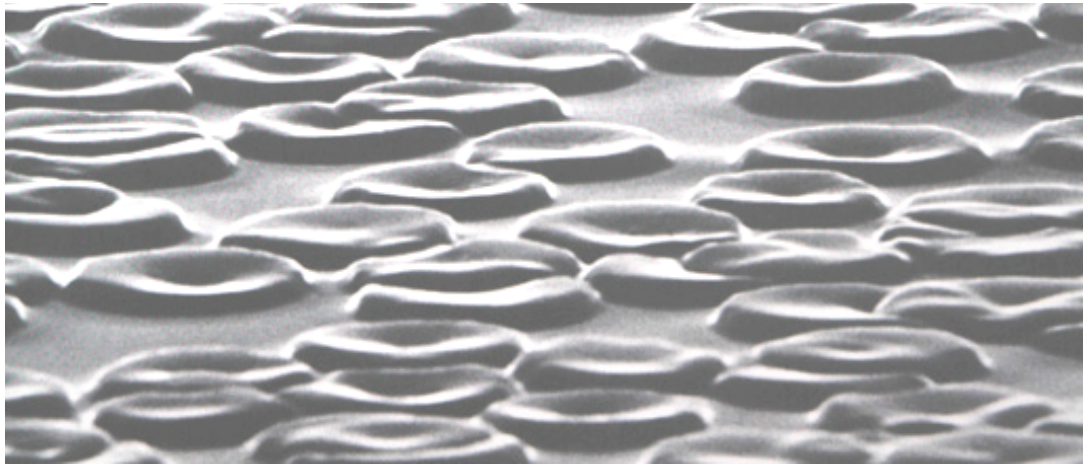

How to Diagnose AML and MDS by Flow Cytometry: ICSH Guidelines for Flow Cytometric Evaluation of Patients with Suspected Acute Myeloid Leukaemia and Myelodysplastic Syndromes

Wolfgang Kern, MLL Munich Leukemia Laboratory

MLL Expert Panel

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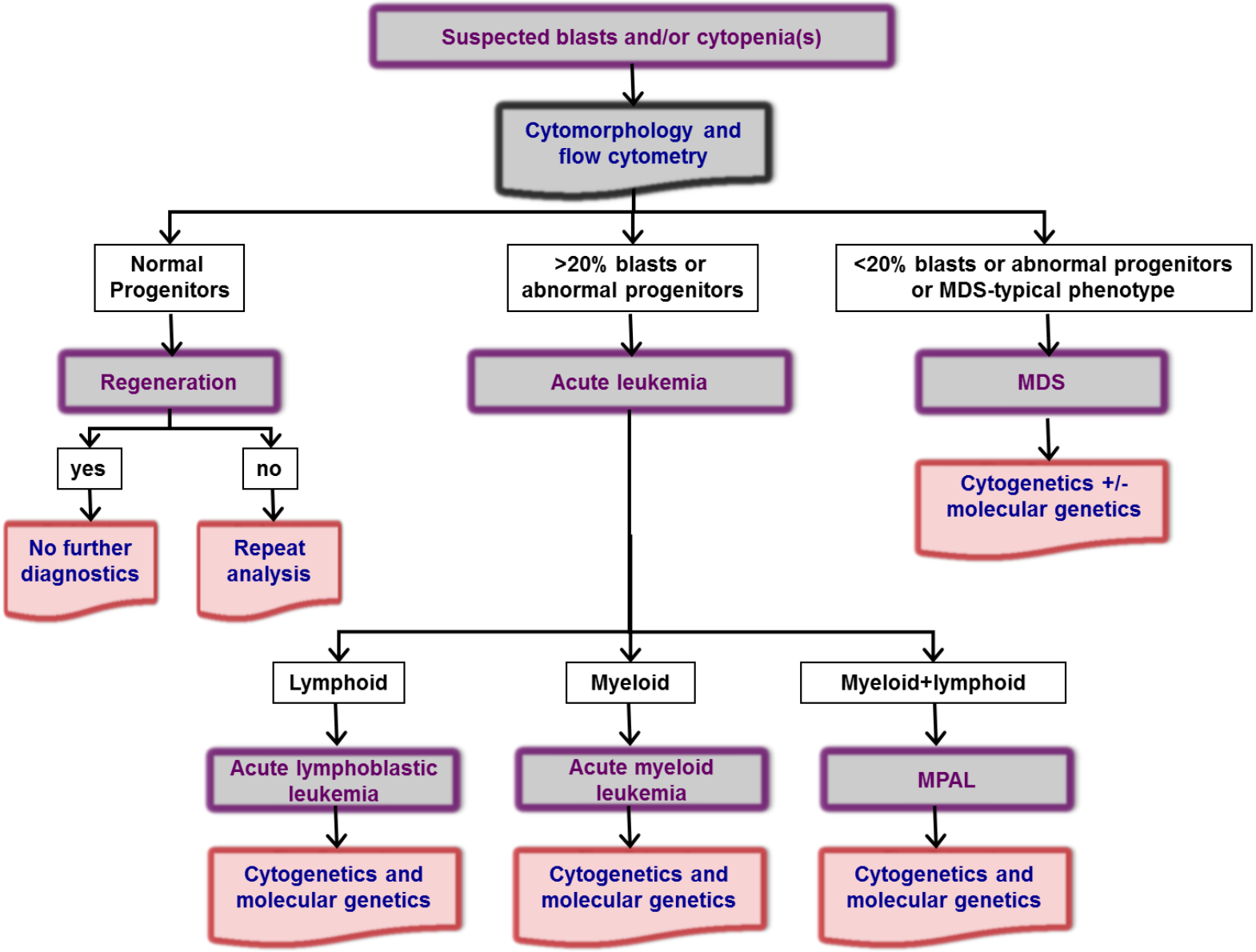
Algorithm-based diagnostic approach



Pre-analytics

Sample requirements

Sample source mainly bone marrow aspirate, anticoagulated

Labeling by ≥2 unique patient identifiers and anatomic source

Test requisition form

•unique patient identifiers, patient's date of birth, sex, diagnosis

•name and location of the physician submitting the specimen

•pertinent information on current medication or recent treatment

•date and time of specimen collection

•source of the specimen

•requested test

•if possible: WBC count; differential; histologic, cytochemical, immunohistochemical, molecular, cytogenetic findings

Pre-analytics

Anticoagulation and Storage Conditions

EDTA, sodium heparin, ACD

EDTA if CBC to be performed in addition

Quick processing needed, sample stable for up to 72 hours

Validation needed

Transportation/storage at 18-25°C, in case of storage at 2-8°C sample must reach room temperature before performing assay

Assessing Specimen Viability and Cell Concentration

Estimation of proportion of viable cells by FSC/SSC or fluorescent DNA-binding dyes

Cell counting to ensure saturating amounts of antibodies

Value of Specimen Cytomorphology

Cytomorphologic evaluation strongly recommended for correlation with MFC data

Essential antigens

Determination of frequency of immature cells

Analysis of CD45/SSC

Use of CD11b/CD14/CD16 for separation of different cell populations

List of antibodies

As recommended in

- Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood 2008; 111: 3941-3967
- Bene MC, Nebe T, Bettelheim P, Buldini B, Bumbea H, Kern W, et al. Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10. Leukemia 2011; 25: 567-574

Myeloid progenitor cells

CD34, CD117, HLA-DR

Cytoplasmic myeloperoxidase most specific marker of myeloid differentiation

Deviations from normal occur in AML and MDS

Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10

MC Béné[1], T Nebe[2], P Bettelheim[3], B Buldini[4], H Bumbea[5], W Kern[6], F Lacombe[7], P Lemez[8], I Marinov[9], E Matutes[10], M Maynadié[11], U Oelschlagel[12], A Orfao[13], R Schabath[14], M Solenthaler[15], G Tschurtschenthaler[16], AM Vladareanu[5], G Zini[17], GC Faure[1] and A Porwit[18]

Acute lymphoblastic leukemia (ALL)

- B lineage: B-I, B-II, B-III, B-IV,[2,10] where, in addition to the degree of differentiation of B-lineage lymphoblastic ALs, the differentiation between B-precursor AL and Burkitt's lymphoma/ leukemia is necessary for important therapeutic consequences (Supplementary Table 1).
- T lineage: T-I, T-II, T-III, T-IV.[11]

Acute myeloblastic leukemia (AML)[9]

- With minimal differentiation (former M0).[12]
- AML with granulocytic or monocytic differentiation.[13]

Acute promyelocytic leukemia (APL)[14]

- Erythroid.[15]
- Megakaryocytic.[16]
- Dendritic cell (DC) precursors.[17–19]
- Basophils and mast cell precursors.[20,21]

The first set of proposed markers: cytoplasmic CD3 (cCD3), myeloperoxidase (MPO), cCD79a and TdT include lineage markers with a cytoplasmic or nuclear localization. It will therefore require cell permeabilization reagents.[28] Cytoplasmic expression of CD3 or CD79a is one of the earliest events occurring upon commitment of a progenitor cell towards the T or B lymphoid lineage, respectively. In normal differentiation, these antigens will be later used to bring the antigen receptor

Essential antigens

Determination of frequency of immature cells

Analysis of CD45/SSC

Use of CD11b/CD14/CD16 for separation of different cell populations

List of antibodies

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Myeloid progenitor cells

CD34, CD117, HLA-DR

Cytoplasmic myeloperoxidase most specific marker of myeloid differentiation

Deviations from normal occur in AML and MDS

Essential antigens

Granulocytic differentiation

CD15, CD11b, CD65

Monocytic differentiation

High expression of CD64 and CD36, CD11b, CD11c

CD14 on mature monocytes

Megakaryocytic differentiation

CD61, CD41, CD42

Erythroid precursors

CD235a, CD71, CD36, CD45

MLL Essential antigens

Characteristic immunophenotype in AML with specific genetic changes

t(8;21)(q22;q22), *RUNX1-RUNX1T1*

inv(16)(p13.1q22) or t(16;16)(p13.1q22), *CBF-MYH11*

t(9;11)(p22;q23), *MLLT3-MLL*

t(6;9)(p23;q34), *DEK-NUP214*

t(1;22)(p13;q13), *RBM15-MKL1*

t(15;17)(q22;q12), *PML-RARA*

AML with minimal differentiation

Discrimination from ALL and MPAL

Leukemia-associated aberrant immunophenotype

Assessment at diagnosis if MRD analysis is intended

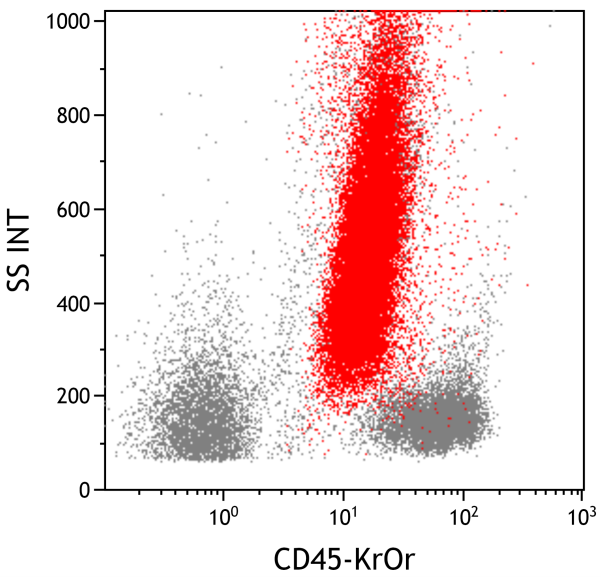
Reference to normal bone marrow populations

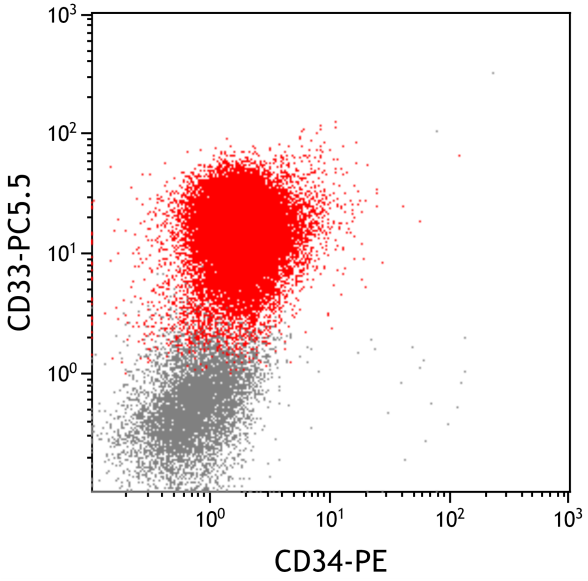
Essential for individual setting in each laboratory

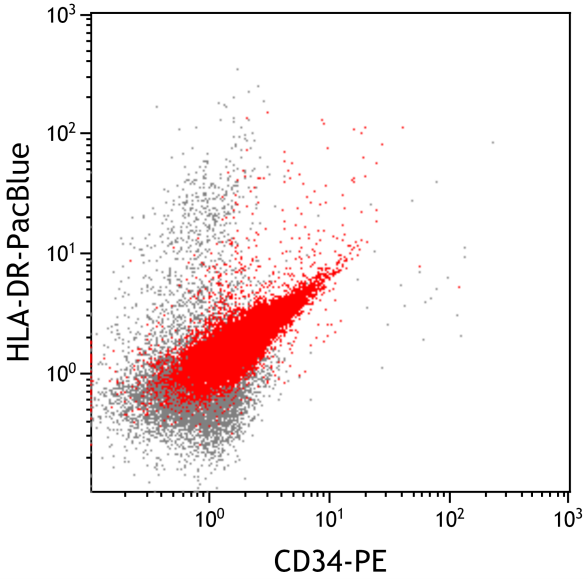
Reference populations

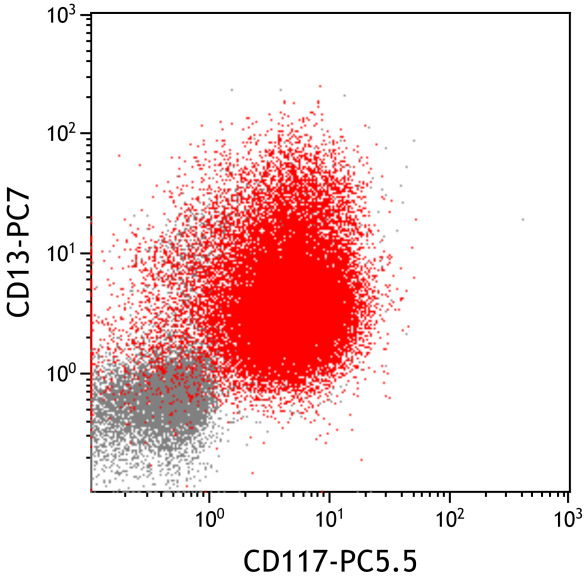
Negative populations to be used, no isotype controls

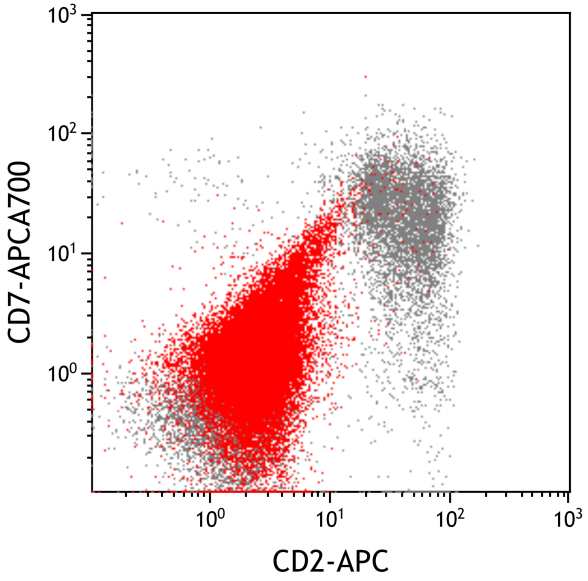
Typical features of AML M3

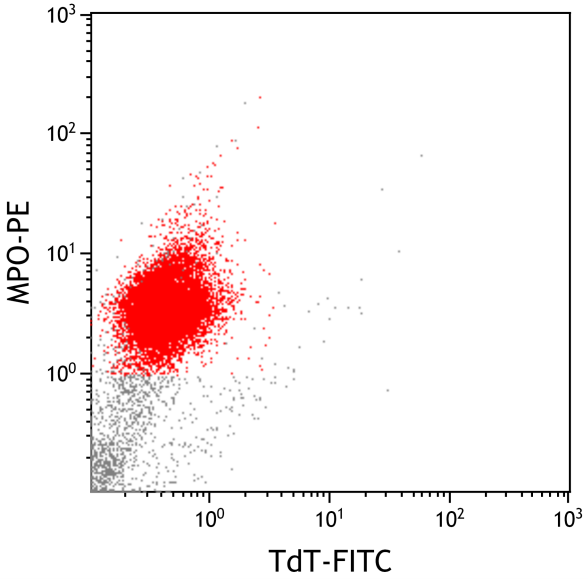




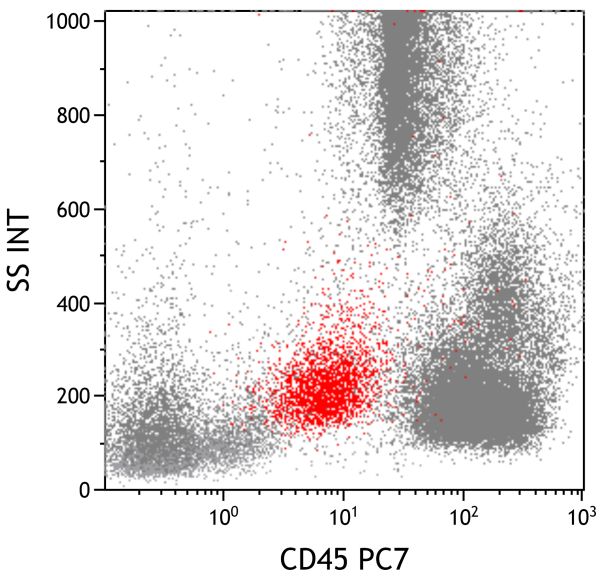


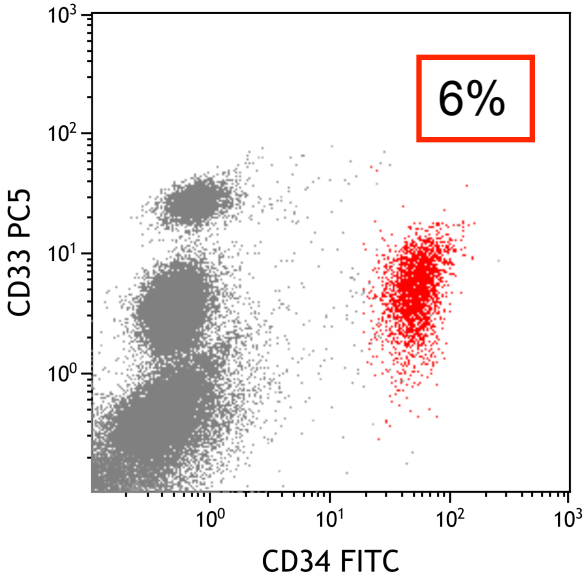


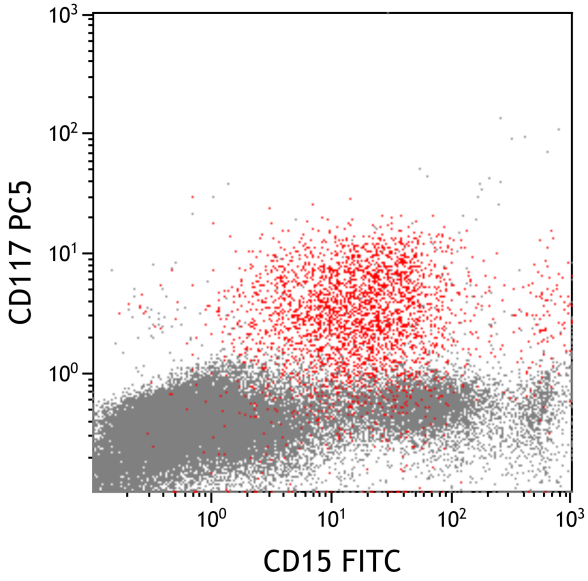


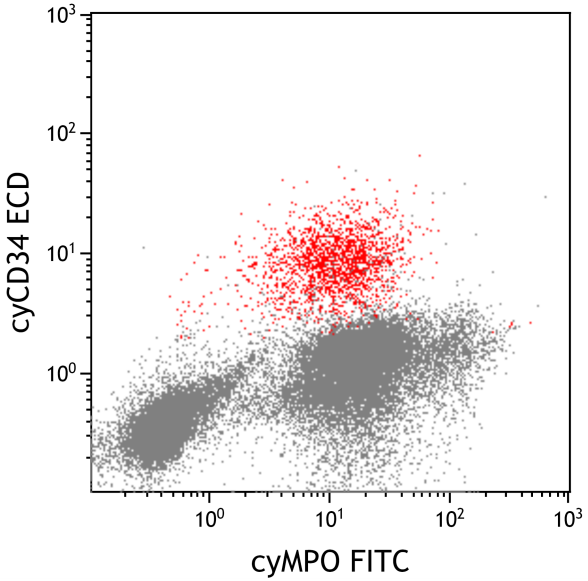


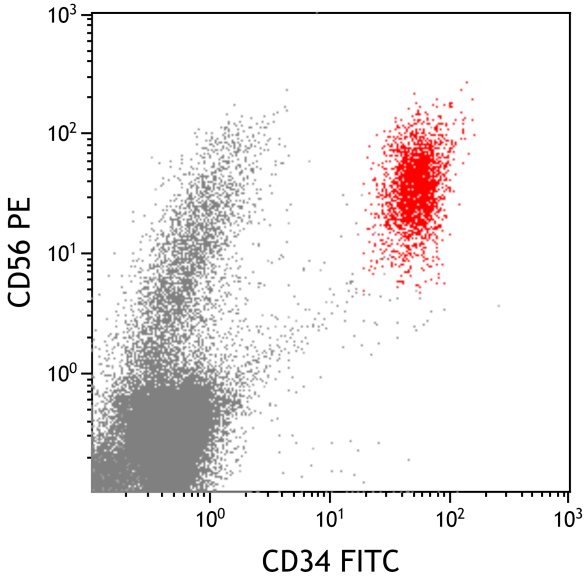
Typical findings in AML with t(8;21)/*RUNX1-RUNX1T1*

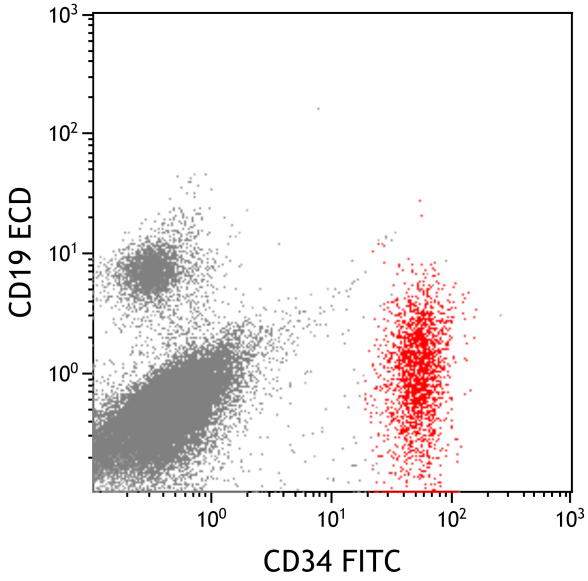












Diagnostic sample analysis

Data Acquisition

Cytometer settings

Compensation procedures

Definition of live gate

Number of cells in live gate $\geq 10^4$ (AML) and $\geq 5 \times 10^4$ (MDS), to be adjusted for MRD

Data analysis

Separation of myeloid progenitor cells and more differentiated cells

Immunological pattern analysis

•myeloid progenitor cells in AML, also more differentiated cells in MDS

Comparison of pathological and normal samples

Minimal residual disease

Technical aspects

Leukemia-associated aberrant immunophenotypes (LAIP) in 95% patients and above in AML, often heterogeneous

Different types of LAIPs to be considered

- cross-lineage expression of lymphoid antigens
- asynchronous co-expression of both progenitor cell and mature antigens
- lack of expression of myeloid antigens
- over-expression of normally expressed antigens

Diagnostic antibody panel to guarantee detection of ≥1 LAIP

“Different from normal” approach in case of no diagnostic immunophenotype available

Sensitivity 1/1,000 to 1/10,000, number of acquired cells to be considered

Relative value in comparison to quantitative PCR to be determined

Incorporating MRD in Clinical Practice

Should be incorporated into treatment protocols

MRD testing demands strong understanding of immunophenotypic features of BM cells under steady-state, pathological and regenerating conditions

MLL Report

Required content

a) a clear, unambiguous identification of the MFC examination

b) identification of the laboratory that issued the report

c) patient identification and location on each page

d) name or other unique identifier of the requester and the requester's contact details

e) date of primary sample collection

f) type of primary sample

g) examination results

•descriptive short text on relevant populations, their size as percentage of the total sample and their antigen expression profiles

•table listing antigens examined and considered absent, partially expressed, dimly expressed or strongly expressed can be added

•Stating the percentage of MPCs in the sample tested for MFC should be accompanied whenever possible by the percentage of blasts observed in the cytomorphologically examined sample

h) comments such as cautionary or explanatory notes

i) identification of the person(s) reviewing the results and authorizing the release of the report

j) date of the report, time of release

k) page number to total number of pages (e.g. "Page 1 of 5", "Page 2 of 5", etc.)

Specific aspects, AML

Adherence to WHO classification

Specific MFC findings should be reported only if clinically relevant

- if indicating highly suggestively a WHO-defined entity
- if LAIP is present which can be applied for MRD monitoring

Summary

- AML
- "consistent with AML“
- alternative MFC conclusion

Reference to cytomorphology

If findings suggest acute promyelocytic leukemia (APL)

- statement on this must be included
- report must be communicated immediately
- genetic testing for t(15;17) and/or for *PML-RARA* must be initiated

Specific aspects, AML

If findings suggest “AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*“ or “AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*“

- may be reported
- should be confirmed by genetic testing

Identified population(s) resulting in the diagnosis of AML should be described and the antigen expression pattern provided

Reports on MRD assessment

- If findings from diagnostic analysis available, report should refer to these and indicate percentages of identified LAIP+ cells both at diagnosis and at follow-up
- If findings from diagnostic analysis not available and MRD-type analysis performed as screening for small CD45/SSC-focused populations with unknown LAIP this should be clearly stated and percentage of identified population should be given
- Estimation of sensitivity of MRD assessment should be provided

Specific aspects, MDS

Assessment recommended according to ELN, but has not been part of diagnostic criteria of MDS according to WHO classification of 2008

Diagnostic finding should be reported as

- "consistent with MDS by flow cytometry"
- "suggestive of MDS by flow cytometry" or
- "no sufficient evidence of MDS by flow cytometry"

Indication for the requirement of repeat analysis, if applicable

Reporting of findings on MDS-typical aberrant antigen expression for each cell compartment according to ELN guidelines

- immature myeloid and monocytic progenitors
- maturing neutrophils
- monocytes
- progenitor B cells
- erythroid compartment

≥3 aberrantly expressed antigens considered necessary to allow "consistent with MDS"

Isolated increase of MPCs >5% also considered sufficient for "consistent with MDS"

MLL Recommended minimal requirements to assess MDS by MFC

Bone marrow subset	*Recommended analyses*	*Aberrancy*
Immature myeloid and monocytic progenitors	Percentage of cells in nucleated cell fraction[a]	Increased percentage
	Expression of CD45	Lack of/decreased/increased
	Expression of CD34	Lack of/decreased/increased
	Expression of CD117	Homogenous under/overexpression
	Expression of HLA-DR	Lack of/increased expression
	Expression of CD13 and CD33	Lack of/decreased/increased
	Asynchronous expression of CD11b, CD15	Presence of mature markers
	Expression of CD5, CD7, CD19, CD56[b]	Presence of lineage infidelity markers
Maturing neutrophils	Percentage of cells as ratio to lymphocytes	Decreased
	SSC as ratio vs SSC of lymphocytes	Decreased
	Relationship of CD13 and CD11b	Altered pattern[c]
	Relationship of CD13 and CD16	Altered pattern[c]
	Relationship of CD15 and CD10	Altered pattern[c]; for example, lack of CD10 on mature neutrophils
Monocytes	Percentage of cells	Decreased/increased
	Distribution of maturation stages	Shift towards immature
	Relationship of HLA-DR and CD11b	Altered pattern[c]
	Relationship of CD36 and CD14	Altered pattern[c]
	Expression of CD13 and CD33	(Homogenous) under/overexpression
	Expression of CD56[b]	Presence of lineage infidelity marker
Progenitor B cells	Enumeration as fraction of total CD34+ based on CD45/CD34/SSC in combination with CD10 or CD19	Decreased or absent
Erythroid compartment[d]	Percentage of nucleated erythroid cells	Increased
	Relationship CD71 and CD235a	Altered pattern[c]
	Expression of CD71	Decreased
	Expression of CD36	Decreased
	Percentage of CD117-positive precursors	Increased

[a]Discrepancies in counts between several definitions indicate aberrancies. [b]To be used with caution, as CD56 can be upregulated upon activation, be aware of normal cut-off values (also in stressed marrow). [c]Altered patterns can include altered distribution of maturation stages and/or altered expression levels of indicated antigens. [d]Under evaluation. Examples of several flow cytometric aberrancies in myelodysplastic syndrome can be found on the European LeukemiaNet website: www.leukemia-net.org.

Westers et al., Leukemia 2012;26:1730-1741

Proposed core markers in the analysis of MDS by MFC

General core markers	*Erythroid*	*Progenitors*	*Maturing neutrophils*	*Monocytes*
CD45	CD45	CD45	CD45	CD45
–	CD71	–	–	–
–	CD235a	–	–	–
CD34	–	CD34	CD34	CD34
CD117	CD117	CD117	CD117	CD117
HLA-DR	–	HLA-DR	HLA-DR	HLA-DR
CD11b	–	CD11b	CD11b	CD11b
CD13	–	CD13	CD13	CD13
CD16	–	–	CD16	CD16
CD33	–	–	CD33	CD33
CD14	–	–	CD14	CD14
–	CD36	–	–	CD36
–	–	–	CD64	CD64
CD7	–	CD7	–	–
CD56	–	CD56	CD56	CD56
CD19	–	CD19	–	–
–	–	CD5	–	–
–	–	–	–	CD2
–	–	CD15	CD15	–
–	–	–	CD10	–

Westers et al., Leukemia 2012;26:1730-1741

ELN recommendations for MDS

Diagnostic tool	Diagnostic value	Priority
Peripheral blood smear	• Evaluation of dysplasia in one or more cell lines • Enumeration of blasts	Mandatory
Bone marrow aspirate	• Evaluation of dysplasia in one or more hematopoietic cell lines • Enumeration of blasts • Enumeration of ring sideroblasts	Mandatory
Bone marrow biopsy	• Assessment of cellularity, CD34+ cells, and fibrosis	Mandatory
Cytogenetic analysis	• Detection of acquired clonal chromosomal abnormalities that can allow a conclusive diagnosis and also prognostic assessment	Mandatory
FISH	• Detection of targeted chromosomal abnormalities in interphase nuclei following repeated failure of standard G-banding	Recommended
Flow cytometry immunophenotyping*	• Detection of abnormalities in erythroid, immature myeloid, maturing granulocytes, monocytes, immature and mature lymphoid compartments	Recommended
SNP-array	• Detection of chromosomal defects at a high resolution in combination with metaphase cytogenetics	Suggested
Mutation analysis of candidate genes	• Detection of somatic mutations that can allow a conclusive diagnosis and also reliable prognostic evaluation	Suggested

Malcovati et al., Blood 2013;122:2943-2964

Quality control

Internal Quality Control

Instrument quality control on a daily basis

Beads and biologic controls

External Quality Assessment

Possible only 4 to 6 time per annum

Awareness of “snap shot”

Personnel

Qualification

Definition of roles to be assigned as well as the respective degree of qualification needed to work on such roles

Selection of personnel accordingly

Professional education of technical staff

- must meet national standards
- shall reflect appropriate education, training, experience and demonstrated skills needed

Professional education of academic personnel responsible for signing off reports

- must meet national standards
- must be specialized in an area that includes diagnostic MFC (hematology, immunology, pathology, clinical chemistry)
- must be trained in laboratory applying MFC for hematological disorders including AML and MDS
- Training should not be <1 year and include ≥500, ideally ≥1,000 cases of PB or BM samples tested in this context

Both technical staff and physicians must have applicable theoretical and practical background and experience

Personnel Introduction to the Organizational Environment

Program to introduce new staff to

- organization, department or area in which the person will work
- terms and conditions of employment
- staff facilities
- health and safety requirements (including fire and emergency)
- occupational health services

Training

Comprehensive training and competency assessment program for all staff

(i) establishment of training objectives

(ii) identification of the methods/tools that will be used in training

(iii) identification of the materials used in training

(iv) establishment of the criteria to be used to assess the effectiveness of training

Training subject to continuous assessment and recorded in a training manual

Algorithm-based diagnostic approach