



FLOW CYTOMETRY

CyAn™ ADP | Multiparameter Flow Cytometry MRD-Detection with 9 Colors, 12 Parameters

Detection of minimal residual disease (MRD) in acute leukemias, especially for acute lymphoblastic leukemia (ALL), is not only of paramount importance for risk assessment and stratification of therapy but also for the understanding of the biological processes underlying treatment response. The mainstay of MRD detection has been the amplification of leukemia specific IgH-rearrangements by polymerase chain reaction (PCR) with a sensitivity of 10^{-4} – 10^{-5} . Although more sensitive than FCM-MRD, molecular methods are very laborious, slower and much more expensive. In many laboratories, multiparameter flow cytometry immunophenotyping has been utilized for MRD detection as well, but detection limits in most approaches are somewhat lower than with PCR (10^{-3} - 10^{-4}). Flow cytometry comprises all the technical features needed for the detection of rare events, and with the implementation of new technologies the CyAn ADP LX 9 Color flow cytometer provides the technical platform to increase sensitivity, speed and reliability of FCM-MRD detection.

Materials and Methods

Setup and Compensation. Single stainings were performed for each fluorescence channel as described below with the mAbs of the Panel using a normal buffy coat. Negative controls for FL-1 SYTO® 16 unstained cells were added after the staining and shortly before acquisition. Compensation was set off and the PMT voltage for each fluorescence channel was adjusted in such a way that unstained cells were positioned in the first log-decade. CompBeads were stained with each of the mAbs as described by the manufacturer. Subsequently, negative control beads were added and the tubes were analyzed in sequence with the same PMT settings. Negative and positive beads were defined for each fluorescence channel by gating and the compensation matrix was calculated. Samples were acquired afterwards without changing or adjusting the accomplished instrument setting. A gating strategy has been developed that permits the removal of the majority of non lymphocyte events, allowing easier identification of the lymphocyte populations and their sub sets.

Cell Preparation and Staining. Heparinized blood and bone marrow specimens from patients with precursor B cell acute lympho-

blastic leukemia (PBC-ALL) were collected at various time points during treatment to assess blast reduction kinetics and minimal residual disease. 7×10^5 – 3×10^6 cells were stained with monoclonal antibodies using 2 μ L of mAb/ 10^5 cells and the nucleic acid stain SYTO 16 according to the panel in Table 1. The tube was vortexed gently and incubated for 10 minutes at room temperature. Afterwards whole blood lysis was performed with FACS Lysing Solution as described by the manufacturer. After centrifugation for 5 minutes with 300 x g, cells were washed once with 5 mL of phosphate buffered saline (PBS) vortexed and pelleted again by centrifugation. After re-suspension in 0.5 mL of PBS, 3×10^5 – 1×10^6 cells were acquired.

Analysis and Gating Strategy

The complete nine color analytical matrix is represented by 36 bivariate dot plots. With addition of FSC and SSC and the cell doublet discriminating parameters pulse area and pulse width of the forward scatter signal 39 bivariate dot plots are needed to visualize all parameters. Eight of these dot plots that enabled the best discrimination between residual blast cells and remaining normal B-cells in the sample were chosen for gating.

Table 1

Panel BCP-ALL MRD on CyAn ADP LX 9 Color									
	FITC	PE	ECD	PC5	PC7	PB	CY	APC	APC-Cy7
CD	SYT016	CD10	CD45	CD13/33	CD34	CD19	CD3	CD58	CD20

The following hierarchical gating strategy (Table 2) is used to define:

1. SYTO 16+ nucleated cells excluding doublets (Figure 1).
2. Enumeration of CD19+ cells (Figure 1).
3. Differentiation between MRD+ blast cells and normal regenerating B cells (Figure 2 and 3).
4. CD19-/CD34+ - progenitor cells (Figure 2 and 3).

Table 2

Name	Expression	Color
SYTO16+ Doublets-	R1 & NOT R6 & NOT R9	Light Purple 16 Shades
Syto16+	R1	Black 16 Shades
CD19total	R1 & R2 & R3 & NOT R4 & NOT R6 & NOT R9	Green 16 Shades
CD19 normal	R1 & R2 & R3 & NOT R4 & R5 & R7	Yellow 16 Shades
CD19- Progenitors	R1 & R13 & NOT R2 & NOT R3	Blue 16 Shades
MRD+Blasts	R1 & R10 & R11 & R12 & R14 & R15 & R16 & R17 & R2 & R3 & NOT R4 & NOT R6 & R8 & NOT R9	Red 16 Shades

Figure 1

Gating Strategy I - Enumeration CD19+ NCs / Exclusion of doublets

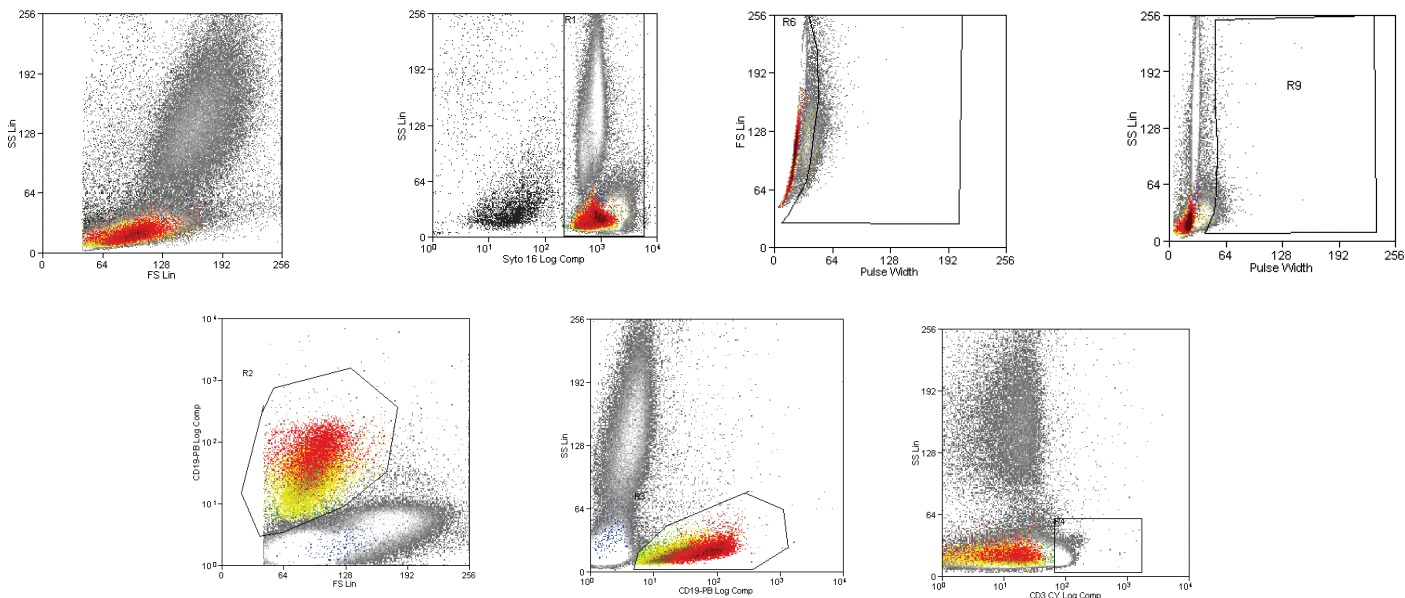


Figure 2

Gating strategy II - Discrimination of MRD+ blast cells and normal B cells
ALL-BFM 2000 Protocol: d15 of treatment

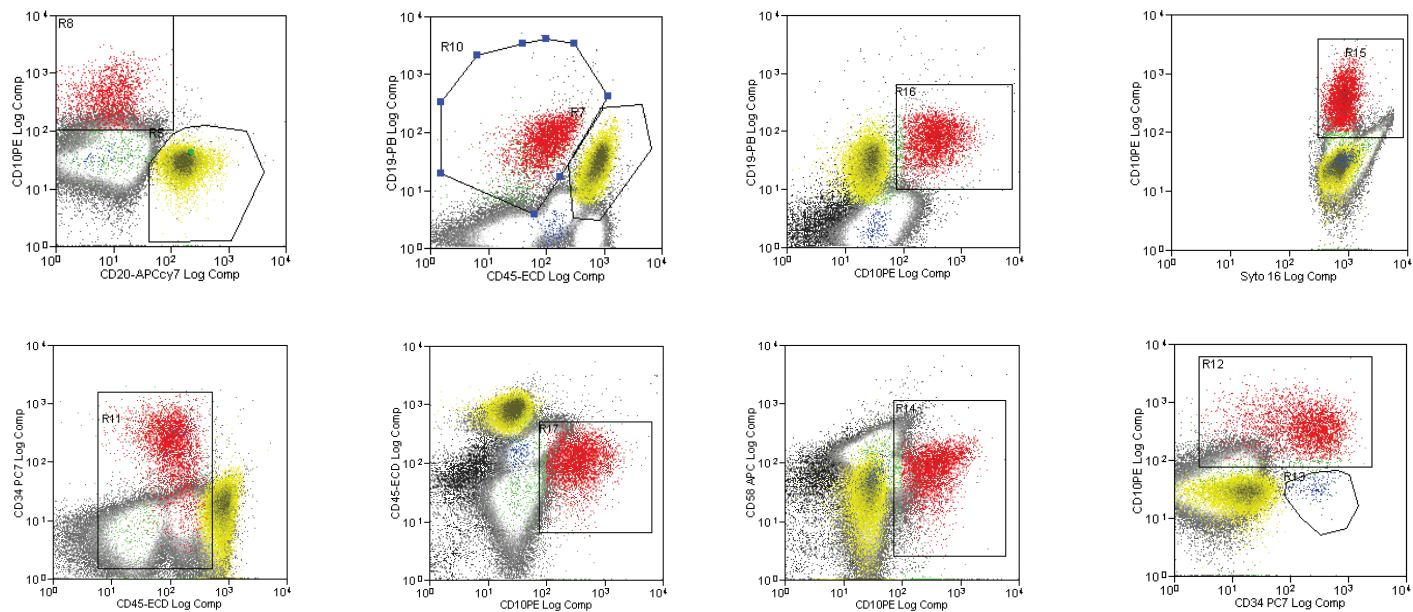
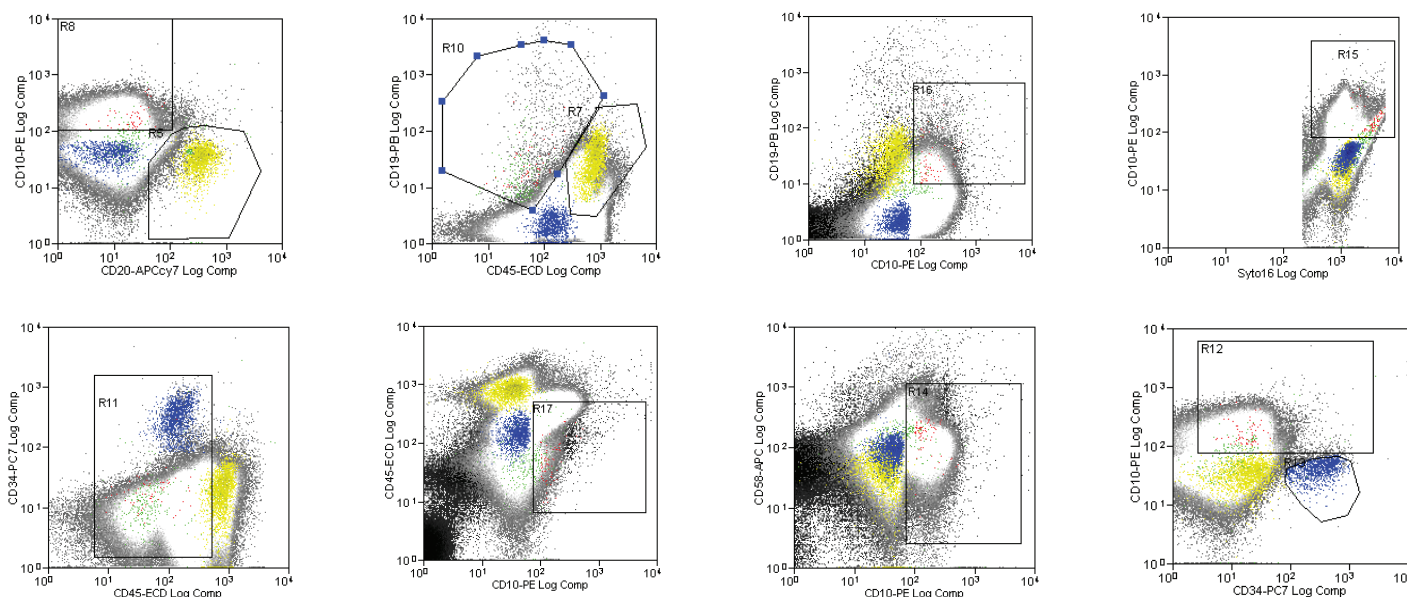


Figure 3
Gating strategy III - Discrimination of MRD+ blast cells and normal B cells
ALL-BFM 2000 Protocol: d33 of treatment



Results

Residual leukemic blast cells defined by the gate *MRD+ blasts* (Table 2) are depicted as red dots in Figures 2, 3 and 4. Quantification of residual blast cells is calculated according to the white blood cell count (WBC/ μ L) and the percentage of cells within the SYTO 16 - gate R1. On day 15 after the first two weeks of chemotherapy, 549 blasts/ μ L are still present in the bone marrow. After a further two weeks of treatment on day 33, three blasts/ μ L remained (Figure 4). Importantly, gates have not been changed or moved between day 15 and day 33 analyses.

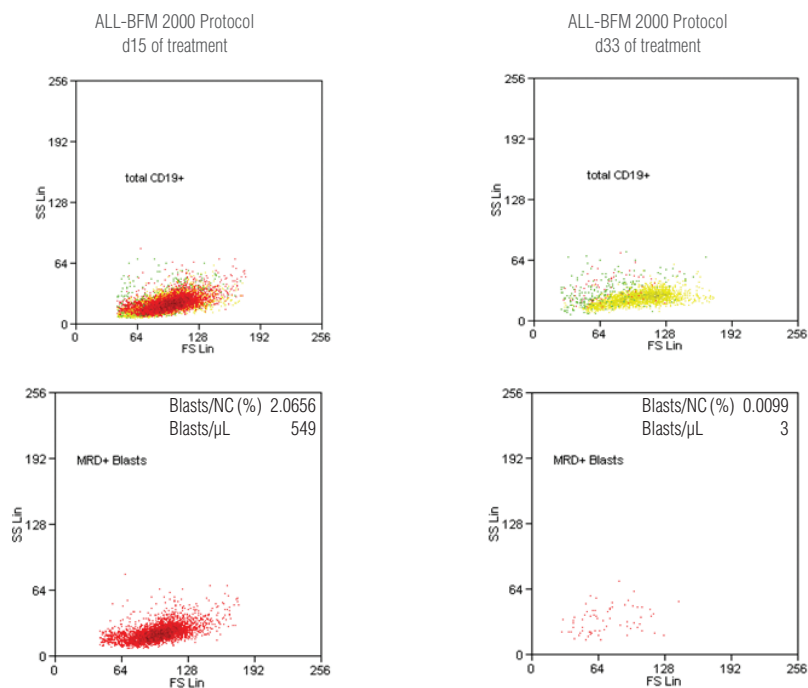
Discussion

The sensitivity of MRD detection with FCM depends to a great extent on the number of cells acquired and the reliable characterization of the leukemia associated immunophenotype, especially the differentiation between normal regeneration, normal B cells and the residual leukemic cells. With this approach of nine colors, 12 parameters in one tube, both sensitivity and reliability of MRD detection are increased, overcoming the drawbacks of the conventional method of sequentially acquiring four-color stainings in three to four tubes. The sample does not need to be aliquoted in four separate tubes; instead, all cells of the sample can be analyzed in one tube, enabling three to four times more cells to be acquired

with one staining. Furthermore, the analysis of nine colors in one tube offers the possibility to combine more parameters crucial for the detection of MRD blast cells.

Figure 4

FCM-MRD Results



References

1. Ratei R, and Ludwig W-D. Flow Cytometry Methods for the Detection of Residual Leukemia. In: Zipf TF, Johnston DA, eds. *Leukemia and Lymphoma*. Totowa, New Jersey: Humana Press; 2002:1-20.
2. Gaipa G, Basso G, Maglia O, Leoni V, Faini A, Cazzaniga G, Bugarin C, Veltroni M, Michelotto B, Ratei R, Coliva T, Valsecchi MG, Biondi A, Dworzak MN. Drug-induced immunophenotypic modulation in childhood ALL: implications for minimal residual disease detection. *Leukemia*. 2005;19:49-56.
3. Dworzak MN, Froschl G, Printz D, Zen LD, Gaipa G, Ratei R, Basso G, Biondi A, Ludwig WD, Gadner H. CD99 expression in T-lineage ALL: implications for flow cytometric detection of minimal residual disease. *Leukemia*. 2004;18:703-708.
4. Veltroni M, De Zen L, Sanzari MC, Maglia O, Dworzak MN, Ratei R, Biondi A, Basso G, Gaipa G. Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: implications for the detection of minimal residual disease in acute lymphocytic leukemia. *Haematologica*. 2003;88:1245-1252.
5. Ratei R, Basso G, Gaipa G, Schrappe M, Dworzak M, Ludwig W, Karawajew L. MRD-Detection by Multiparametric Flow Cytometry in Childhood Precursor B-cell ALL: Predictive Impact of Early Blast Reduction Kinetics in Peripheral Blood (pB) on MRD-Level in Bone Marrow (BM) at Day 33 of the ALL-BFM2000 protocol. *ASH-Meeting. San Diego, USA; 2004*.

Acknowledgments

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TECHNICAL TIPS

- > For compensation of the FL-1 channel with SYTO 16 it is essential to add unstained cells shortly before acquisition in order to have a negative control population.
- > The current approach generates large FCS files with 1×10^6 events acquired. This requires fast computers with a large memory; otherwise analysis will be very slow and tedious.

PRODUCT

CyAn ADP LX 9 Color	CY201
CD10/PE	R0848
CD19/PB	PB985
CD3/CY	CY696

CODE

For research use only – not to be used in diagnostic procedures. Other vendor products used in this application: Becton Dickinson and Invitrogen.

The protocols in this application note might deviate from the normal recommended protocol/specification guidelines that are included with the Dako product or any other non-Dako product.



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