Special article

Updating recommendations of the Brazilian Group of Flow Cytometry (GBCFLUX) for diagnosis of acute leukemias using four-color flow cytometry panels.

Míriam P. Beltrame a,*, Elizabeth Xisto Souto b,c, Mihoko Yamamoto d, Felipe M Furtado e,f, Elaine Sobral da Costa g, Alex Freire Sandes h, Glicínia Pimenta i, Geraldo Barroso Cavalcanti Júnior j, Maria Cláudia Santos-Silva k, Irene Lorand-Metze l, Maura R V Ikoma-Colturato m

a Hospital Erasto Gaertner, Laboratório de Citometria de Fluxo, Curitiba, PR, Brazil
b Hospital do Câncer de Barretos, Barretos, SP, Brazil
c Hospital Brigadeiro, São Paulo, SP, Brazil
d Escola Paulista de Medicina, Universidade Federal de São Paulo (EPM/UNIFESP), São Paulo, SP, Brazil
e Sabin Medicina Diagnóstica, Brasília, DF, Brazil
f Hospital da Criança de Brasília José Alencar, Brasília, DF, Brazil
g Instituto de Puéricula e Pediatría Margatão Gesteira, Universidade Federal do Rio de Janeiro (IPPMG/UFRJ), Rio de Janeiro, RJ, Brazil
h Grupo Fleury - Divisão de Hematologia e Citometria de Fluxo, São Paulo, SP, Brazil
i Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil
j Faculdade de Farmácia, Universidade Federal do Rio Grande do Norte (UFRN), Natal, RN, Brazil
k Universidade Federal de Santa Catarina (UFSC) - Florianópolis, SC, Brazil
l Faculdade de Ciências Médicas, Universidade Estadual de Campinas (FCM Unicamp), Campinas, SP, Brazil
m Hospital Amaral Carvalho, Jau, SP, Brazil

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ABSTRACT

Introduction: Flow cytometry has become an increasingly important tool in the clinical laboratory for the diagnosis and monitoring of many hematopoietic neoplasms. This method is ideal for immunophenotypic identification of cellular subpopulations in complex samples, such as bone marrow and peripheral blood. In general, 4-color panels appear to be adequate, depending on the assay. In acute leukemias (ALs), it is necessary to identify and characterize the population of abnormal cells in order to recognize the compromised lineage and classify leukemia according to the WHO criteria. Although the use of eight- to ten-color immunophenotyping panels is well-established, many laboratories do not have access to this technology. Objective and Method: In 2015, the Brazilian Group of Flow Cytometry (Grupo Brasileiro de Citometria de Fluxo, GBCFLUX) proposed antibody panels designed to allow the precise diagnosis and characterization of AL within available resources. As many Brazilian flow cytometry laboratories use four-color immunophenotyping, the GBCFLUX has updated that...
Results: Recommendations for morphological analysis of bone marrow smears and performing screening panel for lineage(s) identification of AL were maintained from the previous publication. The lineage-oriented proposed panels for B and T cell acute lymphoblastic leukemia (ALL) and for acute myeloid leukemia (AML) were constructed for an appropriate leukemia classification.

Conclusion: Three levels of recommendations (i.e., mandatory, recommended, and optional) were established to enable an accurate diagnosis with some flexibility, considering local laboratory resources and patient-specific needs.

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Introduction

The Brazilian Group of Flow Cytometry (Grupo Brasileiro de Citometria de Fluxo, GBCFLUX) had previously proposed monoclonal antibodies (MoAb) panels to be subsequently validated in an interlaboratory study to assess their effectiveness in the diagnosis and classification of acute leukemia (AL). The new flow cytometers (CF) allow immunophenotyping using 8 or more colors, however, many Brazilian laboratories still work on 4-color multi-parameter flow cytometry (MFC) platforms. The present document aims to update the former reported in 2015. The panels previously proposed were revised, based on a literature review and the extensive experience of the professionals participating in this study. The former goals of the panels were maintained: 1) identification and quantification of abnormal leukemia cells, as well as lineage identification by the screening tubes; 2) disease classification according to the cell maturation stage; 3) identification of leukemia-associated immunophenotypes (LAIPs) to be used for minimal residual disease (MRD) assessments, and; 4) identification of phenotypes associated with molecular alterations with well-recognized prognostic implications.

All the panels were designed at different recommendation levels for diagnosis and classification, allowing flexibility compatible with the local laboratory resources. Three levels of recommendations were considered: mandatory, recommended and optional. Mandatory recommendations contain the minimum criteria for identification, quantification and classification of AL. Recommended level includes markers that are not essential for diagnosis, but are important for leukemia subclassification, prognosis and MRD detection. Optional recommendations include markers useful for MRD evaluation, detection of less frequent leukemia subtypes, associated with molecular or cytogenetic abnormalities, and prognosis, such as CD66c, CD123 and NG2. These panels were validated in the group’s laboratories to assure their effectiveness (see supplementary files).

General recommendations

The recommended pre-analytical and analytical processes have been previously described.

Acute leukemia screening tubes (ALST)

Two tubes are designed to lineage identification of the leukemic blast: B-cell lineage (CD19 and cyC79a), T cell lineage (cyCD3), myeloid (cyMPO) and ambiguous lineage leukemia (Table 1). In addition, some markers were added to improve blast cell identification, such as CD34 as an immaturity marker, CD7, which is very frequent in T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) and in some acute myeloid leukemia (AML), and CD45, that can be used as auxiliary marker for the gating strategy. Compared to the original publication, the 1st tube was maintained and the 2nd tube was slightly modified, with a switch of the CD7 and CD19 fluorochromes. The CD7 is a strong marker and has been combined with fluoroscein isothiocyanate (FITC), according to the classic criterion that highlyexpressed markers must be conjugated with a weak fluorochrome. This is a general rule for choosing MoAb and fluorochromes for a panel. For the same reason, we recommended the CD19 conjugated with phycoerythrin (PE): low density markers should be conjugated with bright fluorochromes.

Based on the immunophenotypic information derived from the ALST tubes, lineage-directed panels [B-cell precursor leukemia (BP-ALL), T-cell precursor leukemia (TP-ALL) and T-ALL] were constructed for optimal acute lymphoblastic leukemia diagnosis and staging, as well as subtyping. The classification of AL is strongly supported by morphological analysis, as the typical findings are characteristic for each lineage. Cytogenetic abnormalities are also important for diagnosis and subtyping. The combination of immunophenotyping with cytogenetic analysis, as well as FISH/ISH and complete cytogenetic analysis (CGH), is frequently used to confirm the diagnosis. The recommended pre-analytical and analytical processes have been previously described.

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acute lymphoblastic leukemia/lymphoma (BCP-ALL) or T-lymphoblastic leukemia (T-ALL) or acute myeloid leukemia/myelodysplastic syndrome (AML/MDS) must be applied in order to provide the final diagnosis (see Tables 1, 2 and 3).

The ALST should be used in all suspected cases of AL, even in those with typical cytological findings, in order to identify the AL lineage and also the ambiguous lineage phenotype (Table 1). However, the screening tube is not enough to reach the final diagnosis. On the other hand, the lineage of some subtypes of leukemia, such as megakaryoblastic leukemia, AML with minimal differentiation, blastic plasmacytoid dendritic cell neoplasm, as well as acute undifferentiated leukemia, cannot be defined by the ALST tube. These AML subtypes often do not express intracytoplasmic myeloperoxidase (cyMPO) nor the lymphoid markers that define the B and T lineage. Thus, in any situation, an expanded assessment should be performed to accurately define the leukemia classification. The rationale for choosing ALST markers has already been described.

### Table 1 – AL Screening Antibody Panel for 4-Color Immunophenotyping.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cyMPO</td>
<td>cy CD79a</td>
<td>CD45</td>
<td>cy CD3</td>
</tr>
<tr>
<td>2</td>
<td>CD7</td>
<td>CD19</td>
<td>CD45</td>
<td>CD 34</td>
</tr>
</tbody>
</table>

FRTC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein/cyanin5; APC: allophycocyanin; Cy: cytoplasmic.

### Table 2 – BCP-ALL Antibodies Panel for 4-Color Immunophenotyping – markers and fluorochromes.

<table>
<thead>
<tr>
<th>Tubes</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mandatory or Essential</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CD20</td>
<td>CD10</td>
<td>CD34</td>
<td>CD19</td>
</tr>
<tr>
<td>2</td>
<td>CyIgM</td>
<td>CD22</td>
<td>CD19</td>
<td>CD123</td>
</tr>
<tr>
<td>3</td>
<td>CD38</td>
<td>CD66c</td>
<td>CD19</td>
<td>CD81</td>
</tr>
<tr>
<td>Recommended when cyIgM+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SmKappa</td>
<td>SmLambda</td>
<td>CD19</td>
<td>SmIgM</td>
</tr>
<tr>
<td>Recommended when CD10negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CD15+CD65</td>
<td>NG2 (71.)</td>
<td>CD34</td>
<td>CD19</td>
</tr>
<tr>
<td>Optional</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>CD58</td>
<td>CD9</td>
<td>CD45 or CD34+</td>
<td>CD19</td>
</tr>
<tr>
<td>7</td>
<td>NuTdT</td>
<td>CD13+CD33</td>
<td>CD45 or CD34+</td>
<td>CD19</td>
</tr>
</tbody>
</table>

FRTC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein/cyanin5; APC: allophycocyanin; Cy: cytoplasmic; Nu: nuclear; Sm: surface membrane* according to expression in the screening panel.

### Table 3 – BCP-ALL molecular abnormalities and related immunophenotypic profiles.

<table>
<thead>
<tr>
<th>Molecular Abnormality</th>
<th>Immunophenotypic expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(9;22)(q34.1;q11.2)</td>
<td>BCR-ABL1 CD19+, CD10+, NuTdT+, CD34+, CD25+, dim expression of CD38.</td>
</tr>
<tr>
<td>t(1;19)(p13.2;q11.2)</td>
<td>KMT2A-rearranged Frequent expression of CD66c, CD13 and CD33, CD19+, CD10+, CD24+, NG2+, CD15/CD65+.</td>
</tr>
<tr>
<td>t(12;21)(p13.2;q22.1)</td>
<td>ETV-RUNX1 CD19+, CD10+, CD34+, CD13+, CD27+. Absence of CD9, CD20, CD66 and CD44.</td>
</tr>
<tr>
<td>ALL with hyperdiploidy</td>
<td></td>
</tr>
<tr>
<td>LLA with hypodiploidy</td>
<td></td>
</tr>
<tr>
<td>t(5;14)(q31.1;q32.1)</td>
<td>IGH/IL3 CD19+, CD10+. Presence of eosinophilia.</td>
</tr>
<tr>
<td>t(1;19)(q23;p13.3)</td>
<td>TCF3-PBX1 CD10+, cyIgM+, CD19+ and CD34+ or dim.</td>
</tr>
</tbody>
</table>


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The differences in the constitution of the tubes, compared to the previous recommendations, are described below (Table 4). The immunophenotypic criteria that are useful in the diagnosis of T-cell neoplasms include the absence, under expression and overexpression of one or more of the pan-T antigens (CD2, SmCD3 and CD5), in addition to the expression of anomalous and cross-lineage antigens. The cytoplasmic CD3 and/or CD7 has been used as a backbone marker for T-cell lineage. Tubes 1 to 3 (Table 3) allow the classification of T-ALL according to the maturation profile of leukemic cells. Tube 4 was designed for later detection of MRD: 1) the CD99 expression is very frequently expressed and recognizes more immature T-ALLs, in addition to being stable after treatment and useful for MRD detection; 2) the CD45RA is expressed only in more immature subtypes of T-ALL and can be useful for detecting MRD. Tube 5 is essential for the identification of ETP-ALL, whose characteristics are the co-expression of cyCD3* and CD5*. The HLA-DR negativity is characteristic of T-ALL, and useful for MRD detection, and the CD44 is an optional marker for MRD detection. CD44 upregulation may be involved in T-ALL leukemogenesis and it has been reported to be highly expressed in pediatric T-ALL. Therefore, these markers may provide additional information for the diagnosis of T-ALL.

In comparison with the first guideline, the fluorochromes CD1a, CD2, CD3, CD4 and CD5 were changed, and CD3 and CD7 were maintained at the same fluorochromes. The HLA-DR negativity is characteristic of T-ALL; and the CD44 is an optional marker for MRD detection. The CD44 upregulation may be involved in T-ALL leukemogenesis and it has been reported to be highly expressed in pediatric T-ALL. Therefore, these markers may provide additional information for the diagnosis of T-ALL.

In comparison with the first guideline, the fluorochromes CD1a, CD2, CD3, CD4 and CD5 were changed, and CD3 and CD7 were maintained at the same fluorochromes in the different tubes as backbone markers to standardize the selections of the gates and to minimize the cost of the panels because these markers are in accordance with the previous recommendations for the MRD panels. The performance of the T-ALL diagnostic panel can be seen in Figure 2S (supplementary files).
Classification panel for acute myeloid leukemia

Acute myeloid leukemia (AML) is classified by the WHO into different categories: AML with recurrent genetic abnormalities; AML with myelodysplasia-related changes, therapy-related myeloid neoplasms; not otherwise specified (NOS), and; myeloid sarcoma and myeloid proliferations associated with Down syndrome.22 Most of them have immunophenotype-associated profiles, diagnosed by multiparametric flow cytometry.23,24

The development of AML from stem cells with specific founder mutations leads to an oligoclonal disease that progresses into a very heterogeneous leukemia at diagnosis.25,26 Table 5 provides information on molecular abnormalities in AML and associated immunophenotypes.24 The immunophenotypic alterations observed in AML are asynchronous antigen expression, antigens under- or over-expression and aberrant/cross lineage marker expression.26,27

Compared to previous recommendations, a few changes have been made. The mandatory panel contains several markers that will contribute to the identification and quantification of blast cells, as well as allow classification according to the lineage differentiation or non-differentiation.

Table 6 is designed to identify the leukemia committed lineage: neutrophil (3 and 4), monocytic (4 and 5) and erythroid (6).

Originally, tube 4 contained CD61 in the 2nd fluorochrome (FL), but this marker was replaced by CD33, which together with the other markers in tubes 3 and 4 (CD11b, CD13, CD15), allows for a better differentiation between leukemic blasts from the neutrophilic and monocytic lineage, including Acute Promyelocytic Leukemia (APL).

### Table 5 – AML molecular abnormalities and related immunophenotypic profiles.

<table>
<thead>
<tr>
<th>Molecular abnormality</th>
<th>Immunophenotypic expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML with t(8;21)(q22;q22.1); RUNX1-RUNXIT1</td>
<td>CD34+, CD117+, CD13high, CD33neg/norm, cMPO+, CD15−/+, CD65−/+, CD19−/+, and/or CD56−, CD11bneg</td>
</tr>
<tr>
<td>APL with t(15;17)(q24.1;q21.2); PML-RARA</td>
<td>CD4+ and/or CD36+ in myeloid blasts. Sometimes CD15+ and/or CD65+ and CD11b+ in the blasts. Presence of monocytic component.</td>
</tr>
<tr>
<td>AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH1</td>
<td>Monocytic markers, sometimes CD4+. CD33+ with CD13−, CD19+. CD13+ CD14+ CD117+, CD34+, CD123+. Basophilia.</td>
</tr>
<tr>
<td>AML with t(9;11)(p21.3;q23.3); KMT2A-MLLT3</td>
<td>Blasts: CD13+ CD117+, CD33 and MPO often negatives. CD11b− CD11c−, CD34+. Aberrant: CD7 e CD123 frequently and CD56 in some cases.</td>
</tr>
<tr>
<td>AML with t(6;9)(p23;q34.1); DEK-NUP214</td>
<td>Megakaryocytic component when present is: CD41+, CD42+, CD61+ and when monocytic present is: CD14 e CD64.</td>
</tr>
<tr>
<td>AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM</td>
<td>CD41+, CD42+ and/or CD61+.</td>
</tr>
</tbody>
</table>


### Table 6 – Monoclonal antibodies combinations recommended for the diagnosis of AML

#### Mandatory/ Essential

<table>
<thead>
<tr>
<th>Tubes</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HLA-DR</td>
<td>CD117</td>
<td>CD45</td>
<td>CD34</td>
</tr>
<tr>
<td>2</td>
<td>CD38</td>
<td>CD56</td>
<td>CD45</td>
<td>CD34</td>
</tr>
<tr>
<td>3</td>
<td>CD16</td>
<td>CD13</td>
<td>CD45</td>
<td>CD11b</td>
</tr>
<tr>
<td>4</td>
<td>CD15</td>
<td>CD33</td>
<td>CD45</td>
<td>CD34</td>
</tr>
<tr>
<td>5</td>
<td>CD300e</td>
<td>CD64</td>
<td>CD45</td>
<td>CD14+CD34</td>
</tr>
<tr>
<td>6</td>
<td>CD36</td>
<td>CD105</td>
<td>CD45</td>
<td>CD71</td>
</tr>
<tr>
<td>7</td>
<td>HLA-DR</td>
<td>CD123</td>
<td>CD45</td>
<td>CD4</td>
</tr>
</tbody>
</table>

#### Recommended

8 CD42b+CD61 CD33 CD45 CD34+CD117 <br> 9 CD45 CD203c CD34 CD122 <br> 10 CD2 CD25 CD45 CD117 <br>

#### Optional

11 CD44 NG2 CD45 CD34 <br> 12 NuTdT CD10 CD45 CD34

FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin.
In tube 5, designed for assess monocytic maturation (CD64, CD36, CD14 and IREM2), CD36 was replaced by CD300e (IREM2), which can distinguish the more mature monocytic compartment (CD14+/CD300e−) from the promonocytes (CD14+/CD300e+). It is a useful combination for subclassifying monocytic and monocytic leukemias. In addition, CD34 was added to CD14 in the 4th FL to identify more immature monocytic precursors and asynchronisms of antigen expressions in this lineage.

In tube 6, designed for erythroid lineage evaluation (CD71, CD36 and CD105), the glycopherin marker present in the first guideline was removed because it did not offer additional information. The CD105 (endoglin) is expressed in the early stages of erythroid differentiation (CD117+/CD45−/CD34+/CD13+/HLA-DR− cells), remains present after the levels of CD71 and CD36 increase and drops gradually after CD117 is lost, so that more mature red cell precursors no longer express CD105. The last mandatory tube (tube 7) is useful for the diagnosis of blastic plasmacytoid dendritic cell neoplasm (BPDCN). Although this is a less frequent type of leukemia, its correct diagnosis is mandatory due to the severity of the disease and the therapeutic impact of this diagnosis. The CD4 included in this tube is useful for the diagnosis of BPDCN and is also expressed in monocytic leukemia. Furthermore, it is a cross-lineage marker that can be useful in the detection of the MRD.

Tubes 8 to 10 are recommended in cases where the tubes described above are not able to subclassify the AML. The recommended tube 8 corroborates the identification of blast cell involvement with the megakaryocytic lineage, as acute megakaryoblastic leukemia can be positive for CD61, CD41 and CD42. The CD41 recommended in the first guideline was replaced by CD42a plus CD61FITC due to the higher frequency of these markers in this leukemia subtype.

Tubes 9 and 10 are intended for the diagnosis of acute basophilic leukemia and mast cell diseases respectively, including mast cell leukemia. The changes in the fluorochromes of CD22 and CD45 in tube 9 were justified by the better performance of CD22 in APC than in FITC and the good performance of CD45 in FITC. In addition, in tube 10 there are the CD2 and CD25 which can be useful markers for MRD purposes and prognosis in AML.

Optional tube 11 contains markers to detect leukemic stem cells (CD44) and immunophenotype associated with KMT2A-MLL rearrangements (NG2). Currently, the optional tube 12 included here is following circumstances: i) the TdT expression in myeloid and lymphoid precursors, ii) for the evaluation of lymphoid precursor cells (type 1), and; iii) the CD10 expression in granulocytic cells in MDS. The CD10 and TdT unrelate to the lymphoid lineage are also good markers for detecting MRD.

The AL screening and AML panel include several markers that detect the aberrant expression of lymphoid-associated antigens (CD19, cyCD79a, CD7, CD2, CD4 and CD56) and asynchronous antigen expression (CD14, CD15, CD16 and CD11b). Some of them can be useful in further MRD assessment. The performance of the AML diagnostic panel can be seen in Figure 3S (supplementary files).

Conclusion

In summary, this document presents updated guidelines for the use of validated 4-color panels considered relevant in the diagnosis of acute leukemia. The GBFLUX Acute Leukemia Committee has revised the monoclonal antibody consensus panel to provide a well-defined diagnosis of acute leukemia for clinical flow cytometry laboratories, including those with limited resources. This technical standardization should improve the quality of the acute leukemia diagnostics among different laboratories. In addition, this work paves the way for multicenter cooperative studies, promoting scientific and technological advances in clinical flow cytometry in Brazil.

Finally, these diagnostic guidelines can be replicated in laboratories that still use 4-color cytometers until they migrate to the 8- to 12-color ones.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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Supplementary materials

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