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Minimal/Measurable Residual Disease Detection in Acute Leukemias by Multiparameter Flow Cytometry

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Abstract

Purpose of Review Minimal or measurable residual disease (MRD) detected by multiparameter flow cytometry (MFC) is an independent prognostic indicator in acute leukemia. However, the predictive value of MFC MRD is affected by technical challenges, interpretive complexities, and inadequate standardization, particularly in acute myeloid leukemia (AML). Here, we critically review the methodological principles of the MFC MRD assay and discuss clinical implications of MRD.

Recent Findings Key components of MFC MRD assays to be discussed include the principles of MFC, panel selection, analysis approaches, level of quantifiable MRD and calculation, reporting, and areas of improvements. Key components of clinical implications include context-dependent detection threshold and the integral role of MRD assessment by MFC in the era of ever-expanding molecular testing.

Summary With advancements in technology and standardization, MFC along with molecular assays will continue to play an important role in MRD assessment to evaluate treatment response and risk stratification.

Keywords Acute myeloid leukemia · Lymphoblastic leukemia · Minimal residual disease detection · Measurable residual disease detection · Immunophenotype · Multiparameter flow cytometry · Real-time quantitative polymerase chain reaction · Next-generation sequencing

Introduction

Acute leukemia is a heterogeneous group of clonal hematopoietic stem cell neoplasms that include various forms of acute myeloid leukemia (AML) and precursor (acute) lymphoblastic leukemia (ALL). Despite improved survival with intensive chemotherapy, acute leukemia variably relapses after remission. Growing evidence demonstrates that the presence of minimal or, more appropriately, *measurable* residual disease (MRD) after induction and/or consolidation chemotherapy is an independent predictor for an increased risk of relapse and shortened survival in AML [1–9] and ALL [10–15]. MRD status is likely related to the genetic characteristics of the particular acute leukemia and intrinsic factors for the particular patient (such as drug metabolism) that affect response to therapy. Detecting MRD thus provides an objective methodology for assessing remission status and for guiding risk-tailored post-remission chemotherapy.

MRD is defined as the persistence of leukemia cells far below the morphology-based threshold of 5% blasts (1 in 20 cells). MRD can be detected at levels as low as $1{:}10^3$ to $1{:}10^6$ of total white blood cells (0.1 to 0.0001%). The most widely used techniques to assess MRD are multiparameter flow cytometry (MFC) and molecular techniques [e.g., real-time quantitative polymerase chain reaction (RT-qPCR) and the newer techniques (next-generation sequencing, NGS) and digital PCR]. The utility of each of these techniques differs depending on the genetics of the particular neoplasm, the timeline of the testing, and the sensitivity of the assay. Sensitivity for MRD analysis ranges typically from 0.1 to 0.001% for MFC and 0.001 to 0.0001% for molecular techniques. At this time, an integrated approach using various combinations of these tests is likely to be the most useful for providing effective and comprehensive assessment of treatment response [3, 16•, 17, 18]. This review focuses on the

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methodological principles of the MFC MRD assay, the clinical implications of MRD, and the current role of MFC MRD in assessing disease response in the era of ever-expanding molecular testing.

Principles of MRD Detection by MFC

Normal hematopoietic cells demonstrate a consistent and reproducible spectrum of antigen expression during maturation. Neoplastic leukemia cells demonstrate patterns of antigen expression similar to their normal myeloid or lymphoid counterparts but also show distinct immunophenotypic deviation from normal [19–22]. This deviated pattern is the precept by which MFC identifies and characterizes acute leukemia, the so-called difference-from-normal (DFN) pattern. Specifically, DFN is the different pattern of marker expression that neoplastic blasts show from normal myeloblasts in AML, normal B-lymphoid progenitors (i.e., hematogones) in B-ALL, and normal T-lymphoid progenitors (i.e., thymocytes) in T-ALL. At initial diagnosis, the DFN establishes a "fingerprint" or "leukemia-associated immunophenotype (LAIP)" for the neoplastic population. This DFN/LAIP is a set of aberrancies and may include (a) the abnormal expression of antigens not typically expressed by the particular cell type, (b) the over/under-expression of normally expressed antigens, and/or (c) the asynchronous expression of normally expressed antigens. The accuracy of MFC MRD analysis can be maximized through a comprehensive integrated approach that utilizes both the LAIP and the DFN.

Methods for MRD Assessment: Samples, Instrument, and Panels

The general protocols for MFC testing including instrument set-up, specimen processing, and data interpretation are available in several excellent publications [23–30]. As MRD testing is a semi-quantitative assay for enumerating rare populations, special cautionary steps on sample type and preparation, instrument set-up, and antibody panels are advisable to ensure high quality and consistent data.

Samples

In AML and B-ALL, MRD levels tend to be one or more logs higher in bone marrow (BM) aspirate than in peripheral blood (PB) [17, 31–34] whereas for T-ALL, MRD levels are comparable in BM and PB [31, 35]. In general, BM aspirate is typically the preferred specimen for MRD analysis. However, PB is a readily obtainable specimen; therefore, it is used at an early time point of treatment to assess the kinetics of leukemia cell clearance in post-induction day 8 B-ALL per the Children's Oncology Group (COG) protocol [11, 36–38]. Collecting BM aspirate is more technically challenging than PB. When obtaining the BM sample, variable degrees of hemodilution are inevitable. Submitting the first BM pull with as low as needed BM volume (typically with only 1–2 ml) for MRD analysis can lessen PB contamination [3, 23]. BM samples should be anticoagulated with ethylenediaminetetraacetic acid (EDTA) or sodium heparin, transported at room temperature, and processed in a timely manner (i.e., ideally within 48 h of collection).

Instrument

During sample acquisition on the cytometer, "carryover" of stained cells from one tube to the next can result in significant artifact and create difficulty in accurate assessment for MRD. Such carryover can be minimized or eliminated by running an aliquot of sheath fluid between each sample aliquot.

Panels

Characterization of hematolymphoid neoplasms and identification of MRD are facilitated through the use of sufficiently broad (preferably ≥ 8 colors) antibody panels at diagnosis and follow-up [2, 19–21, 39].

For AML MRD, several informative monoclonal antibody panels (Table 1) have been recommended by experts [39, 40]. These panels typically include stem cell and progenitor markers (CD34 and CD117), myelomonocytic and erythro-megakaryocytic markers (CD11b, CD13, CD14, CD15, CD33, CD36, and CD64), cross-lineage lymphoid markers (CD2, CD7, CD19, or CD56), and non-lineage specific markers (CD38, CD45, and HLA-DR) [3, 23] (see Fig. 1a for an example of an MFC MRD analysis in AML using the University of Texas Southwestern Medical Center (SWMC) MRD panel (Table 1C)).

As relapses in AML are thought to originate from the outgrowth of leukemic stem cells (LSC), MFC assessment of the frequency of LSC is of importance for relapse prediction. Recently, a one-tube approach for $CD34^{+}/CD38^{-}$ leukemic stem cells including the newer markers CD96 [41], CLL-1 (hMICL) [42, 43], and TIM3 has been proposed [44, 45].

In our experience, assessing MRD in monocytic AML is generally more challenging than in non-monocytic AML, which is largely due to the fact that neoplastic immature monocytes often do not express immature cell markers (e.g., CD34 and CD117) and lack expression of the monocytic marker CD14. However, they typically maintain the expression of CD15, CD33, CD36, and CD64 at levels close to normal mature monocytes [46, 47] with subtle deviation (e.g., an

Table 1 Immunophenotyping panels for the detection of residual/measurable acute myeloid leukemia (AML)

A. Antibodies recommended from ELN working Group [3]

Use the following markers in an MRD panel: CD7, CD11b, CD13, CD15, CD19, CD33, CD34, CD45, CD56, CD117, HLA-DR (backbone: CD45, CD34, CD117, CD13, CD33
If necessary, adding a "monocytic tube" containing: CD64/CD11b/CD14/CD4/CD34/HLA-DR/CD33/CD45

B. Panels from "Flow Cytometric Monitoring of Residual Disease in Acute Leukemia" by B. Wood [39].

	PB	FITC	PE	PE-TR	PCX	PC7	A594	APC	APC-A700	APC7
Tube 1	HLA-DR	CD15	CD33	CD19	CD117	CD13	CD38	CD34	CD71	CD45
Tube 2	HLA-DR	CD64	CD123	CD4	CD14	CD13	CD38	CD34	CD16	CD45
Tube 3	HLA-DR	CD56	CD7	–	CD5	CD33	CD38	CD34	–	CD45

These combinations require a 4-laser flow cytometer having excitation at 407 nm (PB), 488 nm (FITC, PE, PE-TR, PCX, PC7), 594 nm (A594), and 635 nm (APC, A700, APC7).

C. Panels proposed from University Texas Southwestern Medical Center (UTSWMC)

	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-R700	APC-H7	BV421	V500c	BV605
1	CD36	CD34	CD16	CD38	CD11b	CD64	CD7	CD56	CD45	CD13
2	CD15	CD33	-	CD19	CD14	CD34	HLA-DR	CD117	CD45	CD123

These combinations require a 3-laser flow cytometer (BD FACSCanto, 10-color platform) having excitation at 405 nm (BV421, V500, BV605), 488 nm (FITC, PE, PerCP5.5, PC-Cy7), 640 nm (APC, APC-700, APC-H7).

UTSWMC tubes 1C and 2C are designed to allow detailed analysis of nearly all myeloid cells in a sample including maturation spectrums for stem cells/myeloblasts, granulocytes, and monocytes. Expert knowledge of these maturation spectrums and of expected immunophenotypic variation secondary to reactive/regenerative conditions facilitates identification of difference from normal (DFN) for these myeloid populations

• Neoplastic/immature monocytic cells often maintain the expression of CD15, CD33, CD36, and CD64 at the levels close to normal mature monocytes but downregulate to completely lose CD14. The inclusions of CD36/CD64 and CD15/CD33/CD14 in combination with CD56 may allow for identification of immature/neoplastic monocytic cells as the number of neoplastic monocytes defined by $CD14^+$ is frequently lower than that defined by $CD15^+/CD33^+$ and/or $CD36^+/CD64^+$. This discrepancy is indicative of loss of CD14, a common feature of aberrancy. In addition, neoplastic cells often express CD56 in a high proportion of the monocytes

• The inclusion of CD19 in tube 2C allows for identification of possible biphenotypic populations or AML with t(8;21)

increased proportion of $CD15^{bright+}$ cells). Therefore, the inclusion of CD36/CD64 and CD15/CD33/CD14 in combination with CD56 (Table 1C) may aid in identification of immature neoplastic monocytes. A useful technique to identify the neoplastic monocytes is to compare the proportion of monocytes identified by $CD15^+/CD33^{bright+}$ and/or $CD36^+/CD64^+$ versus that identified by $CD14^+$. The proportion of $CD14^+$ monocytes is frequently significantly lower, indicative of CD14 loss, which although not specific is a common feature of neoplastic immature monocytes. Additionally, the CD56 expression when present in a high proportion of the monocytes is often seen on neoplastic monocytes [21].

For ALL MRD, several informative monoclonal antibody panels (Table 2) have been recommended. These panels typically include CD34, CD38, and CD45 and may include TdT. For B-ALL, B cell-related markers (CD10, CD19, CD20, CD22, surface Ig, and CD79a) and myeloid markers (CD13 and CD33) are also included [42]. In an effort to improve discrimination of B lymphoblasts from hematogones in difficult cases, additional markers such as CD9, CD24, CD44, CD58, CD81, and CD123 have been proposed [10]. Furthermore, establishing a CD81/CD58 expression ratio may also aid in identification of B lymphoblasts [50] (see Fig. 1b for an example of an MFC MRD analysis in B-ALL using the UTSWMC MRD panel).

Identifying T-ALL MRD is often easier than B-ALL because normal thymocytes are not generally found outside of the thymus. However, subsets of reactive mature T

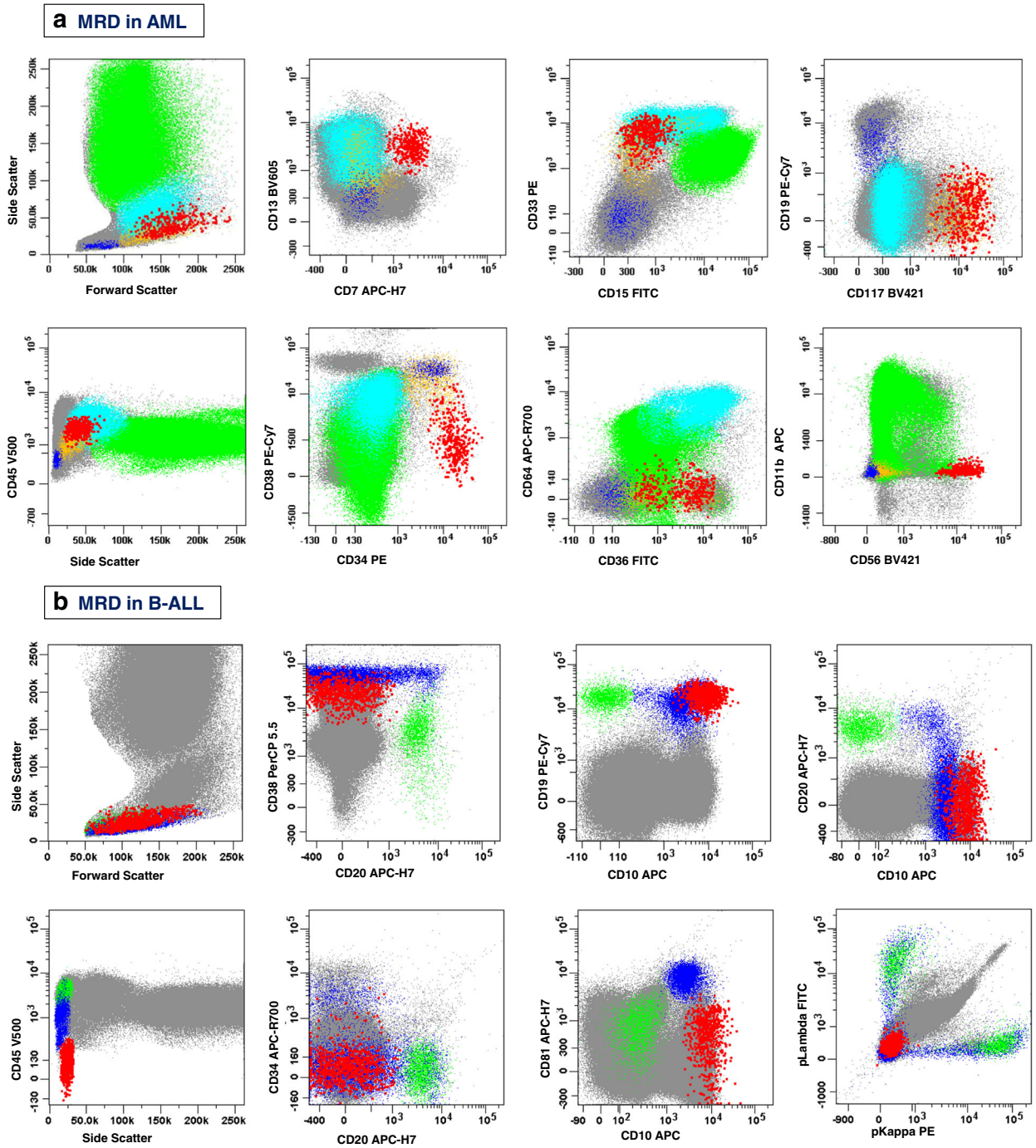

Fig. 1 **a** Minimal residual disease (MRD) in acute myeloid leukemia (AML) using the University of Texas Southwestern Medical Center (UTSWMC) AML MRD panel in Table 1C. Using an integrated "leukemia-associated immunophenotype (LAIP)"-based "different-from-normal (DFN)" approach in a bone marrow (BM) sample with collection of 300,000 events, two populations of $CD34^+$ myeloblasts are identified, one population of normal myeloblasts (in yellow, 0.33% of $CD45^+$ cells), and the other population of residual neoplastic myeloblasts (in red, 0.13% of $CD45^+$ cells). The neoplastic myeloblasts demonstrate the LAIP of $CD7^+$/$CD13^{\text{uniformly strong }+}$/$CD33^{\text{slightly bright }+}$/$CD34^{\text{slightly bright}+}$/$CD38^{\text{dim}+}$/$CD56^+$. This LAIP is distinctly different from the immunophenotype of normal myeloblasts (by DFN). Granulocytes are colored green, monocytes light blue, and stage I $CD34^+$ hematogones blue. **b** MRD in B-lymphoblastic leukemia (B-ALL) using UTSWMC B-ALL MRD panel in Table 2B (tubes 1A and 3). Using a DFN approach in a BM sample with collection of 500,000 events, two populations of immature B-lineage cells are identified: a population of normal hematogones (in blue, 3.0% of total viable cells) and a population of persistent B lymphoblasts (in red, 0.18% of total viable cells). The B lymphoblasts resemble stage 1 hematogones for several markers including CD10, CD20, and surface Ig light chain; however, they show distinct deviation from normal. For instance, the patterns of CD10/CD19 expression and CD10/CD34 expression are abnormal: at the level of intensity of CD10 expression (i.e., $CD10^{\text{bright }+}$), normal expression of CD19 would be dim and CD34 would be positive. Here, the lymphoblasts are $CD10^{\text{bright }+}$ with aberrantly bright expression of CD19 and no expression of CD34. Other aberrancies (DFN) in this case included $CD38^{\text{slightly dim}+}$/$CD45^-$/$CD81^{\text{dim}+\text{ to }-}$. Mature B lymphocytes are colored green

lymphocytes and/or NK lymphocytes may show immunophenotypic features resembling features sometimes identified on T lymphoblasts (e.g., expansions of CD4/CD8 double-positive T cells or the presence of CD117(+) NK cells) in BM and PB, and normal immature T cells can rarely be identified outside the thymus (e.g., ectopic thymic tissue). Therefore, an adequate panel to allow comprehensive DFN/LAIP analysis for T-ALL MRD typically includes CD1a, CD2, surface CD3, CD4, CD5, CD7, CD8, CD10, CD45, and CD56. The inclusion of CD56 is sometimes useful in identifying T-ALL MRD, since normal immature T cells lack CD56. Additionally, CD56 is a negative prognostic marker in T-ALL [51]. Inclusion of cytoplasmic CD3 and the markers of immaturity (CD34 and TdT) may also increase accuracy in T-ALL MRD analysis [52], particularly in difficult cases. Notably, in our experience, the DFN in T-ALL nearly always involves deviation from the normal CD4/CD8 expression pattern and/or the normal CD3/CD1a expression pattern seen on thymocytes [53].

The advent of novel therapeutic agents that target specific cell antigens has created unique challenges for MRD analysis. For instance, chimeric antigen receptor T cell (CART)-19 in B-ALL therapy targets the CD19 molecule expressed by normal B-lineage cells and B lymphoblasts. Following therapy, emerging neoplastic B cell clones often lack expression of CD19. Since CD19 is the most commonly used gating marker for B-lineage cells, this creates a problem for rigid sequential gating analysis. To accommodate for such problems, new MFC MRD approaches include additional gating B cell markers such as CD22, CD24, and/or CD79a. However, further therapeutic intervention directed against such alternative gating markers and heterogeneous antigenic expression among neoplastic populations (e.g., decreased expression of CD22 in *KMT2A*-rearranged B-ALL) [54] can create additional difficulty in MRD analysis. Therefore, a well-designed antibody panel in addition to a flexible analysis that allows identification of MRD based on multiple immunophenotypic characteristics is likely the best approach for identifying MRD in the era of immunologic therapies. Examples of post anti-CD19 MRD panels are presented in Table 2C and can be found in the literature [55].

Methods for MRD Assessment: Data Analysis and Identification of Leukemia Cells

Data Analysis

Initial MFC assessment should focus on data quality by minimizing artifacts through removal of air bubbles, nonviable cells, and doublets [21, 39]. Various analytical software programs are available and can provide similar results; however, a flexible analysis approach such as that described by Shaver et al. and Reichard et al. [56, 57] rather than a rigid sequential gating approach improves the sensitivity and specificity of the analysis. Our laboratory analyzes MFC data by the flexible analysis approach known as cluster analysis using CytoPaint™ Classic software. Cluster analysis requires a high level of expertise but is particularly well suited for analyzing complex immunophenotypic patterns within complex cellular mixtures [21, 57], such as during MRD analysis or analysis of post CAR-T/immunologic therapy-treated specimens. In recent years, a variety of advanced computational tools have been developed to identify specific cell populations in high-dimension (≥ 8 colors) MFC data sets [58] including principal component analysis (PCA) that is implemented in software such as Infinicyt™ [59].

Identification of Leukemia Cells Using an Integrated DFN/LAIP Approach

The recommended strategy for data analysis is to use an integrated DFN/LAIP approach, which is adopted by the European LeukemiaNet (ELN) MRD Working Party [3]. While the LAIP is often useful in subsequent cases to identify persistent and recurrent disease, the LAIP may not be available during subsequent evaluation and neoplastic populations may undergo an immunophenotypic shift after therapy [60–63]. The DFN approach can be applied to all cases with or without diagnostic immunophenotypic information and allows detection of new aberrancies, immunophenotypic shifts [62, 64], clonal evolution, or clonal selection [17, 65, 66]. The integrated "LAIP-based DFN approach" works very well for defining MFC MRD burden and validating the prognostic impact of emerging aberrancies. In practice, MFC MRD analysis most often focuses upon DFN with the assistance of the LAIP when available.

Methods for MRD Assessment: Establishing Sensitivity and Reporting

The sensitivity of the MFC MRD assay is dependent on multiple factors: the number of cells collected, the antibody panels used, the level of immunophenotypic aberrancies on the leukemic blasts (i.e., DFN from normal myeloid or lymphoid progenitors), the number of normal progenitors of similar type present in the background (e.g., normal myeloid progenitors in AML MRD testing and hematogones in B-ALL MRD testing), and the expertise of the analyst. In practice, the higher the degree of the DFN, the easier it is to separate neoplastic cells from normal cells of similar lineage. Routine MFC MRD analysis achieves a sensitivity of 0.01% for ALL and 0.1% for AML.

Table 2 Immunophenotyping panels for the detection of residual/measurable acute precursor lymphoblastic leukemia (ALL)

A. B-ALL MRD panel from the Children's Oncology Group (COG) [11]

	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-R700	APC-H7	BV421	V500c	BV605
1	CD20	CD10	CD38	CD19	CD58	-	CD45	CD56	-	-
2	CD9	CD13 +CD33	CD34	CD19	CD10	-	CD45	CD117	-	-
3	Syto16		CD3	CD19	CD71		CD45			

COG Tubes 1 and 2 are designed to identify B-lymphoblasts by recognizing deviation of antigen expression from normal hematogones (i.e., difference from normal, DFN). Tube 3 is designed to determine the denominator for calculation of the MRD. Detailed description of the analysis strategy and the calculation can be found in Keeney *et al* [48]. These tubes have been shown to be effective with Becton Dickenson and Beckman Coulter instruments and using various analytic softwares.

B. B-ALL MRD Tubes from University Texas Southwestern Medical Center (UTSWMC)

	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-R700	APC-H7	BV421	V500c	BV605
1A	Lambda	Kappa	CD38	CD19	CD10	CD34	CD20	CD5	CD45	-
1B	Lambda	CD22	CD20	CD19	CD5	Drop-in/CD34	CD38	CD10	CD45	Kappa
2	CD10	CD13		CD19	CD33	CD34	CD45	CD117		
3*	CD9	CD24	CD20	CD38	CD10	CD34	CD81		CD45	CD19

UTSWMC B-ALL MRD Tube 1A is currently used to detect MRD through a DFN approach. Tube 2B is a B-cell screening tube developed by ConTexFlo in association with BD Biosciences [49] which will replace tube 1A as the general B-cell screening and MRD tube APC-R700 is empty channel for drop-in CDA34. Tube 2 is included with tube 1 in general B-ALL MRD screening to identify aberrant myeloid antigen expression. *Tube 3 is a reflex B-ALL MRD tube added in difficult cases where the B lymphoblasts closely resembles maturing hematogones for routinely assessed markers.

C. Anti-CD19/CART-19 B-ALL MRD Tubes from University Texas Southwestern Medical Center (UTSWMC)

	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-R700	APC-H7	BV421	V500c	BV605
2	CD10	CD24	-	CD19	CD33	CD34	CD45	CD123	-	CD13

UTSWMC Anti-CD19/CART-19 Tube 2 is added to tube 1a (or 1B) from table B for MRD assessment in patients treated with anti-CD19 therapy. Alternative gating markers for B-lineage cells include CD22 in tube 1B and CD24 (used in combination with orthogonal light scatter to exclude granulocytes) in tube 2. These tubes are performed prior to CART therapy to establish a baseline IP and following CART therapy to monitor for MRD.

D. T-ALL MRD Tubes from the University Texas Southwestern Medical Center (UTSWMC)

	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-R700	APC-H7	BV421	V500c	BV605
1	CD2	CD3	CD5	CD56	CD4	CD64	CD8	CD14	CD45	CD7
2	CD2	CD1a	CD4	CD5	CD10	CD34	CD8	CD3	CD45	CD7

Tube 1D includes CD56 which can aid in MRD analysis and may provide prognostic information. Inclusion of CD14 and CD64 allow for general assessment of myeloid and monocytic populations. Tube 2D contains the combination of CD4/CD8/CD3/CD1a. Evaluation of the normal maturation sequence for these four markers allows identification of DFN in nearly all cases of T-ALL and aids in MRD assessment.

The number of events that must be acquired to achieve this sensitivity depends on the minimal number of events considered to represent a cluster of MRD, which in turn depends on the panel design and the analyst's expertise. Generally, 10–50 events are considered adequate for the lower limit of detection (LOD) [17, 23, 67, 68, 69•, 70], but at least 50 events are required for the lower limit of quantification (LOQ) [71–73]. LOD and LOQ or the total cells that need to be acquired to reach those levels can be obtained from the following calculation adapted from Arroz et al. [73]:

$$\text{LOD or LOQ} = (\text{MRD cluster}/\text{total cells acquired}) \times 100\%$$

The specific cell populations that make up the "total cells acquired" to be used as the denominator in the above equation are poorly standardized between assays and institutions. The current recommendation for AML is to use $CD45^+$ white cells (per 2017 LeukemiaNet (ELN) from an expert panel, [3]) and for ALL is to use $Syto16^+$ nucleated mononuclear cells (per COG protocol) [11].

If using 30 and 50 for the MRD clusters for LOD and LOQ, respectively, we could calculate the number of total cells needed to be acquired to reach a 0.01% test sensitivity:

$$\begin{aligned} \text{LOD} : 0.01\% &= (30/\text{total cells acquired}) \times 100\% \\ \text{Total cells acquired} &= 300,000 \\ \text{LOQ} : 0.01\% &= (50/\text{total cells acquired}) \times 100\% \\ \text{Total cells acquired} &= 500,000 \end{aligned}$$

The College of American Pathologists (CAP) requires that the lower limit of enumeration for MRD analysis be validated and that the validation shows a method for separating the neoplastic population from its normal cell counterpart (e.g., the method for differentiating neoplastic B lymphoblasts from the normal immature B cells known as hematogones). Cardinali and Linden present an excellent description of a dilution study for MRD validation in the Spring 2015 edition of the International Clinical Flow Cytometry Society e-Newsletter [74].

The CAP further requires that the lower limit of enumeration be stated clearly on the assay report. For instance, if the assay was validated to a sensitivity of 0.01%, then the report would state that "The laboratory's lower limit of enumeration for the particular type of acute leukemia is 0.01%." Panels of experts recommend a complete report containing at least the following information:

1. MRD level plus the LOD and LOQ of the assay
2. The immunophenotypic expression profile of neoplastic leukemia cells
3. Cell viability and the quality of sample (e.g., potential hemodilution, cellularity to decide whether the sample is suitable for MRD assay)
4. The proportion of the normal cell counterpart (e.g., percentage of hematogones in a sample evaluated for B-ALL MRD)

For cases where MRD is detected but below the validated LOQ, the result may be reported as MRD identified below the LOQ; therefore, accurate quantification cannot be determined. For cases where neoplastic cells are detected but below the validated LOD, the result may be reported as the neoplastic cells identified but below the LOD; therefore, the significance of which is uncertain, and close clinical follow-up is recommended.

Characteristics of MFC

MFC does not require prior knowledge of the neoplastic cell immunophenotype and, therefore, can be used under various clinical circumstances. Particular advantages of MFC include wide availability in laboratories, rapid results usually available within 24 h of specimen collection, broad applicability for both AML (90% of cases) and ALL (nearly 100% of cases), and a relatively low cost. These attributes allow for efficient risk-adapted clinical management. Furthermore, MFC has the unique ability of providing detailed information regarding neoplastic cells. For instance, MFC can distinguish live neoplastic cells from degenerated or dead cells, can identify potential therapeutic targets, and may provide the leukemogenic potential or "stemness" of neoplastic cells by virtue of its expression of stem cell-associated markers ($CD34^+/CD38^-$). In contrast, molecular assays identify genetic markers without information on the cell type, e.g., live vs. dead cells and mature vs. immature cells. Overall, MFC is a rapid, highly sensitive, and cost-effective technology for MRD analysis, although the sensitivity is generally lower than molecular assays.

When to Choose MFC MRD Assay

MFC and molecular-based MRD assays have different but complementary merits; therefore, the optimal approach for MRD assessment will likely require integration of these assay types for the future: combining MFC and molecular detection techniques at different time points of treatment [3, 4, 16].

MFC MRD assays are the standard of care for MRD assessment at early time points of acute leukemia management in the USA, e.g., at day 8 in B-ALL and end of induction in AML and ALL. However, for AML with specific molecular targets such as fusion transcripts, higher sensitivity molecular assays may be more appropriate for disease monitoring at all time points. The 2017 ELN recommends MRD monitoring for AML with *RUNX1-RUNX1T1*, *CBFB-MYH11*, *PML-RARA*, or mutated

NPM1 be performed through molecular assessment. Whereas, for AML not included in these molecularly defined subgroups, MRD should be assessed by MFC [75]. In B-ALL, several studies have shown relatively good concordance (approximately 90% or greater) between MFC and qPCR for B-ALL with or without fusion transcripts (i.e., *BCR-ABL1*, *KMT2A-AF4*, and *E2A-PBX1* fusion genes) at early time points [76–79].

Therefore, at the present time, choice of early-time-point testing depends upon specific assay availability, disease characteristics, and clinical practice. Depending on disease details and therapeutic plans, the use of MFC and/or qPCR can provide relatively rapid results that enable quick clinical decisions during early assessment of MRD (i.e., end of induction). At later time points, the higher sensitivity of high-throughput NGS can be used to decrease the likelihood of false negative results [17].

Perspectives

From a clinical perspective, the significance of detecting MRD in ALL at levels greater than or equal to 0.01% of mononuclear cells at end of induction or end of consolidation has been established and shows a poor prognosis in B-ALL and T-ALL (although less robust). Interest in developing assays to detect MRD in AML and to determine its clinical significance has increased over recent years [15, 18, 40, 80]. The relevance of different MRD thresholds and the optimal time for MRD assessment is still being determined for AML. The prognostic significance of a low level of positive MRD test may be context dependent: differing across cytogenetic or molecular disease subgroups or time points, treatment regimens, and response criteria. To date, studies have suggested that the greatest clinical significance is at detection levels between 0.01 and 0.1% of total white cells (or 10^{-4} to 10^{-3}) evaluated at either end of induction or consolidation [3, 6, 81–84]. The 2017 ELN recommends the separation of complete remission (CR) for AML into CR-MRD^{negative} and CR-MRD^{positive} subgroups at the level of 0.1% of CD45^{+} cells [16]. The latter subgroup is associated with disease relapse and inferior outcomes and should prompt consideration for changes in therapy.

From a methodological perspective, MFC MRD testing in ALL is relatively well developed and standardized/harmonized over decades of efforts led by the Euroflow consortium and COG [11, 15, 48•, 69•, 85, 86]. However, reproducibility in AML MRD testing remains more of a challenge, as testing protocols and clinical management for MRD are less well developed. There has been a relatively slow embracement of MRD-based definitions of complete remission in AML, which is in part due to lack of consensus regarding standardization of methodology (panels and requiring a high level of expertise in data interpretation), clinically relevant detection threshold, and timing for assessment [3, 17, 18, 87].

It is expected that ongoing improvement for MFC assays will remain a constant theme, as we embrace technological advancement and clinically context-adapted MRD thresholds. For example, while routinely performed MFC MRD assays have a generally lower sensitivity compared to molecular assays [78, 79], in 2017, the EuroFlow consortium developed a high-throughput MFC MRD assay that can achieve MRD sensitivity to 10^{-5} (0.001%) comparable to RT-qPCR [69•]. New technologies will continue to improve MFC MRD analysis and include higher parameters of MFC (10–12 colors), stem cell quantitation, deeper sensitivity of 0.001% comparable to RT-qPCR, and adapted panels directed toward accommodating newer immunologically based therapies such as CAR-T therapy. The clinically relevant thresholds of MRD are likely to be continually adjusted to reflect genetic heterogeneity of leukemia and treatment regimen [5, 6].

Conclusions

In summary, numerous studies lend evidence that presence of MRD detected by MFC assay is an independent risk prognosticator in both ALL and AML. Moving forward, one of the largest challenges for MFC MRD testing will be better test standardization/harmonization for all MFC MRD assays. Ongoing effort is of paramount importance to further improve accuracy and consistency of MFC analytic systems. With continued technologic advancement, MFC along with molecular techniques will play an integral role in assessment of MRD at different time points of treatments to help guide clinical decision.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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