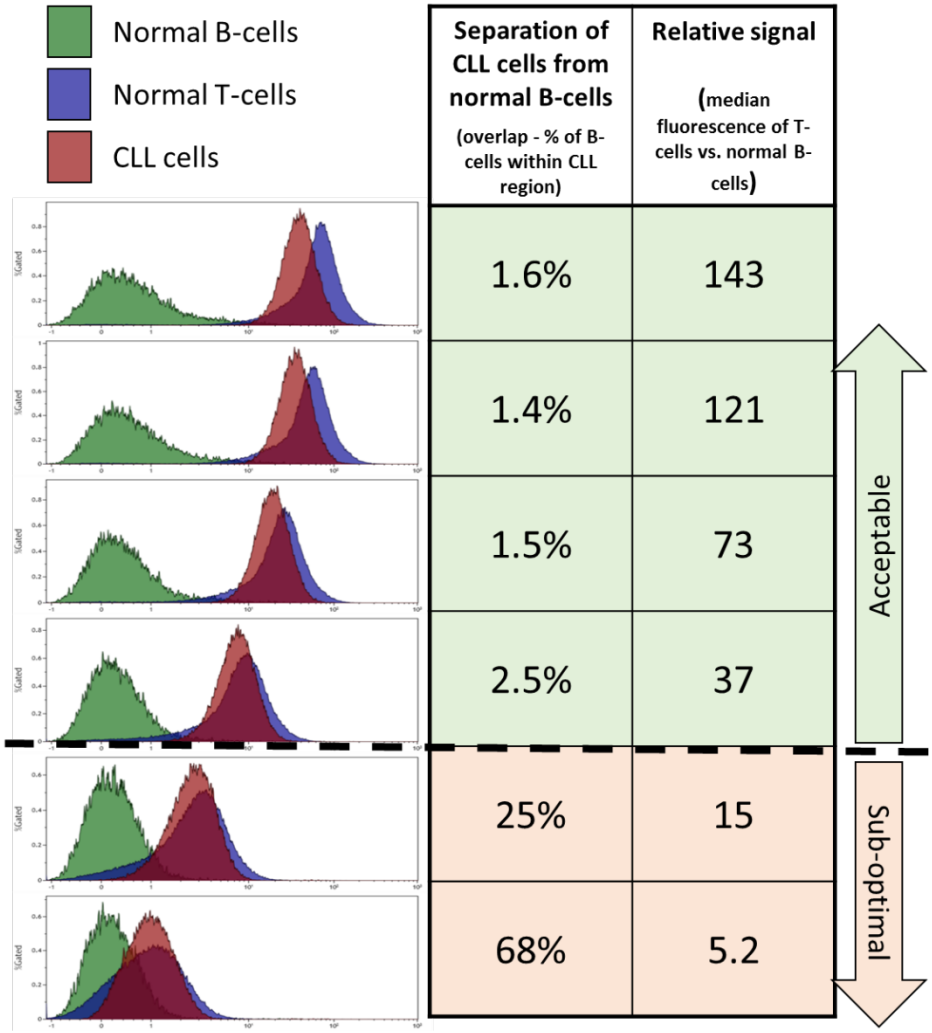
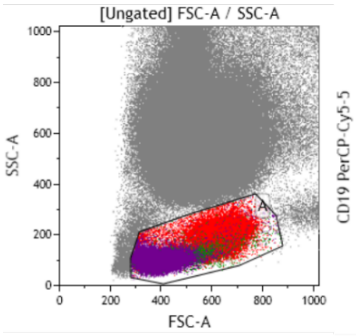


Figure 1. Evaluation of the optimal characteristics of a CD5 reagent

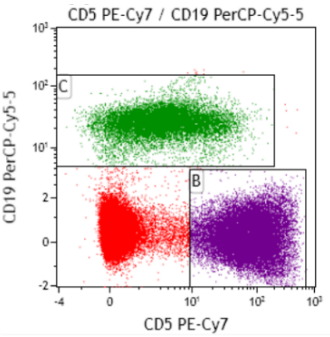
2] **Validation of the proposed optimal relative signal: Diagnosis of CLL.**
The minimum required signal was subsequently evaluated for the diagnosis of CLL in a project to develop harmonized guidelines for the reproducible diagnosis of Chronic Lymphocytic Leukemia by flow cytometry by ERIC & the European Society for Clinical Cell Analysis (ESCCA)(2).

a. A simple gating strategy to identify the expression levels of component markers on control peripheral blood lymphocytes was developed (Fig 2a).
b. The gating strategy was distributed to participating laboratories who tested it on ten historical cases with polyclonal B-cells.
c. The median fluorescence intensity for CD5 expression on defined positive and negative control populations were recorded by ten different laboratories and returned for central analysis.
d. Overall results for median relative signal and range across the ten cases is shown in Figure 2b. The relative signal met the specified target in 63/100 cases. Three of the ten participants had relative signals that were substantially below the target in the majority of cases, while three centers met the specified criteria in at least 8/10 of their cases. The

remaining 4/10 centers had a proportion of borderline cases, i.e. between 2 and 5 of their ten cases had a relative signal below target.



(1) Mononuclear cell (MNC) gate



(2) Identify T-cell and B-cell gates

	Relative CD5 signal
Centre 1	*56.3 (16.2-5892) DK23 APC (DAKO)
Centre 2	*41.1 (17.7-57.2) L17F12 V450 (BD)
Centre 3	44.2 (2.8-102) L17F12 PerCP-Cy5.5 (BD)
Centre 4	237 (52.8-368) L17F12 PE (BD)
Centre 5	*42.9 (15.1-56.7) L17F12 PE-Cy7 (BD)
Centre 6	*37.2 (24.4-105) L17F12 PerCP-Cy5.5 (BD)
Centre 7	**26.2 (17.9-39) L17F12 FITC (BD)
Centre 8	88.6 (51-123) BL1a APC (Coulter)
Centre 9	**5.4 (2.4-45.6) BL1a PE (Coulter)
Centre 10	**16.6 (3-31.5) BL1a APC-AF750 (Coulter)

Figure 2. Simple gating strategy to evaluate relative signal of a proposed minimum antibody panel for CLL diagnosis showing the relative CD5 signal obtained in practice in ten different centers. Centers with 9 or more cases having optimal separation of T-cells from normal B-cells are highlighted in green, centers with 5 or fewer cases having optimal separation are highlighted in red, with intermediate centers [6-8 cases with optimal separation] highlighted in blue.

3]. **Conclusion and comments.** CD5 requires a more stringent specification for MRD analysis than many centers use routinely for diagnosis. A possible explanation is that CD5 is expressed weakly on a proportion of normal B-cells and therefore optimal separation of CLL cells from all normal B-cells requires a greater relative signal than separation of CD5-positive T-cells from CD5-negative background or unstained cells. However, it is possible to obtain a relative signal of >30 in routine multiparameter panels and therefore this should be a target.

- The validation procedure for reagents in CLL diagnosis used the test reagent for gating, however if it is not possible to distinguish a relatively discrete CD5+CD19- population in the majority of normal peripheral blood samples then the reagent is by definition sub-optimal.
- Relative signal depends not only on the reagent quality but on the instrument settings, compensation and laboratory procedure. A reagent with an optimal relative signal in one situation may have sub-optimal results for a different assay or laboratory.. The relative signal should not be the only criteria for acceptability of a CD5 clone &/or fluorochrome. Other applications may require a higher (or lower) relative signal, e.g. reagents with high relative signals may be less suitable, due to increased compensation issues or high background signals. The reagent must be re-validated for each application in each laboratory
- Please submit validation data for CD5 reagents (clones and fluorochromes) in additional applications, or requests for ICCS Q&S group to undertake validation, to info@cytometry.org

4]. **Recommendation for laboratories: assess your CD5 reagents on a number of reactive cases using the simple gating strategy outlined in Figure 2. If the relative signal on T-**

cells vs. B-cells is <30 in more than 20% of cases, re-evaluation of the reagents and procedures is warranted.

Special Thanks to: ICCS, ERIC and ESCCA participants and ICCS Q&S committee

Reviewed and approved by:
Fiona Craig, Mike Keeney & Andrea Illingworth

References:

1. Rawstron AC, Fazi C, Agathangelidis A, Villamor N, Letestu R, Nomdedeu J, et al. A complementary role of multiparameter flow cytometry and high-throughput sequencing for minimal residual disease detection in chronic lymphocytic leukemia: an European Research Initiative on CLL study. Leukemia. 2015 Dec 7;

2. Rawstron AC, Kreuzer K-A, Soosapilla A, Spacek M, Gambell P, McIver-brown N, et al. Reproducible Diagnosis of Chronic Lymphocytic Leukemia (CLL) By Flow Cytometry: An European Research Initiative on CLL (ERIC) & European Society for Clinical Cell Analysis (ESCCA) Harmonisation Project. Blood [Internet]. 2015;126(23). Available from: http://www.bloodjournal.org/content/126/23/4146