Comparison of Euroflow and Conventional Flow Cytometry Protocols for Minimal Residual Disease Detection in Pediatric B-Cell Acute Lymphoblastic Leukemia

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ABSTRACT

Acute Lymphoblastic Leukemia (ALL) is the most frequent hematological malignancy in childhood, and its prognosis is strongly linked to the monitoring of Minimal Residual Disease (MRD). Flow cytometry (FC) is the gold standard for MRD detection, but methods vary widely between laboratories, often leading to discrepancies in sensitivity and reliability. This study aimed to quantitatively and qualitatively compare two flow cytometry protocols—the standardized **EuroFlow** method and a conventional, laboratory-developed protocol—for MRD detection in pediatric B-cell ALL patients. A total of 228 bone marrow samples from 76 pediatric patients at IREN SUR were analyzed. The results, benchmarked against an external reference (INSNSB protocol), showed that 35.5% of patients were MRD-positive, predominantly in the female and older pediatric (11–15 years) subgroups. The EuroFlow protocol demonstrated a consistent **100% accuracy** across all age and sex strata, whereas the conventional method showed diminished accuracy, falling to 83.3% in the female 11–15 years group. Crucially, both protocols maintained **100% specificity (precision)**. Correlation analysis indicated perfect diagnostic concordance for EuroFlow (kappa=1.000) and superior quantitative correlation with the reference for normal B-lymphoblasts (R=0.9798). These findings conclusively establish EuroFlow as the more reliable and robust protocol for MRD detection in a clinical setting, recommending its adoption to standardize diagnostic practice and improve relapse prediction.

Keywords: Acute lymphoblastic leukemia, minimal residual disease, flow cytometry, EuroFlow, standardization.

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) remains the most prevalent cancer among children worldwide. Advancements in multi-agent chemotherapy have dramatically improved five-year survival rates, which now approach 90% in high-income countries. However, relapse continues to be the leading cause of treatment failure and mortality. The accurate and timely detection of residual leukemic cells, known as Minimal Residual Disease (MRD) or Measurable Residual Disease (MRD), is universally recognized as the single most critical prognostic factor in ALL management [7], [9]. MRD status—typically defined by a threshold of $\geq 0.01\%$ or 10^{-4} leukemic cells—guides risk stratification and treatment intensity adjustments, particularly in post-induction and consolidation phases.

Multiparametric Flow Cytometry (MFC) has become the preferred technique for MRD monitoring due to its high sensitivity (capable of detecting 10^{-4} to 10^{-5} cells), speed, and relatively low cost compared to molecular methods like PCR [11]. MFC allows for precise characterization of leukemic cells by simultaneously analyzing multiple antigenic markers to identify aberrant and immature cell populations. It relies on identifying Leukemia-Associated Immunophenotypes (LAIPs) or tracking the aberrant expression patterns of B-cell precursor antigens, such as CD10, CD19, CD34, and CD45, to discriminate between residual leukemic blasts and normal regenerating B-cell precursors [8].

Despite its utility, a major challenge in MFC for MRD is the high variability in results across laboratories, often stemming from differences in antibody panels, sample preparation, compensation settings, and data analysis

strategies [6]. To address this, the European consortium **EuroFlow** developed and validated highly standardized, robust, and reproducible protocols. The EuroFlow approach mandates the use of pre-mixed, highly optimized 8 to 12 color antibody combinations, standardized instrument setup and quality control (using CS&T beads), and the "Bulklysis" technique for sample preparation, ensuring optimal cell recovery and reduced technical variation [10], [12].

This study was designed to provide evidence-based validation for implementing standardized protocols in a regional oncopediatric center. Specifically, we conducted a head-to-head comparison of the standardized EuroFlow protocol against a conventionally used, non-standardized protocol for detecting MRD in pediatric B-cell ALL patients at IREN SUR, with the objective of determining which method provides superior efficiency in terms of accuracy and precision against an external reference laboratory standard (INSNSB protocol).

METHODOLOGY

Study Design and Population

This investigation employed a comparative, cross-sectional design at an explanatory level, analyzing the performance metrics of two flow cytometry protocols. The study population consisted of pediatric patients (aged 3 to 16 years) with confirmed B-cell ALL diagnosis undergoing post-treatment follow-up at the Instituto Regional de Enfermedades Neoplásicas del Sur (IREN SUR), Arequipa, Peru.

A total of 228 bone marrow samples were collected as duplicates from 76 patients during their routine check-ups between July 2023 and June 2025. Inclusion criteria mandated a confirmed B-cell ALL diagnosis and the availability of fresh bone marrow aspirate. Exclusion criteria involved samples with inadequate cellularity or those processed more than 24 hours after extraction. The INSNSB protocol served as the external gold standard for diagnostic validation.

Sample Processing and Flow Cytometry

Bone marrow samples (5–6 mL) were collected in K_3 EDTA tubes. All samples were subjected to routine quality control for cellular viability. One portion of the bone marrow aspirate was processed concurrently via both the conventional and EuroFlow protocols at IREN SUR, while the duplicate was sent to the reference laboratory (INSNSB). Samples were aliquoted, codified, and processed. The FACSCanto II flow cytometer was validated and prepared daily.

1) EuroFlow Protocol:

The standardized EuroFlow approach utilized an optimized 8 to 12 color panel targeting key B-cell lineage markers and LAIPs (e.g., CD10, CD19, CD34, CD38, CD45, CD20, CD81, CD66c/CD123, CD73/CD304). The crucial technical difference was the implementation of the Bulklysis technique. This method involves a bulk red blood cell lysis step followed by staining, which is known to significantly maximize the number of total events collected, improving the statistical power for detecting rare events. A stringent minimum of 1×10^6 events was acquired using a FACSCanto II flow cytometer. Data analysis was performed using the standardized EuroFlow gating strategy within Infinicyt software.

2) Conventional Protocol:

This protocol used a commercial antibody panel with markers similar to the EuroFlow panel. However, sample preparation relied on a traditional lysis and wash method, which often results in lower cell yields compared to Bulklysis. The minimum event acquisition target was lower, typically 5×10^5 events. Analysis was conducted manually using standard gating protocols, which are more susceptible to inter-operator variability.

Statistical Analysis

The primary outcome was the MRD status, dichotomized into Positive ($\geq$ 0.01% leukemic cells) or Negative (<0.01% leukemic cells). Samples were stratified by age group (1–5, 6–10, and 11–15 years) and sex.

Performance metrics were calculated using the INSNSB result as the true status:

- Accuracy: Measures the proportion of correct classifications (True Positives + True Negatives) relative to all cases:

Exactitud=(VP+VN) /(VP+VN+FP+FN)

- Precision (Positive Predictive Value): Measures the probability that a positive test result is truly positive:

Precision=(VP)/(VP+FP)

- Diagnostic Concordance: Assessed using Cohen's Kappa (κ) coefficient.
- Quantitative Correlation: Assessed using the Pearson correlation coefficient (*R*).

RESULTS

Patient Demographics and MRD Prevalence

Among the 76 pediatric patients, 27 were categorized as MRD-positive (≥ 0.01%), resulting in an overall prevalence of 35.5%. Positive cases were slightly higher in females (14 cases) than males (13 cases). The highest frequency of positive MRD was observed in the 6-10 years age group (14 cases) and the 11-15 years group (10 cases).

Quantitative Comparison of Protocols

The mean percentages ($\bar{X}$ ± D.E.) of pathological and normal B-lymphoblasts were compared. While the EuroFlow protocol consistently yielded slightly higher mean values across all subgroups (Pathological: 28.77 ± 37.54 vs. Conventional: 27.85 ± 36.46), statistical testing confirmed no statistically significant difference in the quantitative detection of blasts between the two protocols ($p > 0.05$).

Accuracy and Precision Analysis

The comparative performance against the INSNSB reference protocol showed critical differences in diagnostic accuracy:

Protocol	Overall Accuracy	κ Index (Diagnostic Concordance)
EuroFlow	100%	1.000 (Perfect)
Conventional	97.37%	0.942 (Almost Perfect)

The EuroFlow protocol achieved perfect accuracy (100%) across all age and sex subgroups, indicating zero false negatives (FN). In contrast, the conventional protocol's accuracy dropped in specific subgroups, notably to 90% in males aged 6–10 years and 83.3% in females aged 11–15 years, due to the presence of false negatives. Both protocols demonstrated exceptional precision (100% Positive Predictive Value) across all subgroups.

Correlation Coefficients

The Pearson correlation coefficient (R) for the quantitative detection of B-lymphoblasts showed very high association for all comparisons ($p<0.05$). For Normal B-lymphoblasts, EuroFlow showed a superior correlation with the control (R=0.9798) compared to the conventional protocol (R=0.9460).

DISCUSSION

The primary finding of this study is the statistically and clinically significant superiority of the EuroFlow protocol in terms of diagnostic accuracy and concordance. Achieving a 100% accuracy and perfect Kappa agreement (kappa=1.000) with the gold standard reference protocol positions EuroFlow as the unequivocally reliable method for MRD detection in this cohort.

The observed decline in the conventional protocol's accuracy in specific age and sex subgroups (e.g., 83.3% in females 11-15 years) is critical, as it indicates a propensity for generating false negative results. In ALL monitoring, a false negative result carries a high clinical risk, potentially leading to treatment errors and relapse. The technical advantages of EuroFlow, such as the Bulklysis technique and the standardized gating strategies, are likely responsible for its enhanced ability to reliably detect low-frequency MRD cells across all demographic variations.

While the quantitative results showed no statistically significant difference in the *percentage* of blasts detected ($p>0.05$), the qualitative difference in diagnostic *accuracy* is paramount. The study validates the international push for standardization in flow cytometry. The adoption of the EuroFlow protocol can significantly minimize technical noise and maximize the clinical utility of MRD results, leading to improved, evidence-based clinical decision-making.

CONCLUSIONS

1. The overall prevalence of Minimal Residual Disease ($\geq 0.01\%$) in the studied pediatric B-ALL cohort was 35.5%, with a slight predominance in the female and older pediatric (11–15 years) subgroups.

2. Quantitatively, the EuroFlow and conventional protocols showed equivalent mean percentages of pathological B-lymphoblasts, but only EuroFlow achieved a statistically perfect diagnostic concordance.

3. The EuroFlow protocol demonstrated 100% accuracy and 100% precision across all age and sex strata, confirming its superior reliability.

4. The conventional protocol showed a notable decrease in accuracy (as low as 83.3%) due to false negative results, establishing EuroFlow as the technically superior and clinically more reliable protocol for MRD detection in the B-cell ALL population.

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