Title:

"First proposed panels on acute leukemia for four-color immunophenotyping by flow cytometry from the Brazilian Group of Flow Cytometry - GBCFLUX"

Running title: *"Proposed panels on acute leukemia from GBCFLUX"*

Authors: Maura R V Ikoma[1], Alex F Sandes[2,11], Leandro S Thiago[3], Geraldo B Cavalcanti Júnior[4], Irene G H Lorand-Metze[5], Elaine S Costa[6], Glicinia Pimenta[7,8], Maria C Santos-Silva[9], Nydia S Bacal[10] , Mihoko Yamamoto[11], Elizabeth X Souto[12]

Institutions:

1. Hospital Amaral Carvalho – Jau, SP, Brazil
2. Fleury Group, Division of Hematology, São Paulo-SP, Brazil
3. Brazilian National Cancer Institute (INCa), Cancer Research Center, Rio de Janeiro, RJ, Brazil
4. Universidade Federal do Rio Grande do Norte, RN, Brazil
5. Universidade Estadual de Campinas, SP, Brazil
6. Pediatric Institute IPPMG, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil.
7. Universidade Federal do Rio de Janeiro, RJ, Brazil
8. Laboratórios DASA, Rio de Janeiro, RJ, Brazil
9. Universidade Federal de Santa Catarina, SC, Brazil
10. Hospital Albert Einstein, São Paulo, SP, Brazil
11. Division of Hematology, Department of Clinical and Experimental Oncology, Escola Paulista de Medicina - Universidade Federal de São Paulo (UNIFESP-EPM), São Paulo, Brazil.
12. Laboratórios DASA, São Paulo, SP, Brazil

Primary research format: Regional Spotlight

Correspondence to: Maura R Valério Ikoma, MD, PhD
Address: Rua das Palmeiras, 106 - Jau, São Paulo, Brazil ZIP: 17210120

E-mail: msikoma@uol.com.br

Phone: +55 14 981654884 / Fax: +55 14 36021332

On behalf of GBCFLUX Acute Leukemia Subcommittee**:** Alex F Sandes, Daniela Gregolin Marrese, Elaine S Costa, Eliana Brasil, Elizabeth Souto, Fernanda G P Cunha, Geraldo B. Cavalcanti Jr, Glicínia Pimenta, Irene G H Lorand-Metze, Leandro S Thiago, Maria C S da Silva, Maria C Maioli, Maria M Sales, Mariester Malvezzi, Maura R V Ikoma, Mihoko Yamamoto, Nydia S Bacal, Roseli Antunes, Solange A Pacheco, Tereza C Bortolheiro

Conflict of interest statement: The authors declare that there is no conflict of interest**.**

Text word Count: 3,549
Abstract word count: 163
Figures: 5
Tables: 5

ABSTRACT

Multiparameter flow cytometry (MFC) is a highly sensitive, fast and specific diagnostic technology with a wide range of applicability in hematology. Although well-established eight-color immunophenotyping panels are already available, most Brazilian clinical laboratories are equipped with four-color flow cytometer facilities. Based on this fact, the Brazilian Group of Flow Cytometry (*Grupo Brasileiro de Citometria de Fluxo*, GBCFLUX) for standardization of clinical flow cytometry has proposed an antibody panel designed to allow precise diagnosis and characterization of acute leukemia (AL) within resource-restricted areas. Morphological analysis of bone marrow smears, together with the screening panel, is mandatory for the primary identification of AL. The disease-oriented panels proposed here are divided into three levels of recommendations (mandatory, recommendable and optional) in order to provide an accurate final diagnosis, as well as allow some degree of flexibility based on available local resources and patient-specific needs. The proposed panels will be subsequently validated in an inter-laboratory study to evaluate its effectiveness on the diagnosis and classification of AL. (Assoc editor comm. 2)

Key terms: flow cytometry, acute leukemia panel, acute lymphoblastic leukemia, acute myeloblastic leukemia, GBCFLUX

INTRODUCTION

The Brazilian Group of Flow Cytometry (*Grupo Brasileiro de Citometria de Fluxo*, GBCFLUX) was founded on April 24, 2010, aiming to congregate Brazilian experts in clinical flow cytometry and promote technical and scientific advances on multiparameter flow cytometry (MFC) in Brazil. One of our goals was to standardize all clinical laboratory steps (sample handling, staining protocols, data analysis and interpretation, quality control and data report emissions). In order to reach these aims, several working groups were established. The committee for standardization of diagnosis of acute leukemias collected information on panels used at several institutions and reviewed the recent literature.

In Brazil, most clinical laboratories are equipped with flow cytometers able to analyze three- or four-color combinations of antibodies. In light of this, the GBCFLUX working group has designed disease-oriented antibody panels with four-color antibody combinations based on the most powerful markers able to discriminate acute leukemia (AL) nosological entities.

We looked for the minimum requirements needed to achieve the correct final diagnosis, taking into account the cost-effectiveness acceptable for services with different economic possibilities.

Besides the diagnostic purpose, some of the proposed combinations were designed to provide prognostic information and to be suitable for detection of minimal residual disease (MRD) in clinical studies.

The design of an ideal diagnostic panel should include: i) identification of abnormal blast cells, allowing discrimination between normal/reactive and leukemic cells; ii) lineage identification; iii) disease classification according to the cells maturation stage; iv) identification of leukemia-associated phenotypic markers (LAIPs) to be used in MRD studies; and v) detection of aberrant markers/ phenotypes associated with molecular alterations with well-recognized prognostic implications (1-5).

All panels were designed to accommodate different levels of recommendations for diagnostic accuracy and classification, to allow some degree of flexibility according to the available local laboratory resources. These levels are: i) mandatory, ii) recommendable, and iii) optional. Mandatory recommendations contain the minimum criteria for identification, quantification and classification of AL. Recommendable level includes markers that are not essential for diagnostic but are important for leukemia subclassification. Optional recommendations include markers useful for MRD investigation, detection of rare subtypes of leukemia, alternative classification and markers that may suggest molecular or cytogenetic abnormalities and prognosis.

The fluorochrome combination suggested in the GBCFLUX panels (fluorescein isothiocyanate – FITC, phycoerythrin – PE, peridinin chlorophyll protein/cyanin 5 – PerCP-Cy5.5 , allophycocyanin – APC) were proposed by Becton Dickinson and Beckman Coulter (BC) users and could be applied for laboratories that use any type of flow cytometer equipped with two lasers. Alternatively, BC users could associate the fluorochromes ECD (energy couple dye, PE-Texas Red) and PC5 (phycoerythrin-cyanine 5) to FITC and PE in four-color combinations (supplemental material).

The proposed panels will be subsequently validated in an inter-laboratory study to evaluate its effectiveness on the diagnosis and classification of AL. These recommendations will be updated periodically by the GBCFLUX working group, according to new validated biomarkers data published elsewhere.

GENERAL RECOMMENDATIONS

Relevance of clinical data. It is of utmost importance for the clinical laboratory to receive detailed patient information containing, at minimum: age, gender, peripheral blood (PB) counts, disease phase (diagnosis, minimal residual disease), previous treatment and whether it is a relapse or a secondary transformation.

Morphological analysis. Morphology and flow cytometry immunophenotyping are essential and complementary techniques for the appropriate diagnostic assessment of AL. This makes it desirable to have information about bone marrow (BM) cytology, a description of the blast cells and cytochemistry. However, cell morphology analysis should not be used as a screening panel substitutive, only as additional diagnostic information.

Screening panel. We agreed on panels with three steps: screening, lineage determination and in-depth study with search of aberrant co-expressions including those related to specific molecular changes. This rationale is also found in most of the recent consensus recommendations (1-5). The diagnosis of AL should follow the steps detailed in Figure 1.

ACUTE LEUKEMIA SCREENING PANEL

The main objective of the AL screening panel (ALST) is to provide information about the lineage of the leukemic blasts (Table 1). In addition, ALST allows identification of expression of some lineage infidelity markers, such as the expression of cytoplasmic (Cy) CD79a and CD19 by t(8;21) positive acute myeloid leukemia (AML) patients (6,7), $_{Cy}$CD79 positivity in some T-ALL (8) and CD7 in some AML cases (9).

Based on the immunophenotypic information derived from ALST tubes, lineage-directed panels (BCP-ALL or T-ALL or AML/MDS) must be applied in order to provide the final diagnosis (figures 1 and 2).

However, some cases cannot be diagnosed with the screening tube alone. In these cases, an expanded study must be performed to accurately define the clinical entity. For instance, megakaryoblastic leukemia and minimal differentiated AML frequently do not express $_{Cy}$MPO; and undifferentiated AL may not be detected with an ALST tube.

It is noteworthy that, because an overt cell expansion may occur in reactive conditions or during BM regenerative processes, the discrimination among normal, reactive/regenerative and acute leukemia must be based on an abnormal protein expression pattern and altered light scatter properties (1).

Considering the goal of determining the maturation degree of the expanded compartment, we have included CD45 and CD34 as mandatory markers at this first diagnostic level, since they are the most efficient markers to define immaturity.

The expression of $_{Cy}$CD79a is an early event during B-cell commitment, occurring after $_{Nu}$TdT expression and before acquisition of CD19 protein (10). $_{Cy}$CD79a is a part of the B-cell receptor (BCR) signaling complex, and virtually all B-cell precursor acute lymphoblastic leukemia (BCP-ALL) express this marker (11). In addition, $_{Cy}$CD79a may discriminate between AML, BCP-ALL and T-ALL (1). Overall, $_{Cy}$CD79a is a suitable marker for lineage specification, although its expression may also be found on myeloid or T-cell leukemia. The expression of $_{Cy}$CD79a is followed by the activation of the *PAX5* transcriptional factor gene, a master regulator of B-cell differentiation (12). *PAX5* activates, among other proteins, the expression of CD19. As with $_{Cy}$CD79a, CD19 may be found in non B-cell malignancies (6,7). However, virtually all cases of BCP-ALL express both $_{Cy}$CD79a and CD19.

Regarding T-cell commitment and differentiation, the earliest T-cell precursor ($CD7^+$ / $_{Nu}TdT^+$ / CD34+ / $CD117^+$ / $CD44^+$) migrates into the thymus where T-committed cells will fully differentiate (13). Because CD7 is expressed at pre-thymic differentiation stages during normal hematopoiesis and considering that virtually all T-ALL express CD7, the inclusion of this marker on the

screening panel allows for the identification of early and late T-cell progenitors (14). The acquisition of $_{Cy}$CD3 takes place early in the thymus and is found in nearly all T-ALL cases. But expression of CD7 without that of $_{Cy}$CD3 may be found in AML (9) and, sporadically, in BCP-ALL (15). In the majority of cases with $_{Cy}$CD3 protein expression, the results should be interpreted together with surface membrane (Sm) CD3, because most of T-ALL cases present with a $_{Cy}CD3^{+}/\ _{Sm}CD3^{-/lo}$ pattern (1).

AML comprises the most heterogeneous group, leading to many diagnostic challenges when using limited panels**.** $_{Cy}$MPO was selected for the screening tube based on the finding that this enzyme is expressed in a wide range of myeloid precursors, with the exception of megakaryoblasts, eosinophil precursors and erythroblasts (16,17). Very early myeloid precursors may also lack $_{Cy}$MPO expression, like early $CD34^{+}$ monoblasts. In these cases, if the ALST tubes point towards a non-lymphoid acute leukemia, the AML panel should be performed.

The cases of mixed phenotype acute leukemia (MPAL) are initially detected by the ALST tube, but require more than one complementary panel to complete the diagnosis (18,19).

B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA PANEL

Based on the well-known B-cell differentiation steps, the European Group for the Immunological Characterization of Leukemia (EGIL) (18) proposed a standardized immunological classification for BCP-ALL according to lineage-related markers and maturation stage of leukemic cells (11).

The relevance of flow cytometry when studying lymphoid precursors relies, to a large extent, on the difficulty of distinguishing normal precursor B cells from truly malignant expansions by morphology alone. B-cell precursor malignancies show an abnormal protein expression pattern allowing a clear discrimination between normal/reactive expansions from BCP-ALL (10,11,18,20-22). Because virtually all BCP-ALL cases express CD19, this protein was selected as a backbone marker. CD22 is not lineage-specific because it is expressed on normal mast cells (23) during basophil maturation(24) and on plasmacytoid dendritic cells(25). It was added into the mandatory panel for MRD proposals because its expression is highly variable in leukemic cells and may differentiate them from their normal counterparts.

These so-called mandatory combinations are used to detect B-cell asynchronous differentiation (Table 2) and some frequent lineage infidelity markers: CD13 and CD33 as mandatory (11,26), and CD66c (26) and CD15/CD65 (11) as optional. CD66c is not found in normal maturing B-cells and

its expression in BCP-ALL is not affected by chemotherapy (26). It should be stressed that the homogeneous expression of $CD34^{bright}$ and $CD10^{bright}$, the $CD45^{dim}$ and $CD38^{dim}$ expression, and the presence of myeloid markers CD13 and CD33 are associated with the presence of the *BCR-ABL* fusion gene, especially if the CD66c protein is expressed (27,28). CD66c is also expressed in hyperdiploid BCP-ALL cases.

When peripheral blood is examined, the presence of circulating cells expressing proteins not usually found in circulating lymphoid cells may indicate cells with an ectopic phenotype (e.g., $CD19^{+}/CD10^{+}/_{Nu}TdT^{+}$). These represent malignant cells.

In general, the BCP-ALL is classified into four major groups based on the presence/absence of some key proteins (CD10, $_{Cy}$IgM, $_{Sm}$IgM), according to the score proposed by the EGIL group (18). The majority of Pro-B cases ($CD19^{+}$/ $CD10^{-}$/ $_{Nu}TdT^{+}$/ $CD34^{+}$) represent 5% of pediatric and 10% of adult cases. The B-II subtype ($CD19^{+}/CD10^{+}/_{Cy}IgM^{-}$) is the most common presentation form, with 75% and 50% of pediatric and adult cases, respectively (18,29). Blast cells $_{Cy}IgM^{+}$, expressing or not CD10, are classified as Pre-B ALL (18) and comprise approximately 15% and 10% of pediatric and adult BCP-ALL cases, respectively.

The B-IV type is related to the Burkitt lymphoma (18) and deserves further comment. In cases where leukemic blasts present a mature B-cell phenotype ($CD19^{+}/TdT^{-}/CD34^{-}$ /$CD10^{+}/CD20^{+}/_{Sm}IgM^{+}$) and surface-membrane

light chains of immunoglobulin ($_{Sm}\kappa$ or $_{Sm}\lambda$), the sample should be reoriented to a panel designed to investigate mature B-cell lymphomas in order to establish a correct diagnosis.

CD10, CD19, CD20, CD34, $_{Nu}$TdT and CD38 combinations (Table 2: tubes 1 and 3) are highly efficient to discriminate malignant from normal/regenerative/reactive immature B-cells. These combinations could be useful in MRD assessment. CD58 protein is an interesting LAIP marker because it is highly expressed on malignant B-cells but is expressed at low levels in normal/regenerating B-cell precursors and mature B-cells (30,31).

CD10 is expressed at very early stages during B-cell ontogeny, and the absence of CD10 expression is considered an aberrant phenotype. Interestingly, pre-B ALL lacking CD10 expression (32) and adult patients with pre-B ALL (33) show a high frequency of cytogenetic abnormalities involving chromosome band 11q23. In those cases, the samples should be investigated for the expression of CD15/CD65 and NG2 (Table 2: tube 6), because this phenotype correlates with MLL rearrangements independently of the partner gene (33-35) (figure 3). It is noteworthy that the expression of CD15/CD65 and/or NG2 are not considered specific for *MLL* rearrangements (35) and thus, molecular biology studies are needed to confirm the presence of the genetic abnormality. Because BCP-ALL with MLL rearranged is the most common ALL in infants <1 year of age, the use of CD15/CD65 and NG2 is recommended for this group.

T-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA PANEL

T lymphoblastic leukemia/lymphoblastic lymphoma are neoplasms derived from immature lymphoid cells of T-lineage involving BM and PB (T-acute lymphoblastic leukemia, T-ALL) or presenting with primary involvement of thymus, nodal or extranodal sites (T-acute lymphoblastic lymphoma, T-LBL).

For this diagnosis, this panel should be used when ALST identifies blast cells expressing CD7 and/or $_{Cy}$CD3. In addition to the lineage assessment, the panel was constructed to apply immunophenotypic sub-classification systems according to the T-cell maturation stages and detect LAIPs for later investigation of minimal residual disease.

Identification and quantification of blast cells. CD7 antigen has been classically considered the earliest T-cell-associated marker expressed during T-cell maturation. It is usually present at high levels and is an excellent marker for blast cell gating strategy. To exclude mature residual T-cells from the analysis, CD7 is combined with surface CD3 or CD5. However, this strategy is not capable of discriminating residual NK cells from lymphoblasts.

The immature lymphoid marker $_{Nu}$TdT is expressed in more than 90% of T-ALL cases (14) and, combined with CD7 and $_{Cy}$CD3, is a powerful strategy to correctly identify and quantify the blast cells (Table 3).

Diagnosis. To evaluate the immature nature of blast cells, $_{Nu}$TdT and CD1a were included in the mandatory panel and CD99 in the recommended panel. CD34 was already assessed by the ALST. To specify the T-lineage maturation stage of blast cells CD2, $_{Sm}$CD3, CD4, CD5, CD7, CD8, CD10, TCRαβ and TCRγδ were included. The choice of these antibodies, along with fluorochrome combinations, was based on literature review and the experience of the expert group (36-41).

T-cell development occurs primarily in the thymus, and the identification of immature T-cells in PB, BM or tissues other than thymus is *per se* abnormal. - Virtually all T-ALL cases have immunophenotypic abnormalities that distinguish the leukemic cells from normal thymocytes (14). This can be extremely helpful in the differential diagnosis of thymic hyperplasia, when flow cytometry of mediastinal tissue is performed (36).

$_{Cy}$CD79a and CD10 expression have been observed in approximately 10-40% (37) and 40-50% (38) of T-ALL cases, respectively. These markers should not be considered as evidence of B-cell differentiation in the absence of CD19 expression.

The recommended and optional tubes contain the myeloid markers CD13, CD33 and CD117. Myeloid markers are expressed in 19-30% of cases (39,40). Its presence does not exclude the diagnosis of T-ALL/LBL, nor does it indicate a mixed phenotype T/myeloid leukemia in the absence of myeloperoxidase expression (38). Many markers characteristic of T-cell lineage, such as CD7 and

CD2, and even $_{Cy}$CD3, may also be seen in NK cell precursors (41). Thus, it may be very difficult to distinguish the rare true precursor NK lymphoblastic leukemia/lymphoma from T-ALL/LBL that expresses only immature markers. CD56, while characteristic of NK-cells, is not lineage-specific, as it can be found on other hematological malignancies (42,43) and is commonly found in solid tumors(44). Moreover, CD56 expression does not exclude T-ALL (38). In this regard, the expression of myeloid markers in T-ALL but not in NK-ALL may be helpful in this situation (45). The diagnosis of precursor NK lymphoblastic leukemia may be considered in a case where CD56 is found together with immature T-cell markers, but only if (1) B-cell and myeloid antigens are lacking, (2) *TCR* and *Ig* receptor genes are in the germline configuration and (3) blastic plasmacytoid dendritic cell neoplasm (BPDCN) has been excluded (45)**.**

The presence of CD123, an interleukin-3 receptor associated with $_{Cy}$CD3, CD4, CD19 and $_{Cy}$MPO expression, could be helpful on the differential diagnosis of BPDCN with expression of T-lineage markers (e.g., CD2 and CD7). CD123$^{dim/int}$ expression is found in 16% of T-ALL cases and is more common in immature T-ALL (46). Despite the low number of cases evaluated, CD123 expression was associated with remission failure after chemotherapy induction (46,47). Although there was no consensus about the use of CD123 in T-ALL, it may be an important marker for MRD detection.

Classification. T-ALLs are classified according to their maturation arrest in comparison to normal T-cell ontogeny. The EGIL classification divides T-ALL into four stages based on CD2, $_{Sm}$CD3, CD5, CD7 , CD8, CD1a and TCR expression: pro-T (or TI), pre-T (TII), cortical T (TIII) and mature/medullary T (TIV) ALL(18,38). Pro-T ALL typically shows co-expression of $_{Cy}$CD3 and CD7 in the absence of other T-cell associated markers (i.e., $CD2^-$, $_{Sm}CD3^-$, $CD5^-$, $CD8^-$ and $CD1a^-$). In addition to $_{Cy}$CD3 and CD7, pre-T ALL cases express surface CD2, CD5 and/or CD8 in the absence of CD1a. As normal cortical thymocytes, blast cells from cortical T-ALL express CD1a (figure 4). The medullary T-ALL phenotype ($_{Sm}CD3^+$, $CD1a^-$, $CD4^+$ or $CD8^+$) is more frequently observed among patients presenting T-LBL. Some groups have shown a correlation between the stages of EGIL classification and survival (48-50). T-ALL tends to show a more immature immunophenotype compared with T-LBL, but the groups usually overlap (51,52).

Our panel was designed to allow the application of EGIL classification. In both TIII and TIV T-ALL, surface expression of CD3 may be associated with expression of TCR of either the TCRαβ or TCRγδ type. Therefore, combinations including TCR are only recommended if blasts express surface CD3.

Recently, a new subtype of T-ALL, originated from early T-cell precursors (ETPs), was described. This is a subset of thymocytes representing recent immigrants from the BM to the thymus, which retain multilineage differentiation

potential (53,54). This subtype was identified by using a set of genes differentially expressed in ETPs by gene expression profile; it presents a distinct immature phenotype characterized by a lack of CD1a and CD8 expression, weak CD5 expression with <75% positive blasts, and expression of one or more myeloid or stem/progenitor cell markers on at least 25% of blasts (CD117, CD34, HLA-DR, CD13, CD33, CD11b and/or CD65) (53). ETP-ALL comprises 20-30% of T-ALL cases and presents a poor prognosis when treated with standard chemotherapy (53). The recommended and optional combinations, together with ALST, permit the identification of this entity by co-expression of $_{Cy}CD3^{+}$, $CD5^{-/lo}$, $CD1a^{-}$, $CD8^{-}$ with stem/progenitor-cell/myeloid markers (CD34, CD117, CD13 and CD33).

Leukemia-associated immunophenotype (LAIP) markers. Evaluation of LAIPs at a diagnosis of T-ALL/LBL is essential for MRD assessment by flow cytometry. Because virtually no immature T-cells are detected in normal PB and BM samples, the presence of immature T-cells on these tissues is highly suspicious for leukemia. Therefore, the identification of intra-thymic phenotypes in PB or BM is considered an important LAIP. Such ectopic phenotype could be evaluated by the presence of precursor T-cell proteins (e.g., CD1a, $_{Nu}$TdT, CD34 and CD99) or the absence/dim expression of surface CD3.

In addition, the low expression/lack of T-cell proteins (CD2, CD5, CD4 and CD8), overexpression of CD7 and infidelity of lineage markers ($_{Cy}$CD79a,

CD13, CD33 and CD117) are frequently detected in T-ALL and could be promptly assessed by the proposed panel.

ACUTE MYELOID LEUKEMIA PANEL

AML is a heterogeneous group of clonal hematologic disorders that affect multiple cell lineages at several maturation stages. According to the World Health Organization's (WHO's) 2008 classification, the AML diagnostic work-up includes recurrent genetic abnormalities, AML with myelodysplasia-related changes and AML not otherwise specified (55). These abnormalities are associated with distinct clinical, morphological and immunophenotypic characteristics, defining specific pathological entities.

AML immunophenotypic profiles are highly heterogeneous, probably due to the genetic diversity. An aberrant immunophenotypic pattern can be found in 85% to 90% (56-58) of AML cases. These altered phenotypes are due to asynchronous expressions (80%), lineage-infidelity phenotype (27%) or aberrant marker expression (20%) (56).

We wanted to propose a panel that could detect and classify AML, especially focusing on the lineage assessment and maturation profile of blast cells (Table 4). The mandatory panel contains several markers that contribute to the identification and quantification of blast cells (CD45, CD34, CD117 and HLA-

DR) (59), and enable characterization of immature cells into the major myeloid compartments: neutrophil (CD117, CD13, CD33 and CD15), monocytic (CD64, CD36, CD4 and CD14), erythroid (CD71, CD235a and CD36) and megakaryocytic lineage (CD61 and CD36) (figure 5). In addition, screening and AML mandatory panels include several markers that are able to detect aberrant expression of lymphoid-associated antigens (CD19, $_{Cy}$CD79a, CD7, CD2 and CD56) and asynchronous antigen expression (CD14, CD15, CD16 and CD11b), which are essential to further MRD assessment (60,61).

The optional panel contains a combination of CD105, CD45, CD36 and CD71, allowing a more complete evaluation of erythroid lineage. CD105 (endoglin) is expressed in the early stages of erythroid differentiation ($CD117^{+}$/ $CD45^{++}$/$CD34^{-}$/$CD13^{-}$/HLA-DR^{-} cells); it remains present after the increasing of CD71 and CD36 and gradually decreases in more mature red cell precursors (1,62). Moreover, the optional panel corroborates the commitment of blast cells to the megakaryocytic lineage, as megakaryoblastic acute leukemia can be positive only for CD61 or CD42 (16).

If the lineage-commitment of blast cells cannot be defined after completing the mandatory panel, additional tubes should be performed to evaluate the less frequent myeloid lineages (pDC, basophils and mast cells). BPDCN could be suspected in $CD4^{+}$/$CD56^{+}$ cases that lack expression of CD34 and lineage-specific markers ($_{Cy}$MPO, CD64, CD14, CD61, $_{Cy}$CD3, $_{Cy}$CD79a and CD19)

(42,63). BPDCN can also express CD36 and NG2 (64). Commitment with the pDC lineage is confirmed by the strong expression of CD123 associated with HLA-DR^{+} (65). CD117$^{-/lo}$ basophils also highly express CD123, but they are usually HLA-DR negative. They also express CD203c in virtually all cases (66). Mast cell leukemia should be suspected in cases with a CD117^{+++}/HLA-DR^{-}/CD34^{-} phenotype, and expression of CD203c, CD25 and CD2 can be used to identify aberrant mast cell components (23).

Some specific immunophenotypic patterns may suggest recurrent genetic abnormalities. Thus, this may be of great help to direct further specific analysis by fluorescent *in situ* hybridization (FISH) or molecular biology (Table 5) (67-71).

CONCLUSION

The consensus presented here represents the initiative of GBCFLUX to provide clinical laboratories with a frame of reference for a systematic diagnostic approach. This effort aims to propose a similar working schedule for different laboratories in order to provide consistent quality in the diagnosis of acute leukemia in our country, as the basis for multicentric clinical studies and inter-laboratory quality control.

ACKNOWLEDGMENTS

The authors thank Ms Marcimara Penitenti (Hospital Amaral Carvalho Jau) for data collection and tabulation.

REFERENCES

1. van Dongen JJ, Lhermitte L, Bottcher S, Almeida J, van der Velden VH, Flores-Montero J, Rawstron A, Asnafi V, Lecrevisse Q, Lucio P and others. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 2012;26(9):1908-75.
2. Bene MC, Nebe T, Bettelheim P, Buldini B, Bumbea H, Kern W, Lacombe F, Lemez P, Marinov I, Matutes E and others. Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10. Leukemia 2011;25(4):567-74.
3. Wood BL, Arroz M, Barnett D, DiGiuseppe J, Greig B, Kussick SJ, Oldaker T, Shenkin M, Stone E, Wallace P. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: optimal reagents and reporting for the flow cytometric diagnosis of hematopoietic neoplasia. Cytometry B Clin Cytom 2007;72 Suppl 1:S14-22.
4. Ruiz-Arguelles A, Rivadeneyra-Espinoza L, Duque RE, Orfao A. Report on the second Latin American consensus conference for flow cytometric immunophenotyping of hematological malignancies. Cytometry B Clin Cytom 2006;70(1):39-44.
5. Haycocks NG, Lawrence L, Cain JW, Zhao XF. Optimizing antibody panels for efficient and cost-effective flow cytometric diagnosis of acute leukemia. Cytometry B Clin Cytom 2011;80(4):221-9.
6. Kita K, Nakase K, Miwa H, Masuya M, Nishii K, Morita N, Takakura N, Otsuji A, Shirakawa S, Ueda T and others. Phenotypical characteristics of acute myelocytic leukemia associated with the t(8;21)(q22;q22) chromosomal abnormality: frequent expression of immature B-cell antigen CD19 together with stem cell antigen CD34. Blood 1992;80(2):470-7.
7. Tiacci E, Pileri S, Orleth A, Pacini R, Tabarrini A, Frenguelli F, Liso A, Diverio D, Lo-Coco F, Falini B. PAX5 expression in acute leukemias: higher B-lineage specificity than CD79a and selective association with t(8;21)-acute myelogenous leukemia. Cancer Res 2004;64(20):7399-404.
8. Asnafi V, Beldjord K, Garand R, Millien C, Bahloul M, LeTutour P, Douay L, Valensi F, Macintyre E. IgH DJ rearrangements within T-ALL correlate with cCD79a expression, an immature/TCRgammadelta phenotype and absence of IL7Ralpha/CD127 expression. Leukemia 2004;18(12):1997-2001.

9. Macedo A, Orfao A, Vidriales MB, Lopez-Berges MC, Valverde B, Gonzalez M, Caballero MD, Ramos F, Martinez M, Fernandez-Calvo J and others. Characterization of aberrant phenotypes in acute myeloblastic leukemia. Ann Hematol 1995;70(4):189-94.
10. Dworzak MN, Fritsch G, Froschl G, Printz D, Gadner H. Four-color flow cytometric investigation of terminal deoxynucleotidyl transferase-positive lymphoid precursors in pediatric bone marrow: CD79a expression precedes CD19 in early B-cell ontogeny. Blood 1998;92(9):3203-9.
11. Lucio P, Parreira A, van den Beemd MW, van Lochem EG, van Wering ER, Baars E, Porwit-MacDonald A, Bjorklund E, Gaipa G, Biondi A and others. Flow cytometric analysis of normal B cell differentiation: a frame of reference for the detection of minimal residual disease in precursor-B-ALL. Leukemia 1999;13(3):419-27.
12. Cobaleda C, Schebesta A, Delogu A, Busslinger M. Pax5: the guardian of B cell identity and function. Nat Immunol 2007;8(5):463-70.
13. Cavalcanti Junior GB, Savino W, Pombo-de-Oliveira MS. CD44 expression in T-cell lymphoblastic leukemia. Braz J Med Biol Res 1994;27(9):2259-66.
14. Porwit-MacDonald A, Bjorklund E, Lucio P, van Lochem EG, Mazur J, Parreira A, van den Beemd MW, van Wering ER, Baars E, Gaipa G and others. BIOMED-1 concerted action report: flow cytometric characterization of CD7+ cell subsets in normal bone marrow as a basis for the diagnosis and follow-up of T cell acute lymphoblastic leukemia (T-ALL). Leukemia 2000;14(5):816-25.
15. Hussein S, Gill KZ, Sireci AN, Colovai AI, Small T, Emmons FN, Murty VV, Bhagat G, Alobeid B. Aberrant T-cell antigen expression in B lymphoblastic leukaemia. Br J Haematol 2011;155(4):449-56.
16. Gassmann W, Loffler H. Acute megakaryoblastic leukemia. Leuk Lymphoma 1995;18 Suppl 1:69-73.
17. Orfao A, Ortuno F, de Santiago M, Lopez A, San Miguel J. Immunophenotyping of acute leukemias and myelodysplastic syndromes. Cytometry A 2004;58(1):62-71.
18. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, van't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995;9(10):1783-6.
19. Costa ES, Thiago LS, Otazu IB, Ornellas MH, Land MG, Orfao A. An uncommon case of childhood biphenotypic precursor-B/T acute lymphoblastic leukemia. Pediatr Blood Cancer 2008;50(4):941-2.
20. Ciudad J, Orfao A, Vidriales B, Macedo A, Martinez A, Gonzalez M, Lopez-Berges MC, Valverde B, San Miguel JF. Immunophenotypic

analysis of CD19+ precursors in normal human adult bone marrow: implications for minimal residual disease detection. Haematologica 1998;83(12):1069-75.

21. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdaal NA, van Dongen JJ. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. Cytometry B Clin Cytom 2004;60(1):1-13.
22. Patkar N, Alex AA, B B, Ahmed R, Abraham A, George B, Vishwabandya A, Srivastava A, Mathews V. Standardizing minimal residual disease by flow cytometry for precursor B lineage acute lymphoblastic leukemia in a developing country. Cytometry B Clin Cytom 2012;82(4):252-8.
23. Escribano L, Diaz-Agustin B, Lopez A, Nunez Lopez R, Garcia-Montero A, Almeida J, Prados A, Angulo M, Herrero S, Orfao A. Immunophenotypic analysis of mast cells in mastocytosis: When and how to do it. Proposals of the Spanish Network on Mastocytosis (REMA). Cytometry B Clin Cytom 2004;58(1):1-8.
24. Han K, Kim Y, Lee J, Lim J, Lee KY, Kang CS, Kim WI, Kim BK, Shim SI, Kim SM. Human basophils express CD22 without expression of CD19. Cytometry 1999;37(3):178-83.
25. Martin-Martin L, Almeida J, Hernandez-Campo PM, Sanchez ML, Lecrevisse Q, Orfao A. Immunophenotypical, morphologic, and functional characterization of maturation-associated plasmacytoid dendritic cell subsets in normal adult human bone marrow. Transfusion 2009;49(8):1692-1708.
26. Kalina T, Vaskova M, Mejstrikova E, Madzo J, Trka J, Stary J, Hrusak O. Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of CD66c distinct from other myeloid antigens. BMC Cancer 2005;5:38.
27. Owaidah TM, Rawas FI, Al Khayatt MF, Elkum NB. Expression of CD66c and CD25 in acute lymphoblastic leukemia as a predictor of the presence of BCR/ABL rearrangement. Hematol Oncol Stem Cell Ther 2008;1(1):34-7.
28. Tabernero MD, Bortoluci AM, Alaejos I, Lopez-Berges MC, Rasillo A, Garcia-Sanz R, Garcia M, Sayagues JM, Gonzalez M, Mateo G and others. Adult precursor B-ALL with BCR/ABL gene rearrangements displays a unique immunophenotype based on the pattern of CD10, CD34, CD13 and CD38 expresssion. Leukemia 2001;15(3):406-14.
29. Alves GV, Fernandes AL, Freire JM, Paiva Ade S, Vasconcelos RC, Sales VS, Lemos TM, Gil EA, Freire FH, Cavalcanti e Silva DG and others.

Flow cytometry immunophenotyping evaluation in acute lymphoblastic leukemia: correlation to factors affecting clinic outcome. J Clin Lab Anal 2012;26(6):431-40.

30. 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(11):1245-52.
31. Chen JS, Coustan-Smith E, Suzuki T, Neale GA, Mihara K, Pui CH, Campana D. Identification of novel markers for monitoring minimal residual disease in acute lymphoblastic leukemia. Blood 2001;97(7):2115-20.
32. Gleissner B, Goekbuget N, Rieder H, Arnold R, Schwartz S, Diedrich H, Schoch C, Heinze B, Fonatsch C, Bartram CR and others. CD10- pre-B acute lymphoblastic leukemia (ALL) is a distinct high-risk subgroup of adult ALL associated with a high frequency of MLL aberrations: results of the German Multicenter Trials for Adult ALL (GMALL). Blood 2005;106(13):4054-6.
33. Ludwig WD, Rieder H, Bartram CR, Heinze B, Schwartz S, Gassmann W, Loffler H, Hossfeld D, Heil G, Handt S and others. Immunophenotypic and genotypic features, clinical characteristics, and treatment outcome of adult pro-B acute lymphoblastic leukemia: results of the German multicenter trials GMALL 03/87 and 04/89. Blood 1998;92(6):1898-909.
34. Hrusak O, Porwit-MacDonald A. Antigen expression patterns reflecting genotype of acute leukemias. Leukemia 2002;16(7):1233-58.
35. Borowitz MJ, Chan JKC. B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities. In: Swerdlow S.H.; Campo EH, N.L.; Jaffe, E.S.; Pileri, S.A., Stein, H., Thiele, J.; Vardiman, J.W., editor. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: International Agency for Research on Cancer.; 2008. p 171-175.
36. Gorczyca W, Tugulea S, Liu Z, Li X, Wong JY, Weisberger J. Flow cytometry in the diagnosis of mediastinal tumors with emphasis on differentiating thymocytes from precursor T-lymphoblastic lymphoma/leukemia. Leuk Lymphoma 2004;45(3):529-38.
37. Pilozzi E, Pulford K, Jones M, Muller-Hermelink HK, Falini B, Ralfkiaer E, Pileri S, Pezzella F, De Wolf-Peeters C, Arber D and others. Co-expression of CD79a (JCB117) and CD3 by lymphoblastic lymphoma. J Pathol 1998;186(2):140-3.
38. Borowitz MJ, Chan JKC. T lymphoblastic leukaemia/lymphoma. In: Swerdlow S.H.; Campo EH, N.L.; Jaffe, E.S.; Pileri, S.A., Stein, H., Thiele, J.; Vardiman, J.W., editor. WHO classification of tumours of

haematopoietic and lymphoid tissues. 4th ed. Lyon: International Agency for Research on Cancer.; 2008. p 176-178.

39. Khalidi HS, Chang KL, Medeiros LJ, Brynes RK, Slovak ML, Murata-Collins JL, Arber DA. Acute lymphoblastic leukemia. Survey of immunophenotype, French-American-British classification, frequency of myeloid antigen expression, and karyotypic abnormalities in 210 pediatric and adult cases. Am J Clin Pathol 1999;111(4):467-76.
40. Uckun FM, Gaynon PS, Sensel MG, Nachman J, Trigg ME, Steinherz PG, Hutchinson R, Bostrom BC, Sather HN, Reaman GH. Clinical features and treatment outcome of childhood T-lineage acute lymphoblastic leukemia according to the apparent maturational stage of T-lineage leukemic blasts: a Children's Cancer Group study. J Clin Oncol 1997;15(6):2214-21.
41. Spits H, Lanier LL, Phillips JH. Development of human T and natural killer cells. Blood 1995;85(10):2654-70.
42. Sandes AF, Castro IN, Miura TE, Maekawa YH, Cozac AP, Chauffaille ML, Rizzatti EG. Bone marrow infiltration by cells resembling plasmablasts in a patient with blastic plasmacytoid dendritic cell neoplasm. J Hematopathol 2011;4(2):123-126.
43. Baer MR, Stewart CC, Lawrence D, Arthur DC, Byrd JC, Davey FR, Schiffer CA, Bloomfield CD. Expression of the neural cell adhesion molecule CD56 is associated with short remission duration and survival in acute myeloid leukemia with t(8;21)(q22;q22). Blood 1997;90(4):1643-8.
44. Ferreira-Facio CS, Milito C, Botafogo V, Fontana M, Thiago LS, Oliveira E, da Rocha-Filho AS, Werneck F, Forny DN, Dekermacher S and others. Contribution of multiparameter flow cytometry immunophenotyping to the diagnostic screening and classification of pediatric cancer. PLoS One 2013;8(3):e55534.
45. Borowitz MJ, Bene MC, Harris NL, Porwit A, Matutes E. Acute leukaemias of ambiguous lineage. In: Swerdlow S.H.; Campo EH, N.L.; Jaffe, E.S.; Pileri, S.A., Stein, H., Thiele, J.; Vardiman, J.W., editor. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: International Agency for Research on Cancer.; 2008. p 150-155.
46. Lhermitte L, de Labarthe A, Dupret C, Lapillonne H, Millien C, Landman-Parker J, Hermine O, Baruchel A, Sigaux F, Macintyre E and others. Most immature T-ALLs express Ra-IL3 (CD123): possible target for DT-IL3 therapy. Leukemia 2006;20(10):1908-10.
47. Asnafi V, Buzyn A, Thomas X, Huguet F, Vey N, Boiron JM, Reman O, Cayuela JM, Lheritier V, Vernant JP and others. Impact of TCR status and genotype on outcome in adult T-cell acute lymphoblastic leukemia: a LALA-94 study. Blood 2005;105(8):3072-8.

48. Czuczman MS, Dodge RK, Stewart CC, Frankel SR, Davey FR, Powell BL, Szatrowski TP, Schiffer CA, Larson RA, Bloomfield CD. Value of immunophenotype in intensively treated adult acute lymphoblastic leukemia: cancer and leukemia Group B study 8364. Blood 1999;93(11):3931-9.
49. Marks DI, Paietta EM, Moorman AV, Richards SM, Buck G, DeWald G, Ferrando A, Fielding AK, Goldstone AH, Ketterling RP and others. T-cell acute lymphoblastic leukemia in adults: clinical features, immunophenotype, cytogenetics, and outcome from the large randomized prospective trial (UKALL XII/ECOG 2993). Blood 2009;114(25):5136-45.
50. Vitale A, Guarini A, Ariola C, Mancini M, Mecucci C, Cuneo A, Pane F, Saglio G, Cimino G, Tafuri A and others. Adult T-cell acute lymphoblastic leukemia: biologic profile at presentation and correlation with response to induction treatment in patients enrolled in the GIMEMA LAL 0496 protocol. Blood 2006;107(2):473-9.
51. Weiss LM, Bindl JM, Picozzi VJ, Link MP, Warnke RA. Lymphoblastic lymphoma: an immunophenotype study of 26 cases with comparison to T cell acute lymphoblastic leukemia. Blood 1986;67(2):474-8.
52. Patel JL, Smith LM, Anderson J, Abromowitch M, Campana D, Jacobsen J, Lones MA, Gross TG, Cairo MS, Perkins SL. The immunophenotype of T-lymphoblastic lymphoma in children and adolescents: a Children's Oncology Group report. Br J Haematol 2012;159(4):454-61.
53. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D, Cheng C, Su X, Rubnitz JE, Basso G and others. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. Lancet Oncol 2009;10(2):147-56.
54. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, Easton J, Chen X, Wang J, Rusch M and others. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature 2012;481(7380):157-63.
55. Vardiman JW, Brunning R, Arber D, LeBeau MM, Porwit A, Tefferi A, Bloomfield CD, Thiele J. Introduction and overview of the classification of the myeloid neoplasms. In: Swerdlow S.H.; Campo EH, N.L.; Jaffe, E.S.; Pileri, S.A., Stein, H., Thiele, J.; Vardiman, J.W., editor. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: International Agency for Research on Cancer.; 2008. p 18-30.
56. San Miguel JF, Vidriales MB, Lopez-Berges C, Diaz-Mediavilla J, Gutierrez N, Canizo C, Ramos F, Calmuntia MJ, Perez JJ, Gonzalez M and others. Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups

and may contribute to postinduction treatment stratification. Blood 2001;98(6):1746-51.

57. Buccisano F, Maurillo L, Spagnoli A, Del Principe MI, Fraboni D, Panetta P, Ottone T, Consalvo MI, Lavorgna S, Bulian P and others. Cytogenetic and molecular diagnostic characterization combined to postconsolidation minimal residual disease assessment by flow cytometry improves risk stratification in adult acute myeloid leukemia. Blood 2010;116(13):2295-303.
58. Pereira FG, Metze K, Costa FP, Lima CS, Lorand-Metze I. Phenotypic quantitative features of patients with acute myeloid leukemia. Neoplasma 2006;53(2):155-60.
59. Sandes AF, Kerbauy DM, Matarraz S, Chauffaille Mde L, Lopez A, Orfao A, Yamamoto M. Combined flow cytometric assessment of CD45, HLA-DR, CD34, and CD117 expression is a useful approach for reliable quantification of blast cells in myelodysplastic syndromes. Cytometry B Clin Cytom 2013;84(3):157-66.
60. Bahia DM, Yamamoto M, Chauffaille Mde L, Kimura EY, Bordin JO, Filgueiras MA, Kerbauy J. Aberrant phenotypes in acute myeloid leukemia: a high frequency and its clinical significance. Haematologica 2001;86(8):801-6.
61. Bahia Kerbauy DM, Kimura EY, Chauffaille ML, Bordin JO, Kerbauy J, Yamamoto M. The singular value of CD34 and CD117 expression for minimal residual disease detection in AML. Leuk Res 2003;27(11):1069-70.
62. Buhring HJ, Muller T, Herbst R, Cole S, Rappold I, Schuller W, Zhu X, Fritzsch U, Faul C, Armeanu S and others. The adhesion molecule E-cadherin and a surface antigen recognized by the antibody 9C4 are selectively expressed on erythroid cells of defined maturational stages. Leukemia 1996;10(1):106-16.
63. Thiago LS, Sandes AF. Diagnostic Challenges of CD4+/CD56+/CD123+ hematological neoplasms. An Bras Dermatol 2013;88(4):679.
64. Bueno C, Montes R, Martin L, Prat I, Hernandez MC, Orfao A, Menendez P. NG2 antigen is expressed in CD34+ HPCs and plasmacytoid dendritic cell precursors: is NG2 expression in leukemia dependent on the target cell where leukemogenesis is triggered? Leukemia 2008;22(8):1475-8.
65. Bueno C, Almeida J, Lucio P, Marco J, Garcia R, de Pablos JM, Parreira A, Ramos F, Ruiz-Cabello F, Suarez-Vilela D and others. Incidence and characteristics of CD4(+)/HLA DRhi dendritic cell malignancies. Haematologica 2004;89(1):58-69.
66. Staal-Viliare A, Latger-Cannard V, Didion J, Gregoire MJ, Lecompte T, Jonveaux P, Rio Y. CD203c /CD117-, an useful phenotype profile for

acute basophilic leukaemia diagnosis in cases of undifferentiated blasts. Leuk Lymphoma 2007;48(2):439-41.

67. Hurwitz CA, Raimondi SC, Head D, Krance R, Mirro J, Jr., Kalwinsky DK, Ayers GD, Behm FG. Distinctive immunophenotypic features of t(8;21)(q22;q22) acute myeloblastic leukemia in children. Blood 1992;80(12):3182-8.

68. Haas OA, Koller U, Grois N, Nowotny H. Immunophenotype of hematologic neoplasms with a translocation t(8;21). Recent Results Cancer Res 1993;131:361-8.

69. Orfao A, Chillon MC, Bortoluci AM, Lopez-Berges MC, Garcia-Sanz R, Gonzalez M, Tabernero MD, Garcia-Marcos MA, Rasillo AI, Hernandez-Rivas J and others. The flow cytometric pattern of CD34, CD15 and CD13 expression in acute myeloblastic leukemia is highly characteristic of the presence of PML-RARalpha gene rearrangements. Haematologica 1999;84(5):405-12.

70. Petrovici K, Graf M, Hecht K, Reif S, Pfister K, Schmetzer H. Use of NG2 (7.1) in AML as a tumor marker and its association with a poor prognosis. Cancer Genomics Proteomics 2010;7(4):173-80.

71. Munoz L, Nomdedeu JF, Villamor N, Guardia R, Colomer D, Ribera JM, Torres JP, Berlanga JJ, Fernandez C, Llorente A and others. Acute myeloid leukemia with MLL rearrangements: clinicobiological features, prognostic impact and value of flow cytometry in the detection of residual leukemic cells. Leukemia 2003;17(1):76-82.

ANNEXES

Legends to tables:

Table 1. Acute leukemia screening antibody panel for 4-color immunophenotyping

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCPCy5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin; Cy: cytoplasmic.

Table 2. BCP-ALL antibody panel for 4-color immunophenotyping

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCPCy5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin; Cy: cytoplasmic; Nu: nuclear; Sm: surface-membrane

*Markers for identification and quantification of blast cells: CD19, CD45. Markers for diagnostic definition: CD10, CD19, CD20, CD22, cCD79a, nTdT. Markers for classification: CD10, IgM ($_{Cy}$IgM and $_{Sm}$IgM), $_{Sm}$Kappa, $_{Sm}$Lambda. Markers for identification of lineage-infidelity: CD13, CD33, CD66c, CD15/CD65. Identifying phenotypes associated with well-defined cytogenetic/molecular aberrations: 7.1 (NG2), CD33, CD13, CD66c. Markers for MRD detection: CD10, CD20, CD22, CD34, CD38, CD58, $_{Nu}$TdT, CD45, CD66c.

Table 3. T-ALL/LBL antibody panel for 4-color immunophenotyping

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCPCy5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin; Cy: cytoplasmic; Nu: nuclear; Sm: surface-membrane

Table 4. Antibody combinations recommended for the diagnosis of AML

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCPCy5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin.

*Tube 1: blast cell quantification; tube 2: neutrophilic maturation; tube 3: monocytic maturation; tube 4: erythroid lineage; tube 5: megakaryocitic lineage and myeloid markers; tube 6: LAIP markers; tube 7: identification of abnormal blast cells; tube 8: erythroid maturation; tube 9: megakaryocitic complementation and myeloid markers; tube 10: immunophenotype associated with 11q23 abnormalities; tube 11: plasmacytic dendritic cell and basophilic lineage; tube 12: mast cell and basophilic lineage; tube 13: mast cell lineage.

Table 5. Immunophenotypic characteristics of AML with specific genetic abnormalities.

Legend to figures

Figure 1. Diagnostic workup for immunophenotypic characterization of acute leukemias. AML – acute myeloid leukemia; T-ALL – T-cell precursor acute lymphoblastic leukemia/lymphoma; BCP-ALL – B-cell precursor acute lymphoblastic leukemia/lymphoma.

Figure 2. Representative bivariate dot plots illustrating the GBFLUX acute leukemia screening antibody panel for 4-color immunophenotyping. Dot plots show an AML case (panels A-B) and a BCP-ALL case (panels C-D): FSC x SSC and cyMPO vs. cyCD79a respectively.

Figure 3. Representative bivariate dot plots illustrating the GBFLUX antibody panel for BCP-ALL characterization using 4-color immunophenotyping. Dot plots show a ALL case presenting a MML rearrangement (panels A-E); FSC x SSC; CD15/CDw65 vs. NG2 ;CD58 vs. CD66c ; CD13 vs. CD22 and SSC vs. cyIgM respectively.

Figure 4. Representative bivariate dot plots illustrating the GBFLUX antibody panel for T-ALL characterization using 4-color immunophenotyping. Dot plots

show a cortical T-ALL case (panels A-D): CD7 vs. $_{cy}$CD3; CD10 vs. $_{Nu}$TdT; CD1a vs. CD7; and CD8 vs. CD4 vs CD8 respectively.

Figure 5. Representative bivariate dot plots illustrating the GBFLUX antibody panel for AML characterization using 4-color immunophenotyping. Dot plots show an AML case presenting monocytic differentiation (panels A-E); SSC vs. CD45; SSC vs. CD34; SSC vs CD117; CD64 vs. CD36; and CD64 vs. CD14 respectively.

Table 1. Acute leukemia screening antibody panel for 4-color immunophenotyping

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**PerCP-Cy5.5**	**APC**
Mandatory				
1.	$_{Cy}$MPO	$_{Cy}$CD79a	CD45	$_{Cy}$CD3
2.	CD19	CD7	CD45	CD34

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin; Cy: cytoplasmic.

Table 2. BCP-ALL antibody panel for 4-color immunophenotyping

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**PerCP-Cy5.5**	**APC**
Mandatory				
1.	CD34	CD20	CD19	CD10
2.	$_{Cy}$IgM	CD13	CD19	CD22
3.	$_{Nu}$TdT	CD33	CD19	CD38
Recommended when $_{cy}IgM^{+}$				
4.	$_{Sm}$Kappa	$_{Sm}$Lambda	CD19	$_{Sm}$IgM
Optional				
5.	CD58	CD66c	CD45	CD19
6.	CD15 /CD65	NG2	CD45	CD19

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin; Cy: cytoplasmic; Nu: nuclear; Sm: surface-membrane

*Markers for identification and quantification of blast cells: CD19, CD45. Markers for diagnostic definition: CD10, CD19, CD20, CD22, cCD79a, nTdT. Markers for classification: CD10, IgM ($_{Cy}$IgM and $_{Sm}$IgM), $_{Sm}$Kappa, $_{Sm}$Lambda. Markers for identification of lineage-infidelity: CD13, CD33, CD66c, CD15/CD65. Identifying phenotypes associated with well-defined cytogenetic/molecular aberrations: 7.1 (NG2), CD33, CD13, CD66c. Markers for MRD detection: CD10, CD20, CD22, CD34, CD38, CD58, $_{Nu}$TdT, CD45, CD66c.

Table 3. T-ALL/LBL antibody panel for 4-color immunophenotyping

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**PerCP-Cy5.5**	**APC**
Mandatory				
1.	$_{Nu}$TdT	CD7	cyCD3	CD10
2.	CD8	CD7	CD4	$_{Sm}$CD3
3.	CD2	CD1a	CD5	CD7
Recommended when $_{Sm}CD3^{+}$				
4.	TCRαβ	CD7	$_{Sm}$CD3	-
5.	TCRγδ	CD7	$_{Sm}$CD3	-
or	TCRαβ	TCRγδ	$_{Sm}$CD3	CD7
Recommended in all cases				
6.	CD7	CD13	$_{Sm}$CD3	CD117
7.	CD7	CD99	$_{Sm}$CD3	CD56
Optional				
8.	CD7	CD33	$_{Sm}$CD3	CD123

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin; Cy: cytoplasmic; Nu: nuclear; Sm: surface-membrane

Table 4. Antibody combinations recommended for the diagnosis of AML.

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**PerCP-Cy5.5**	**APC**
Mandatory				
1.	HLA-DR	CD117	CD45	CD34
2.	CD16	CD13	CD45	CD11b
3.	CD36	CD64	CD45	CD14
4.	CD71	CD235a	CD45	CD33
5.	CD15	CD61	CD45	CD13
6.	CD2	CD56	CD45	CD4
7.	CD38	CD117	CD45	CD34
Optional				
8.	CD36	CD105	CD45	CD71 or CD235a
9.	CD41	CD42b	CD45	CD33 or CD13
10.	CD65	NG2	CD45	-
Recommended when lineage could not be defined with previous tubes				
11.	HLA-DR	CD123	CD45	CD38
12.	CD22	CD203c	CD45	CD117
13.	CD2	CD25	CD45	CD117

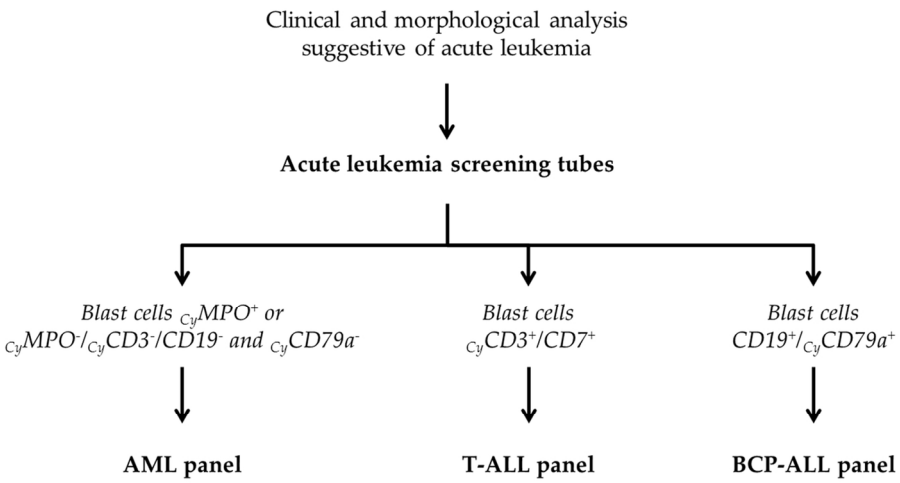
Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy5.5: peridinin chlorophyll protein/cyanin 5; APC: allophycocyanin.

*Tube 1: blast cell quantification; tube 2: neutrophilic maturation; tube 3: monocytic maturation; tube 4: erythroid lineage; tube 5: megakaryocitic lineage and myeloid markers; tube 6: LAIP markers; tube 7: identification of abnormal blast cells; tube 8: erythroid maturation; tube 9: megakaryocitic complementation and myeloid markers; tube 10: immunophenotype associated with 11q23

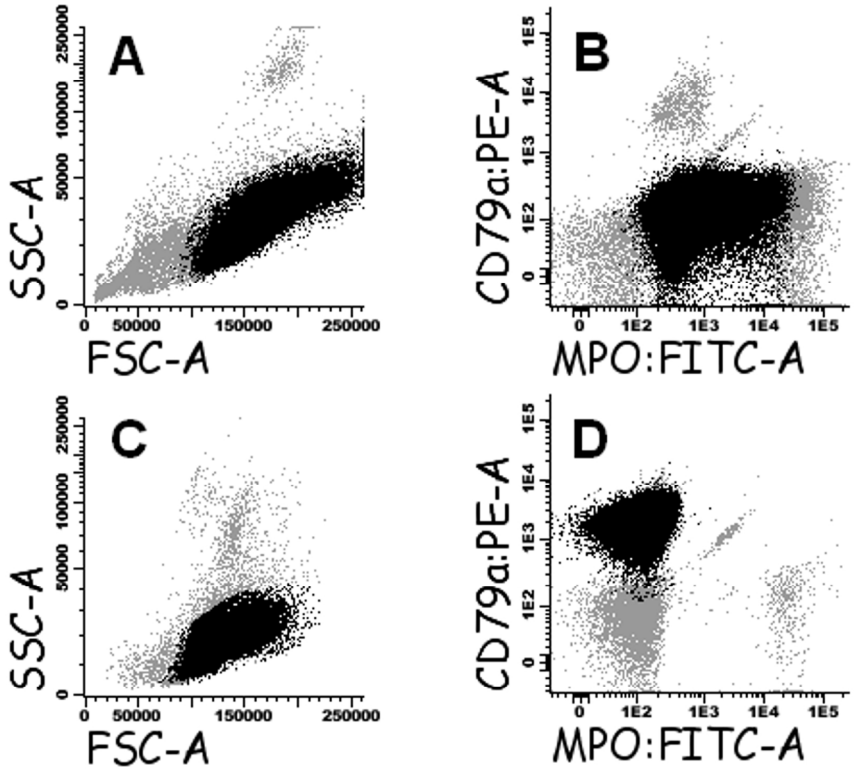
abnormalities; tube 11: plasmacytic dendritic cell and basophilic lineage; tube 12: mast cell and basophilic lineage; tube 13: mast cell lineage.

Table 5. Immunophenotypic characteristics of AML with specific genetic abnormalities.

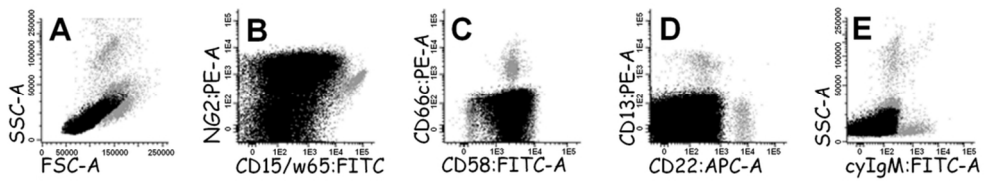
Genetic abnormalities	Immunophenotypic characteristics
t(8;21)(q22;q22); ***RUNX1-RUNX1T1***	$CD34^{+\ high}$, $HLA\text{-}DR^{+}$, MPO^{+}, $CD13^{+}$, $CD33^{+low}$, $CD19^{+}$, $CD79a^{+/-}$, $CD56^{+/-}$
Inv(16) (p13.1q22) or t(16;16)(p13.1q22) or t(16;16)p13.1;q22); ***CBFB-MYH11***	$CD34^{+\ high}$, $CD117^{+}$, $CD13^{+}$, $CD15^{+}$, $CD65^{+}$, MPO^{+high}, $CD2^{-/+low}$ Monoblastic antigens (variable expression): CD4, CD11b, CD14, CD36, CD64, lisozyme
t(15;17)(q22;q12); ***PML-RARα***	$CD34^{-/low}$, $CD15^{-/low}$, CD13 heterogeneous, CD33 and MPO homogeneous, $HLA\text{-}DR^{-}$, $CD2^{+}$ (microgranular morphology)
t(9;11)(p22;q23); ***MLLT3-MLL*** (AML with 11q23 abnormalities)	$CD33^{+\ high}$, $CD65^{+\ high}$, $CD13^{+\ low}$, $CD34^{-/+}$, $CD117^{-/+}$, $NG2^{+}$, $CD56^{+}$ Monoblastic antigens (variable expression): CD4, CD11b, CD11c, CD14, CD36, CD64, lisozyme



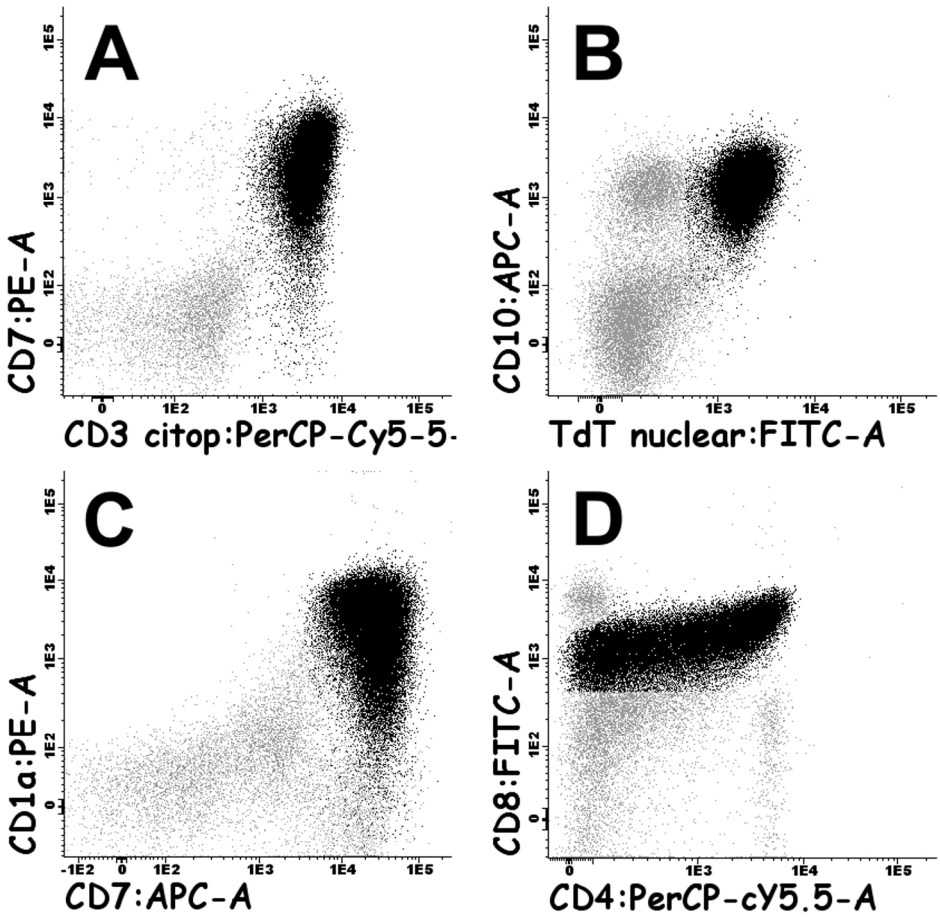
131x70mm (300 x 300 DPI)



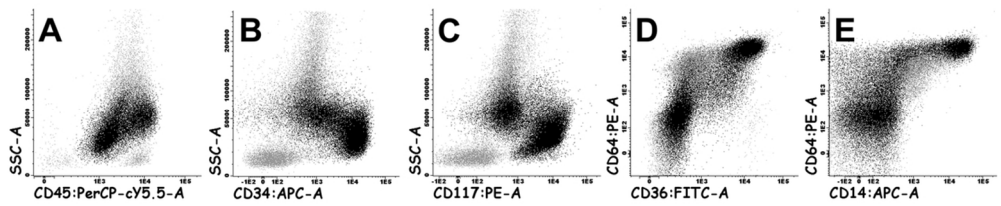
161x130mm (300 x 300 DPI)



91x16mm (300 x 300 DPI)



191x184mm (300 x 300 DPI)



107x23mm (300 x 300 DPI)

SUPPLEMENTAL MATERIAL FOR:

"First proposed panels on acute leukemia for four-color immunophenotyping by flow cytometry from the Brazilian Group of Flow Cytometry - GBCFLUX"

Authors: Maura R V Ikoma[1], Alex F Sandes[2,11], Leandro S Thiago[3], Geraldo B Cavalcanti Júnior[4], Irene G H Lorand-Metze[5], Elaine S Costa[6], Glicinia Pimenta[7,8], Maria C Santos-Silva[9], Nydia S Bacal[10] , Mihoko Yamamoto[11], Elizabeth X Souto[12]

Institutions:

1. Hospital Amaral Carvalho – Jau, SP, Brazil
2. Fleury Group, Division of Hematology, São Paulo-SP, Brazil
3. Brazilian National Cancer Institute (INCa), Cancer Research Center, Rio de Janeiro, RJ, Brazil
4. Universidade Federal do Rio Grande do Norte, RN, Brazil
5. Universidade Estadual de Campinas, SP, Brazil
6. Pediatric Institute IPPMG, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil.
7. Universidade Federal do Rio de Janeiro, RJ, Brazil
8. Laboratórios DASA, Rio de Janeiro, RJ, Brazil
9. Universidade Federal de Santa Catarina, SC, Brazil
10. Hospital Albert Einstein, São Paulo, SP, Brazil
11. Division of Hematology, Department of Clinical and Experimental Oncology, Escola Paulista de Medicina - Universidade Federal de São Paulo (UNIFESP-EPM), São Paulo, Brazil.
12. Laboratórios DASA, São Paulo, SP, Brazil

CONTENTS

Table S1. Alternative acute leukemia screening antibody panel for 4-color immunophenotyping for Beckman Coulter users

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**ECD**	**PC5**
Mandatory				
1.	$_{Cy}$MPO	$_{Cy}$CD79a	CD45	$_{Cy}$CD3
2.	CD7	CD34	CD45	CD19

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; ECD: energy couple dye, PE-Texas Red; PC5: phycoerythrin-cyanine 5; cy: cytoplasmic;

Table S2. Alternative BCP-ALL antibody panel for 4-color immunophenotyping for Beckman Coulter users

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**ECD**	**PC5**
Mandatory				
1.	CD34	CD20	CD19	CD10
2.	$_{Cy}$IgM	CD13	CD19	CD33
3.	$_{Nu}$TdT	CD22	CD19	CD38
Recommended when cyIgM$^+$				
4.	$_{Sm}$IgM	-	CD19	-
5.	$_{Sm}$Kappa	$_{Sm}$Lambda	CD19	-
Optional				
5.	CD58	CD66c	CD45	CD19
6.	CD15/CD65	NG2	CD45	CD19

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; ECD: energy couple dye, PE-Texas Red; PC5: phycoerythrin-cyanine 5; Cy: cytoplasmic; Nu: nuclear; Sm: surface-membrane

.

*Markers for identification and quantification of blast cells: CD19, CD45. Markers for diagnostic definition: CD10, CD19, CD20, CD22, cCD79a, nTdT. Markers for classification: CD10, IgM ($_{Cy}$IgM and $_{Sm}$IgM), $_{Sm}$Kappa, $_{Sm}$Lambda. Markers for identification of lineage-infidelity: CD13, CD33, CD66c, CD15/CD65. Identifying phenotypes associated with well-defined cytogenetic/molecular aberrations: 7.1 (NG2), CD33, CD13, CD66c. Markers for MRD detection: CD10, CD20, CD22, CD34, CD38, CD58, $_{Nu}$TdT, CD45, CD66c.

Table S3. Alternative T-ALL/LBL antibody panel for 4-color immunophenotyping for Beckman Coulter users

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**ECD**	**PC5**
Mandatory				
1.	$_{Nu}$TdT	CD7	$_{Cy}$CD3	CD10
2.	CD8	CD4	$_{Sm}$CD3	CD7
3.	CD2	CD1a	CD5	CD7
Recommended when $_{Sm}CD3^{+}$				
4.	TCRαβ	CD7	$_{Sm}$CD3	-
5.	TCRγδ	CD7	$_{Sm}$CD3	-
or	TCRαβ	TCRγδ	$_{Sm}$CD3	CD7
Recommended in all cases				
6.	CD7	CD13	$_{Sm}$CD3	CD117
7.	CD7	CD99	$_{Sm}$CD3	CD56
Optional				
8.	CD7	CD123	$_{Sm}$CD3	CD33

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; ECD: energy couple dye, PE-Texas Red; PC5: phycoerythrin-cyanine 5; Cy: cytoplasmic; Nu: nuclear; Sm: surface-membrane

Table S4. Alternative antibody combinations recommended for the diagnosis of AML for Beckman Coulter users

Tubes	*Fluorochromes and Markers*			
	FITC	**PE**	**ECD**	**PC5**
Mandatory				
1.	HLA-DR	CD117	CD45	CD34
2.	CD16	CD13	CD45	CD11b
3.	CD36	CD64	CD45	CD14
4.	CD71	CD235a	CD45	CD33
5.	CD15	CD61	CD45	CD13
6.	CD2	CD56	CD45	CD4
7.	CD38	CD117	CD45	CD34
Optional				
8.	CD36	CD105	CD45	CD117
9.	CD41	CD42b	CD45	CD33 or CD13
10.	CD65	NG2	CD45	-
Recommended when lineage could not be defined with previous tubes				
11.	HLA-DR	CD123	CD45	CD38
12.	CD22	CD203c	CD45	CD117
13.	CD2	CD25	CD45	CD117

Abbreviations: FITC: fluorescein isothiocyanate; PE: phycoerythrin; ECD: energy couple dye, PE-Texas Red; PC5: phycoerythrin-cyanine 5.

*Tube 1: blast cell quantification; tube 2: neutrophilic maturation; tube 3: monocytic maturation; tube 4: erythroid lineage; tube 5: megakaryocitic lineage and myeloid markers; tube 6: LAIP markers; tube 7: identification of abnormal blast cells; tube 8: erythroid maturation; tube 9: megakaryocitic complementation and myeloid markers; tube 10: immunophenotype associated with 11q23 abnormalities; tube 11: plasmacytic dendritic cell and basophilic lineage; tube 12: mast cell and basophilic lineage; tube 13: mast cell lineage.

.