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DESCRIPTION

[0001] The invention relates to the field of flow cytometry and more particularly to a panel of antibody reagents conjugated to fluorescent compounds. They find their use in the immunophenotypic characterization of normal, reactive, regenerating and neoplastic cells in peripheral blood (PB), bone marrow (BM), pleural effusions, ascitis, cerebrospinal fluid (CSF), vitreous humor, synovial fluid, bronchoalveolar lavage, urine, spleen, liver, lymph node, and other tissue samples. Flow cytometric immunophenotyping of normal, reactive, regenerating, and malignant cells (in particular leukocytes) is currently being used for many applications in medicine, including immunology, hematology and oncology. Among others, said applications include monitoring of the immune system; diagnosis and classification of primary immunodeficiencies; immunophenotyping of leukemias, lymphomas and plasma cell dyscrasias; monitoring of low frequencies of malignant leukocytes as measure for treatment effectiveness; diagnosis and monitoring of clonal disorders such as paroxysmal nocturnal hemoglobinuria (PNH) and mastocytosis; evaluation of hematopoiesis or lymphopoiesis in different clinical conditions, e.g. in healthy individuals, after stem cell transplantation or gene therapy (using hematopoietic precursor cells as target); and evaluation of the composition and quality of cell products to be used for therapeutic purposes.

[0002] The conventional diagnostic process in flow cytometric immunophenotyping is typically based on the usage of panels of antibodies. At present different, overlapping panels of antibodies are recommended for specific applications. Such panels are driven either by the medical indication (e.g.: screening for cytopenias, characterization of lymphocytosis), by disease (e.g.: acute leukemia diagnosis, lymphoma diagnosis) or disease status (e.g.: diagnostic classification of ALL vs. monitoring of ALL for evaluation of treatment effectiveness). Examples of recommended antibody panels are the European Leukemia Net (ELN) (1), the 2006 Bethesda International Consensus (2) panels for different subtypes of hematological malignancies, the EGIL panels for lineage assignment and subclassification of acute leukemias (3), the European Myeloma Network (EMN) panels for diagnosis, classification and monitoring of plasma cell dyscrasias (4), the ERIC (5) and Matutes (6) score panels for immunophenotypic subclassification of chronic lymphocytic leukemia and hairy cell leukemia vs. other chronic lymphoproliferative disorders.

[0003] These recommended antibody panels are mostly comparable between different countries and study groups, but never fully identical. Importantly, no or limited information has been provided by the various networks and study groups on how the antibodies should preferably be combined into a panel of antibody reagents conjugated with specific fluorescence compounds. Only simple lists of antibodies have been proposed, even when diagnostic laboratories can simultaneously stain a sample aliquot with 2, 3, 4, 5, 6, 7, 8 or more antibodies conjugated with different fluorescent dyes having fluorescence emissions that can be measured separately. Variations between centres around the world are due to the usage of:

- Comparable but not identical lists of antibodies and antibody clones;
- The same antibodies conjugated with different fluorochromes that show different sensitivities. For example, an antibody reagent might provide a negative result if the antibody is combined with a low-sensitive fluorochrome, while a positive result could be obtained if an antibody reagent consisting of the same antibody combined with a different, more sensitive fluorochrome, is used to stain the same cells.
- Simultaneous assessment of different combinations of antibody reagents.
- Variable and frequently suboptimal or inappropriate strategies for the identification of the cell population(s) of interest, such as the CD45 gating in acute myeloblastic leukemias.

[0004] As a consequence, the seemingly comparable antibody panels do not result in comparable diagnosis of clinical samples. In fact, the reproducibility between diagnostic laboratories is not more than 70%. Unfortunately, diagnoses are missed because of inappropriate discrimination between normal and malignant

cells or regenerating bone marrow is erroneously diagnosed as recurrence of acute leukemia.

[0005] In US Patent No. 5,047,321, Loken and Terstappen disclosed a procedure for the multiparameter analysis of cellular components in PB and BM. With the procedure described, these inventors could distinguish several cellular components of PB and BM, count the number of cells within each component, and provide a differential analysis of each of them based on the combined use of the LDS-751 (Exciton) DNA-dye, the thiazol orange (TO, Molecular Probes, Inc) RNA-dye, a fluorescently labeled anti-CD45 monoclonal antibody, forward light scatter (FSC) and sideward light scatter (SSC). This approach allowed the specific identification of nucleated red cells, erythrocytes, reticulocytes, platelets, lymphocytes, monocytes, neutrophilic granulocytes, basophilic granulocytes, eosinophilic granulocytes, and precursors of all nucleated hematopoietic cells. However, the described multiparameter analysis could not specifically differentiate between normal, reactive, regenerating and neoplastic cells coexisting in the same sample, neither could this procedure further characterize these groups of cells. A further refinement of this patent was described later on in U.S. Patent No 6,287,791 by Terstappen and Chen, but they did not show any improvement in characterizing the different populations of leukocytes.

[0006] More recently, Orfao et al. described in US. Patent No. 7,332,295 a procedure for the multidimensional leukocyte differential analysis of PB, BM and other body fluids, which specifically allowed identification of dendritic cells and their subsets in addition to nucleated red cells, lymphocytes, monocytes, neutrophilic granulocytes, basophilic granulocytes, eosinophilic granulocytes and hematopoietic precursors of all nucleated cells. Furthermore, in U.S. Patent No 5,538,855, Orfao et al. described a procedure that allowed a more detailed analysis of the lymphoid compartments through the simultaneous identification of up to 12 different subsets of T, B and NK-cells in PB, BM and lymph node samples. In the patent, Orfao et al. used a combined staining for the CD3, CD19, CD56 (and/or CD16), CD4 and CD8 antigens in a 3-color single staining, where pairs of monoclonal antibodies conjugated with the same fluorochrome were used. Nevertheless, through this approach they could neither further characterize the identified cell subsets nor discriminate between normal and neoplastic cells; furthermore, some relevant subsets of lymphoid and non-lymphoid cells (e.g. the TCRγδ+ T-cells) present in a normal PB, BM or other tissues and body fluids, could not be specifically detected.

[0007] None of the referred procedures allowed directly for a more detailed characterization of the cells identified, including discrimination between normal, reactive, regenerating, and neoplastic/clonal cell populations. Such characterization and discrimination requires the use of a greater number of stainings associated with distinguishable fluorescence emissions and of flow cytometer instruments capable of measuring a higher number of different fluorescence emissions.

[0008] Many other publications show that, based on specific immunophenotypic profiles, normal cells can be discriminated from their neoplastic counterparts, and that the discrimination capabilities (sensitivity for discriminating normal from clonal/malignant cells) increase with the number of fluorochrome- conjugated antibody reagents used to stain the same sample. Some reports also show that normal and reactive cells as well as maturing cells during BM regeneration, may show distinct immunophenotypic profiles, but these cells could not be clearly discriminated from malignant cells. Importantly, panels of antibody combinations that have been proposed thus far can not efficiently discriminate between normal, reactive, regenerating cell populations and their neoplastic counterparts in a systematic way and, at the same time, allow for a clear-cut discrimination between different diagnostic disease categories through an extended immunophenotypic characterization of the malignant cells.

[0009] With the introduction of the new generation of multi-laser flow cytometers in diagnostic laboratories, the potential space for building different panels even with the same list of antibodies, has exponentially increased due to the possibility of using a larger number of different fluorescent compounds and simultaneously measure a higher number of fluorescence emissions in single cells. In U.S. Patent No. 7,321,843, Orfao, Pedreira and Sobral da Costa propose a new approach based on mathematical calculation procedures (e.g. the closest

neighbour principle), where flow cytometry list mode data files containing information for each single cell measured about an unlimited number of parameters could be generated, after several aliquots of a sample stained with different, partially overlapping, combinations of monoclonal antibodies had been measured in a flow cytometer. The utility of this procedure could only be maximised with the increased multicolour capabilities of the most recent flow cytometers. This is because these procedures require the design of efficient panels of combinations of monoclonal antibody reagents where common backbone markers are combined with other additional markers which vary for each combination of monoclonal antibodies in a panel. In parallel, the same authors also described (U.S. Patent No. 7,507,548) a procedure for comparison of flow cytometry data from a case against a reference data file which would allow direct comparison of e.g. normal vs. neoplastic cells. However, in both patents, the authors failed to propose a comprehensive antibody panel that could be thoroughly and systematically applied to the diagnosis, classification, staging and/or monitoring of leukaemia, lymphoma and plasma cell dyscrasias.

[0010] Thus, existing protocols do not teach about how to specifically combine the widely recommended monoclonal antibodies in a panel; they propose different panels for the diagnostic classification and monitoring of minimal disease; they focus in single, uniform disease groups; they show a limited efficiency once widely tested; they do not provide a way for direct combination of the information measured for several aliquots from a single sample; and/or they have failed in systematically distinguishing normal/reactive versus clonal/neoplastic cells based on their immunophenotypic properties, in specific diseases such as chronic T and NK lymphoproliferative disorders (e.g. in the differential diagnoses of large granular lymphocytic leukemia).

[0011] The present inventors recognized these difficulties, and set out to design improved, well-defined antibody panels that avoid misinterpretation and over-interpretation of results. After careful selection of the relevant markers, design of appropriate combinations of antibodies in multi-color tubes, and the selection of suited fluorochromes (based on need for brightness, compensation, stability, etc.), a set of antibody reagents was developed. The studies were complemented with extensive multicentric evaluation of the consensus panels in order to reshape and achieve an optimal efficiency.

[0012] Accordingly, the invention provides in one embodiment a reagent composition for flow cytometric immunophenotyping of leukocytes comprising at least eight distinct fluorochrome-conjugated antibodies comprising a set of at least three identification antibodies for the identification of a leukocyte population of interest and at least four characterization antibodies for further characterization and/or classification of said leukocyte population. A reagent composition as provided herein comprises a panel of antibodies directed against the following combinations of markers: cyCD3, CD45, cyMPO, cyCD79a, CD34, CD19, CD7 and CD3 (ALOT reagent composition).

[0013] Braylan et al. (Cytometry, Vol. 46, no. 1, pp.23-27, 2001) relates to reagents for flow cytometric phenotyping of lymphocytes. A set of ten markers (CD10, CD19, CD13, CD33, CD34, CD45, CD7, CD14, CD3 and HLA-DR) is taught to be essential for evaluating acute leukemia. Additional markers can be added. However, Braylan *et al.* fails to disclose a single reagent composition comprising antibodies against the specific marker combination of the present invention.

[0014] In a preferred embodiment of the present invention, the composition comprises monoclonal antibodies. CD stands for cluster designation and is a nomenclature for the identification of specific cell surface antigens defined by monoclonal antibodies. Abbreviations used are as follows: cyIgκ = cytoplasmic IgG kappa chain; cyIgλ = cytoplasmic IgG lambda chain; β2micro =β_2 microglobulin; cyMPO = cytoplasmic myeloperoxidase. (Monoclonal) antibodies against the indicated markers can be commercially obtained from various companies, including Becton/Dickinson (BD) Biosciences, Dako, Beckman Coulter, CYTOGNOS, Caltag, Pharmingen, Exbio, Sanquin, Invitrogen, and the like.

[0015] Suitable fluorochromes for conjugating antibodies are known in the art. As will be understood, the

fluorochromes used within a reagent composition should be distinguishable by flow cytometry. The fluorochromes are preferably selected for brightness, limited spectral overlap and limited need for compensation, stability, etc. The following panel of fluorochromes is of particular use in a reagent composition according to the invention: (1) pacific blue (PacB) or Horizon V450, (2) pacific orange (PacO) or AMCA, (3) fluorescein isothiocyanate (FITC) or Alexa488, (4) phycoerythrin (PE), (5) peridinin chlorophyl protein/cyanine 5.5 (PerCP-Cy5.5), PerCP or PE-TexasRed, (6) phycoerythrin/cyanine7 (PE-Cy7), (7) allophycocyanine (APC) or Alexa647, and (8) allophycocyanine/H7 (APC-H7), APC-Cy7, Alexa680 or Alexa700. After multiple testing rounds, the present inventors observed that very good results can be obtained if the following fluorochromes are chosen: Pacific Blue or Horizon V450, Pacific Orange, fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyl protein/cyanine 5.5 (PerCp-Cy5.5), PE-Cy7, allophycocyanine (APC), and APC-H7.

[0016] The reagent composition can be used as such, e.g. for the screening of a lymphoid disease. See Figure 2 for a schematic overview of exemplary applications of the various reagent compositions. The invention thus also relates to diagnostic kits comprising one or more reagent compositions. However, the compositions are also advantageously used in combination with one or more further reagent compositions, in particular reagent compositions designed for the further screening and classification of the disease. The expression "in combination with" does not refer to the physical combination or mixing of the reagent compositions, but to their application in separate (consecutive) analysis steps and combination of the data thus obtained. For example, a screening tube used in combination with a characterization tube involves two separate analytical steps on separate aliquots of the same biological sample, each using one of the reagent compositions, followed by data recording and evaluation.

[0017] Therefore, the invention also relates to a set of at least two reagent compositions, said set comprising a reagent composition as described herein above, and at least one further reagent composition comprising distinct fluorochrome-conjugated antibodies. Thus, both reagent compositions comprise a distinct panel of antibodies, although some antibodies might be present in both compositions. It is very convenient if the panel of distinct fluorochromes is essentially the same for each of the reagent compositions, and that up to eight different fluorochromes are used in total.

[0018] In one embodiment, a set of at least two reagent compositions comprising a first reagent composition as defined herein above (ALOT reagent), and at least one further reagent composition comprising distinct fluorochrome-conjugated antibodies directed against one of the following combinations of markers:

1. (i) CD20, CD45, CD58, CD66c, CD34, CD19, CD10 and CD38
2. (ii) IgK, CD45, cyIgμ, CD33, CD34, CD19, IgM, CD117 and Igλ, wherein the antibodies against IgM and CD117 are conjugated to the same fluorochrome
3. (iii) CD9, CD45, terminal deoxynucleotidyl transferase (TdT), CD13, CD34, CD19, CD22 and CD24
4. (iv) CD21, CD45, CD15, CDw65, NG2, CD34, CD19, CD123 and CD81, wherein the antibodies against CD15 and CDw65 are conjugated to the same fluorochrome
5. (v) cyCD3, CD45, TdT, CD99, CD5, CD10, CD1a and CD3
6. (vi) cyCD3, CD45, CD2, CD117, CD4, CD8, CD7 and CD3
7. (vii) cyCD3, CD45, TCRγδ, TCRαβ, CD33, CD56, cyTCRβ and CD3
8. (viii) cyCD3, CD45, CD44, HLADR, CD45RA, CD123 and CD3
9. (ix) HLADR, CD45, CD16, CD13, CD34, CD117, CD11b and CD10
10. (x) HLADR, CD45, CD35, CD64, CD34, CD117, immune receptor expressed by myeloid cell 2 (IREM2) and CD14
11. (xi) HLADR, CD45, CD36, CD105, CD34, CD117, CD33 and CD71
12. (xii) HLADR, CD45, CD15, chondroitin sulfate proteoglycan (NG2), CD34, CD117, CD7 and CD38
13. (xiii) HLADR, CD45, CD42a, CD61, CD203c, CD34, CD117, CD123 and CD4, wherein the antibodies against CD42a and CD61 are conjugated to the same fluorochrome
14. (xiv) HLADR, CD45, CD41, CD25, CD34, CD117, CD42b and CD9
15. (xv) or

HLADR, CD45, TdT, CD56, CD34, CD117, CD22 and CD19.

[0019] Exemplary sets of reagent compositions include at least the reagent composition as recited in tube#20 of Table 2 herein below, together with at least one reagent composition as recited in any one of Tables 3-5. Also provided is a set of reagent compositions comprising at least the reagent composition as recited in tube #20 of Table 2, the reagent composition as recited in tube #1 of Table 1, and the reagent compositions as recited in tubes #29, 30, 31 and 32 of Table 5.

[0020] A further aspect relates to a diagnostic kit for flow cytometric immunophenotyping of leukocytes, wherein the kit comprises one or more sets of at least two reagent compositions described above. The kit may in addition comprise other useful components, such as instructions for use, sample preparation reagent, buffer, and/or control samples.

[0021] Reagent compositions, sets and diagnostic kits provided herein find their application in various fields. For example, the proposed panels can be applied as a whole or only partially depending on the nature of the sample, medical indication or the specific goal. The panel may use specific monoclonal antibody reagents by a single or several different manufacturers, in combination with different cell preparation techniques for staining of cell surface only and/or cell surface plus intracellular markers. Similarly, the reagent compositions may be upgraded into a panel including combinations containing monoclonal antibody reagents conjugated with > 8 different fluorochromes where the same backbone markers are maintained and combined with additional or similar markers. An antibody composition may also act as a core panel to which new combinations are added with different goals, including monitoring of the immune system.

[0022] Also provided is a diagnostic kit for identification and characterization of immature lymphoid cells, comprising the ALOT reagent composition together with at least one reagent composition for detecting B-cell precursor ALL (BCP-ALL) comprising antibodies against CD20, CD45, CD58, CD66c, CD34, CD19, CD10 and CD38; Igκ, CD45, cyIgμ, CD33, CD34, CD19, IgM, CD117 and Igλ, wherein the antibodies against IgM and CD117 are conjugated to the same fluorochrome; CD9, CD45, TdT, CD13, CD34, CD19, CD22 and CD24; or CD21, CD45, CD15, CDw65, NG2, CD34, CD19, CD123 and CD81, wherein the antibodies against CD15 and CDw65 are conjugated to the same fluorochrome.

[0023] In a further aspect, a diagnostic kit for identification and characterization of immature lymphoid cells comprises the ALOT reagent composition together with at least one reagent composition for detecting T-cell precursor ALL (T-ALL) comprising antibodies against cyCD3, CD45, TdT, CD99, CD10, CD1a and CD3; cyCD3, CD45, CD2, CD117, CD4, CD8, CD7 and CD3; cyCD3, CD45, TCRγδ, TCRαβ, CD33, CD56, TCR βF1 and CD3; or cyCD3, CD45, CD44, HLADR, CD45RA, CD123 and CD3.

[0024] In still a further aspect, a diagnostic kit is provided for identification and characterization of myeloid cells, comprising the ALOT reagent composition together with at least one reagent composition for detecting acute myeloid leukemia (AML), myelodysplastic syndrome (MDS)/ chronic myeloproliferative disorder (MPD), comprising antibodies against HLADR, CD45, CD16, CD13, CD34, CD117, CD11b and CD10; HLADR, CD45, CD35, CD64, CD34, CD117, IREM2 and CD14; HLADR, CD45, CD36, CD105, CD34, CD117, CD33 and CD71; HLADR, CD45, CD15, NG2, CD34, CD117, CD7 and CD38; HLADR, CD45, CD42a, CD61, CD203c, CD34, CD117, CD123 and CD4, wherein the antibodies against CD42a and CD61 are conjugated to the same fluorochrome; HLADR, CD45, CD41, CD25, CD34, CD117, CD42b and CD9; HLADR, CD45, CD41, CD25, CD34, CD117, CD42b and CD9; or HLADR, CD45, TdT, CD56, CD34, CD117, CD22 and CD19.

[0025] The invention also relates to a method for flow cytometric immunophenotyping of leukocytes, comprising the steps of providing a biological sample comprising leukocytes and contacting at least a portion (aliquot) of the sample with a reagent composition provided herein. Any type of (human) sample known or

suspected to contain leukocytes may be used. For example, the sample is peripheral blood, bone marrow, tissue sample such as lymph nodes, adenoid, spleen, or liver, or other type of body fluid such as cerebrospinal fluid, vitreous fluid, synovial fluid, pleural effusions or ascitis. Preferably, the method comprises contacting a first aliquot of said sample with a first reagent composition of a set according to the invention and contacting at least a second aliquot of said sample with a further reagent composition of said set; analyzing leukocytes in said aliquots in a flow cytometer; and storing and evaluating the data obtained. Typically, step (c) comprises the use of software for data integration and multidimensional analysis of flow cytometry files. Very suitable for use in a method of the invention is the software commercially available from CYTOGNOS SL (Salamanca, Spain) under the tradename INFINICYT™. The INFINICYT™ software can automatically combine the immunophenotypic information of the selected cell populations from multiple tubes according to the so-called nearest neighbor calculations in which individual cells from one aliquot of a sample are matched with corresponding individual cells from another aliquot of the same sample, according to their backbone markers and scatter profile. The INFINICYT procedure can transform the herein presented 8-color EuroFlow panels into 12, 16, or ≥ 20-color immunostainings, dependent on the number of tubes per panel and the number of backbone markers per tube. The antibody panels and the INFINICYT software can be used in combination with all currently available flow cytometers that allow 8-color immunostainings, such as FACSCanto™ II, FACSAria, LSRII, DAKO CyAn™,Gallio, etc.

LEGEND TO THE FIGURES

[0026]

Figure 1. Composition of three Categories of antibody panels

Figure 2. Diagnostic flow diagram showing potential applications of the EuroFlow antibody panels.

DETAILED DESCRIPTION

1. Introduction

[0027] The present study was performed by the The EuroFlow Consortium (LSHB-CT-2006-018708) who initiated the project "Flow Cytometry for Fast and Sensitive Diagnosis and Follow-up of Haematological Malignancies", which includes the design of standardized multicolor immunophenotyping protocols for diagnosis, classification, and monitoring of leukemias, lymphomas and plasma cell dyscrasias. A key innovative component of these protocols are the EuroFlow panels of antibody combinations (EuroFlow panels). This EuroFlow invention relates among others to panels of combinations of antibody reagents (reagent compositions), which can be used to define normal, reactive, regenerating and malignant hematopoietic cells in a standardized way. As such, they allow for a comprehensive immunophenotypic diagnosis, classification, staging and monitoring of both chronic and acute leukemias, myelodysplastic syndromes, myeloproliferative disorders, mastocytosis, paroxysmal nocturnal haemoglobinuria, lymphomas and plasma cell dyscrasias. For the first time, the proposed panels are not based on subjective expert opinions, but they have been tested prospectively and modified for improving the answer to the most frequent medical indications of flow cytometry immunophenotyping. In turn, these panels are designed in an innovative way to be applied in combination with both conventional data analysis approaches and new interactive and semi-automated data analysis procedures in which information on single cells is combined for all parameters derived from the measurement of staining a sample with the antibody panel.

[0028] In order to establish these panels, the following sequential steps were performed:

1. 1. Evaluation of all relevant markers used in the field for their usefulness or added value
    - Markers proposed by European networks, such as ELN, EMN, ERIC;
    - Markers proposed by the 2006 Bethesda International Consensus in US;
    - New markers for mature B-cell malignancies as proposed by Rawstron et al.
2. 2. Design and selection of combinations of markers (≥6), which can recognize normal vs. reactive vs. regenerating vs. abnormal/malignant cells within a specific compartment of hematopoietic cells (cell lineage, differentiation pathway, maturation stage and/or functional subset).
3. 3. Evaluation of the proposed panels of combinations of antibody reagents in primary samples from healthy subjects and patients.
4. 4. Repeated testing and optimization of the combinations of antibody reagents (choice of marker, choice of antibody clone, and choice of fluorochrome) based on uncommitted goals and room for improvement.
5. 5. Evaluation of the optimized versions of the EuroFlow panels on large series of well-defined patient samples and samples from healthy controls.

[0029] Compared with pre-existing knowledge, this is the first design of a comprehensive panel (after prospectively evaluated in a multicentric way), which allows both discrimination between normal, reactive, regenerating and clonal/neoplastic cells and classification, staging and monitoring of clonal/neoplastic haematopoietic disorders, providing a clear indication about: 1) those markers required to be stained in common for appropriate and reproducible identification of all cell populations of interest in all stained aliquots of a sample, and 2) how they should be combined with further characterization markers in specific combinations of fluorochrome-conjugated antibody reagents. In addition, information about the goals of each combination is also given as indication about when and how to apply it. The invention was made only after extensive antibody panel testing and several redesigning cycles.

[0030] The EuroFlow panels of reagents are composed of subsets of one or multiple combinations (named tubes) of antibodies conjugated with eight or more fluorescent compounds, each of said combinations of reagents having different goals. The EuroFlow panels consist of three different categories: Category 1, Category 2 and Category 3 antibody panels (Figure 1).

2. Categories of antibody panels

[0031] The following purposes are pursued by the EuroFlow panels of antibody combinations:
The Category 1 antibody panels aim at identification and characterization of different subsets of mature lymphoid cells, including normal, reactive, regenerating and neoplastic B-, T-, NK-cells and plasma cells, particularly in samples where a clonal and/or neoplastic lymphoid disorder is suspected because of e.g. lymphocytosis, lymph node enlargement, splenomegaly, monoclonal serum component, unexplained neurological symptoms, etc. The Category 2 antibody panels aim at identification and characterization of normal, reactive, regenerating and neoplastic immature (or early maturing) T- and/or B-lymphoid cells, particularly in samples suspected of containing neoplastic lymphoid precursors. The Category 3 antibody panels aim at identification and characterization of normal, reactive, regenerating and neoplastic immature, maturing and matured myeloid cells, particularly in samples suspected of containing neoplastic myeloid cells or neoplastic cells expressing myeloid-associated markers.

[0032] Within each of said three Categories, some of the combinations of antibody reagents are aimed to be used in a single-tube screening step with more broad aims, while others are more likely applied in multi-tube classification steps when more specific target populations have already been identified in the screening step.

2.1 Category 1 antibody panels

[0033] The Category 1 antibody panels are composed of three screening tubes aimed at the initial identification and characterization of the specific subgroups of mature lymphoid cells present in samples which contain normal or high cell counts (e.g. normal peripheral blood) and low cell counts (e.g. vitreous humor), respectively, and four different sets of multi-tube antibody combinations aiming at further characterization of B-, T-, NK-cells and plasma cells. The Category 1 screening tubes may be used to screen for the presence of clonal and neoplastic T, B- or NK-cells and plasma cells in samples with relatively high and low cell counts, respectively. Typical examples of low cell count samples are fine needle aspirates (FNA), cerebrospinal fluid (CSF), and vitreous humor.

[0034] The above mentioned screening tubes are now termed as lymphoid screening tube for high cell count samples (abbreviated as LST), small sample tube (SST) for low cell count samples, and a separate plasma cell screening tube (PCST). In turn, the other four sets of tubes are devoted, among other uses, to further characterize the identified clonal or neoplastic B-, T-, NK-cells and plasma cells in patients with different B-, T- and NK-cell chronic lymphoproliferative disorders (abbreviated as BCLPD, TCLPD and NKCLPD, respectively) and plasma cell dyscrasias (abbreviated as PCD), respectively.

2.2 Category 2 antibody panels

[0035] The Category 2 antibody panels consist of a screening tube devoted to the initial identification and characterization of immature lymphoid vs. non-lymphoid precursors, and two sets of tubes for further detailed characterization of B-cell and T-cell precursors. Among other applications, the screening tube may be used for the classification of acute leukemias into lymphoid vs. non-lymphoid versus undifferentiated versus biphenotypic or bilineage acute leukemias. This tube might also be used for the discrimination between normal/regenerating and neoplastic/malignant B-cell precursors in the bone marrow, peripheral blood and other tissues and T-cell precursors in the thymus, respectively. Consequently, this screening tube is now termed as acute leukemia orientation tube (abbreviated ALOT). The other two sets of tubes may be used, among other applications, for the characterization of lymphoid blast cells showing B-cell and T-cell commitment in patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) and T-ALL as well as in patients with acute leukemia showing an undifferentiated or biphenotypic/bilineage phenotype.

2.3 Category 3 antibody panels

[0036] Finally, the Category 3 antibody panels are composed of a screening tube which aims at discriminating between lymphoid and non-lymphoid precursor cells and which is identical to the screening tube described above in Category 2 (ALOT tube), and one additional set of tubes aiming at the characterization of maturing non-lymphoid cells from the earliest hematopoietic precursors to mature myeloid cells, including the different maturation stages of cells committed to the erythroid, megakaryocytic, monocytic, neutrophilic, eosinophilic, basophilic, mast cell and dendritic cell lineages as well as the more immature, uncommitted hematopoietic precursors and stromal cells in case they are present in the sample (e.g. in BM). Among other uses, this set of tubes may be applied for the characterization of neoplastic/clonal diseases and other disorders in which the cells of interest (e.g.: clonal/neoplastic, altered/aberrant cells) display myeloid differentiation such as in patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), chronic myeloproliferative disorder (MPD), paroxysmal nocturnal haemoglobinuria (PNH), mastocytosis, idiopatic cytopenia of undetermined significance (ICUS)- and/or acute leukemia with myeloid-associated markers as it occurs in biphenotypic and bilineage acute leukemias. This group of tubes is abbreviated from now on as AML/MDS/MPD.

2.4 Types of markers used in EuroFlow panels

[0037] In each tube of the EuroFlow panels, two types of antibody reagents are combined: 1) reagents which mainly aim at identifying the precise cell populations of interest present in the sample (Backbone markers), which also provide additional information about the phenotypic characteristics of said cell populations, and; 2) reagents mainly devoted to further characterization/classification of said cell populations as well as other groups of cells in the sample (Characterization markers).

[0038] Typically, backbone markers are repeated in each tube of the following sets of tubes: AML/MDS/MPD, BCP-ALL, T-ALL, B-CLPD, T-CLPD, NK-CLPD, PCD). In addition, backbone markers of the B-CLPD set of tubes are also common to the LST and SST screening tubes, backbone markers of the PCD set of tubes are common to the PCST, and backbone markers of the BCP-ALL and the T-ALL sets of tubes are also common to the ALOT; finally, two of the backbone markers in the AML panel (i.e.: CD34 and CD45) are also common to the ALOT tube.

[0039] In turn, characterization markers are combined in a comprehensive way in each tube, so that they would allow distinguishing normal, reactive regenerating vs clonal/neoplastic cells, even when present in low numbers and in case of neoplastic cells they would allow further diagnosis, subclassification, staging and monitoring of acute and chronic leukemias, lymphomas and plasma cell dyscrasias. For this purpose in multi-tube panels, each tube is devoted to a specific aim related to full characterization and monitoring of a disease entity if combined with the information from the corresponding screening tube (e.g. for the diagnosis, staging and monitoring of CLL, tube#4 of the B-CLPD multi-tube panel in combination with the LST will suffice) or for specific disease -associated information (e.g. tube# 24 in the BCP-ALL multi-tube panel shown in Table 3 is specifically devoted for distinguishing between normal/reactive and regenerating versus malignant B-cell precursors and minimal residual disease monitoring in BCP-ALL).

2.5 INFINICYT software tools for optimal application of the EuroFlow panels

[0040] The antibody panels according to the invention can be used in combination with the INFINICYT software tools which are commercially available. The software is based on recently described procedures for generating files with an unlimited number of parameters through merging data files and calculating the information derived from the measurement of markers in one sample aliquot to the individual cells measured in other aliquots of the same sample, using different combinations of antibody reagents which only have partial overlap (US 7,321,843), as well as for comparisons between different samples or different groups of samples (US 7,507,548).

3. Composition of the Category 1 antibody panels for mature lymphoid cells

[0041] In the multi-tube combinations of antibody reagents of the Category 1 antibody panels for mature lymphoid cells the selected common backbone markers vary between the four different panels and consist of (antibody CD plus fluorochrome compound number): 1) CD20-1, CD45-2 and CD19-6 for B-CLPD; 2) CD4-1, CD45-2, CD3-5 and CD8-8 for T-CLPD; 3) CD45-2, CD3-5, CD56-6 and CD19-8 for NK-CLPD, and; 4) CD45-1, CD138-2, CD38-3 and CD19-6 for PCD. In addition, the backbone markers used in the B-CLPD tube are also used in the LST and SST screening tubes, and the backbone markers of the PCD tubes are also used in the PCST.

[0042] The backbone markers in each of these multi-tube combinations of antibody reagents aim at providing

delineation of the groups of cells of interest and the specific identification of neoplastic cells in diagnostic and follow-up samples containing enough numbers of tumor cells. In addition, for each of the four multi-tube combinations, backbone markers are combined with a variable number of additional characterization markers which further contribute to the discrimination between normal, reactive, regenerating and neoplastic/clonal B-, T, NK-cells and plasma cells, respectively, even when these are present in minimal numbers (e.g. minimal residual disease monitoring and disease staging), as well as for the distinction between clonal/neoplastic cells from different disease categories.

3.1. The lymphoid screening tube (LST), the small sample tube (SST), and the plasma cell screening tube (PCST)

[0043] The EuroFlow LST reagent composition was designed and approved for evaluation of several suspected clinical conditions, such as lymphocytosis, lymph node enlargement, splenomegaly, monoclonal serum components, unexplained neurological symptoms, unexplained cytopenias, etc. (Figure 2). The composition of an exemplary LST tube is provided in Table 1. This tube detects aberrant mature lymphocyte populations of B, T and NK lineage.

[0044] In any of the Tables of the present application, fluorochrome number 1 corresponds to pacific blue (PacB) or Horizon V450, number 2 to pacific orange (PacO) or AMCA, number 3 to fluorescein isothiocyanate (FITC) or Alexa488, number 4 to phycoerythrin (PE), number 5 to peridinin chlorophyl protein/cyanine 5.5 (PerCP-Cy5.5), PerCP or PE-Texas Red, number 6 to phycoerythrin/cyanine7 (PE-Cy7), number 7 to allophycocyanine (APC) or Alexa647, and number 8 to allophycocyanine/H7 (APC-H7), APC-Cy, Alexa680 or Alexa700. A preferred combination is pacific blue (PacB), pacific orange (PacO), fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyl protein/cyanine 5.5 (PerCP-Cy5.5), phycoerythrin/cyanine7 (PE-Cy7), allophycocyanine (APC), and allophycocyanine/H7 (APC-H7).

TABLE 1. Single tube EuroFlow screening combinations for normal and malignant/ aberrant mature lymphoid cells

Fluorochrome / Tube#	1	2	3	4	5	6	7	8
1								
(LST)	$CD20^{BB}$ and CD4	$CD45^{BB}$	Igλ and CD8	Igκ and CD56	CD5	$CD19^{BB}$ and TCRγδ	CD3	CD38
2								
(SST)	$CD20^{BB}$	$CD45^{BB}$	CD8 and Igλ	CD56 and Igκ	CD4	$CD19^{BB}$	CD3 and CD14	CD38
3								
(PCST)	$CD45^{BB}$	$CD138^{BB-}$	$CD38^{BB}$	CD56	β2micro	$CD19^{BB}$	CyIgκ	CyIgλ
BB =These markers act as Backbone markers when LST or SST results are combined with the results obtained with multi-tube EuroFlow classification combination for B-CLPD or when the PCST results are combined with the results obtained with the 2-tube EuroFlow classification combination for PCD; LST = lymphoid screening tube; SST = small sample tube; PCST= plasma cell screening tube.								

4. Composition of the Category 2 panel for immature lymphoid cells

4.1. The acute leukemia orientation tube (ALOT)

[0045] The EuroFlow ALOT tube was designed for assessment of the nature of immature blast cell populations in acute leukemia patients (B, T versus non-lymphoid, undifferentiated or mixed phenotype acute leukemias) and consequent orientation towards the most appropriate complementary antibody panel(s): BCP-ALL, T-ALL, and/or AML/MDS/MPD. The composition of the ALOT tube is provided in Table 2; the markers CD45-2, CD34-5 and CD19-6 act as backbone markers when the information of the ALOT is combined with the BCP-ALL panel (e.g.: using the INFINICYT software) and the cyCD3-1, CD45-2 and CD3-8 markers act also as common backbone markers when the information of the ALOT is combined with the T-ALL panel; in addition CD45-2 and CD34-5 are also common backbone markers to the AML/MDS/MPD multi-tube panel.

TABLE 2. Single tube EuroFlow combination for lineage assignment of blast cells in acute leukemias

Tube#	1	2	3	4	5	6	7	8
20 (ALOT)	cyCD3^{BB}	CD45^{BB}	cyMPO	cyCD79a	CD34^{BB}	CD19^{BB}	CD7	CD3^{BB}
BB =These markers act as Backbone markers when results are combined with the results obtained with multi-tube EuroFlow classification combinations for BCP-ALL or T-ALL (see Table 3 and Table 4, respectively);								

[0046] The ALOT is not suitable for exclusion of a hematological malignancy, because the ALOT antibody combination is not sufficient for that purpose. However, when the ALOT is combined with the LST and 4 tubes of the AML/MDS protocol (tube # 29, 30, 31 and 32), virtually all types of hematological malignancies can be detected (not classified) or excluded (Figure 2).

4.2. The multi-tube antibody panel for B-cell precursor acute lymphoblastic leukemia (BCP-ALL)

[0047] The EuroFlow BCP-ALL tube aims at the recognition and classification of all classically defined BCP-ALL (pro-B-ALL, common-ALL, pre-B-ALL) or alternative BCP-ALL classifications, including immunophenotypic classifications associated with well-defined molecular aberrations, such as specific fusion genes. The information obtained with the BCP-ALL tube set needs to be combined with ALOT, based on the backbone markers CD45-2, CD34-5 and CD19-6 and using appropriate data analysis software (e.g. via the INFINICYT software) (Table 3).

TABLE 3. Multi-tube EuroFlow classification combinations for B-cell precursor ALL (BCP-ALL)

Tube#	1	2	3	4	5	6	7	8	Aim
21	CD20	CD45^{BB}	CD58	CD66c	CD34^{BB}	CD19^{BB}	CD10	CD38	Diagnosis and classification of BCP-ALL; Detection of LAP markers
22	Igκ	CD45^{BB}	CyIgμ	CD33	CD34^{BB}	CD19^{BB}	IgM and CD117	Igλ	Diagnosis and classification of BCP-ALL; Detection of phenotypes assiciated with molecular aberrations
23	CD9	CD45^{BB}	TdT	CD13	CD34^{BB}	CD19^{BB}	CD22	CD24	Diagnosis and classification of BCP-ALL; Detection of phenotypes assiciated with molecular aberrations; Detection of LAP markers

Tube#	1	2	3	4	5	6	7	8	Aim
24	CD21	$CD45^{BB}$	CD15 and CDw65	NG2	$CD34^{BB}$	$CD19^{BB}$	CD123	CD81	Subclassification of BCP-ALL; Detection of LAP markers
BB = Backbone markers. The tubes can also be successfully applied for disease staging and monitoring.									

4.3. The multi-tube antibody panel for T-cell acute lymphoblastic leukemia (T-ALL)

[0048] The EuroFlow T-ALL panel consists of four tubes and uses cyCD3-1, CD45-2, and CD3-8 as backbone markers (see Table 4) in common with the ALOT. The T-ALL panel aims at the recognition and classification of all classically defined T-ALL (immature T-ALL, common thymocytic T-ALL, mature T-ALL) or alternative T-ALL classification, e.g. based on TCR protein expression (cyTCRβ, TCRαβ, TCRγδ) or based on association with well-defined molecular aberrations.

TABLE 4. Multi-tube EuroFlow classification combinations for T-ALL

Tube#	1	2	3	4	5	6	7	8	Aim
25	$cyCD3^{BB}$	$CD45^{BB}$	TdT	CD99	CD5	CD10	CD1a	$CD3^{BB}$	Diagnosis of T-ALL; Identification of LAP markers
26	$cyCD3^{BB}$	$CD45^{BB}$	CD2	CD117	CD4	CD8	CD7	$CD3^{BB}$	Diagnosis of T-ALL, classification of T-ALL, and determine the maturation stage of arrest; Identification of LAP markers
27	$cyCD3^{BB}$	$CD45^{BB}$	TCRγδ	TCRαβ	CD33	CD56	Cy TCRβ	$CD3^{BB}$	Diagnosis of T-ALL and determine the maturation stage of arrest; Identification of LAP markers
28	$cyCD3^{BB}$	$CD45^{BB}$	CD44	CD13	HLADR	CD45RA	CD123	$CD3^{BB}$	Subclassification of T-ALL
BB = Backbone markers. The tubes can also be successfully applied for disease staging and monitoring.									

5. Composition of the Category 3 panel for myeloid cells

[0049] The EuroFlow AML/MDS/MPD panel comprises two complementary marker combinations (tubes 29-32 and tubes 32-35; see Table 5), all of them containing HLA-DR-1, CD45-2, CD34-5 and CD117-6 as backbone markers (Table 5). The first set of tubes (tubes 29-32) is designed to preferably be used in combination with EuroFlow ALOT in order to aim at the detection and classification (lineage assignment and maturation) of

myeloid malignancies, such as in AML and MDS, with a major focus on immature neutrophilic lineage (tube 30), monocytic lineage (tube 31), and erythroid lineage (tube 32). These four tubes also contribute to the detection of APL (tube 32), PNH (tubes 29 and 30) and other aberrant myeloid phenotypes.

[0050] The second set of tubes (tubes 33-35) provides additional information about megakaryocytic, basophilic, plasmacytoid dendritic lineages (tube 33), as well as relevant information for the diagnosis of mastocytosis in association (or not) with AML/MDS (tube 34). Tube 35 further characterizes AML/MDS, and it is particularly focused on the aberrant expression of lymphoid-associated markers and abnormal lymphoid maturation.

TABLE 5. Multi-tube EuroFlow classification combinations for AML/MDS/MPD

Tube#	1	2	3	4	5	6	7	8	Aim
29	$HLADR^{BB}$	$CD45^{BB}$	CD16	CD13	$CD34^{BB}$	$CD117^{BB}$	CD11b	CD10	Diagnosis of AML
30	$HLADR^{BB}$	$CD45^{BB}$	CD35	CD64	$CD34^{BB}$	$CD117^{BB}$	IREM2	CD14	Diagnosis and subclassification of AML and PNH especially focussed on neutrophilic lineage
31	$HLADR^{BB}$	$CD45^{BB}$	CD36	CD105	$CD34^{BB}$	$CD117^{BB}$	CD33	CD71	Diagnosis and subclassification of AML especially focussed on monocytic lineage
32	$HLADR^{BB}$	$CD45^{BB}$	CD15	NG2	$CD34^{BB}$	$CD117^{BB}$	CD7	CD38	Diagnosis and subclassification of AML and APL especially focussed on erythroid lineage
33	$HLADR^{BB}$	$CD45^{BB}$	CD42a plus CD61	CD203c	$CD34^{BB}$	$CD117^{BB}$	CD123	CD4	Diagnosis and subclassification of AML especially focussed on megakaryocytic, basophilic, and plasmacytoid dendritc lineages
34	$HLADR^{BB}$	$CD45^{BB}$	CD41	CD25	$CD34^{BB}$	$CD117^{BB}$	CD42b	CD9	Characterization of AML/MDS
35	$HLADR^{BB}$	$CD45^{BB}$	TdT	CD56	$CD34^{BB}$	$CD117^{BB}$	CD22	CD19	Characterization of AML/MDS

BB = Backbone markers; This multi-tube set of antibody combinations is not only useful for AML, MDS and MPD detection, but also for mastocytosis, PNH, ICUS. The tubes can also be successfully applied for disease staging and monitoring.

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PATENTKRAV

1. Reagenssammensætning til strømningscytometrisk immunfænotypebestemmelse af leukocytter omfattende mindst otte særskilte fluorochrom-konjugerede antistoffer, der omfatter et sæt af mindst tre identifikationsantistoffer til identifikation af en leukocytpopulation af interesse og mindst fire karakteringsantistoffer til yderligere karakterisering og/eller klassifikation af leukocytpopulationen, hvor antistofferne er rettet mod følgende kombination af markører:

cyCD3, CD45, cyMPO, cyCD79a, CD34, CD19, CD7 og CD3.

2. Sæt af mindst to reagenssammensætninger, hvilket sæt omfatter en første reagenssammensætning ifølge krav 1, og mindst én yderligere reagenssammensætning, der omfatter særskilte fluorochrom-konjugerede antistoffer rettet mod én af følgende kombinationer af markører:

(i) CD20, CD45, CD58, CD66c, CD34, CD19, CD10 og CD38

(ii) Igκ, CD45, cyIgμ, CD33, CD34, CD19, IgM, CD117 og Igλ, hvor antistofferne mod IgM og CD117 er konjugeret til det samme fluorochrom

(iii) CD9, CD45, TdT, CD13, CD34, CD19, CD22 og CD24

(iv) CD21, CD45, CD15, CDw65, NG2, CD34, CD19, CD123 og CD81, hvor antistofferne mod CD15 og CDw65 er konjugeret til det samme fluorochrom

(v) cyCD3, CD45, TdT, CD99, CD5, CD10, CD1a og CD3

(vi) cyCD3, CD45, CD2, CD117, CD4, CD8, CD7 og CD3

(vii) cyCD3, CD45, TCRγδ, TCRαβ, CD33, CD56, cyTCRβ og CD3

(viii) cyCD3, CD45, CD44, CD13, HLADR, CD45RA, CD123 og CD3

(ix) HLADR, CD45, CD16, CD13, CD34, CD117, CD11b og CD10

(x) HLADR, CD45, CD35, CD64, CD34, CD117, IREM2 og CD14

(xi) HLADR, CD45, CD36, CD105, CD34, CD117, CD33 og CD71

(xii) HLADR, CD45, CD15, NG2, CD34, CD117, CD7 og CD38

(xiii) HLADR, CD45, CD42a, CD61, CD203c, CD34, CD117, CD123 og CD4, hvor antistofferne mod CD42a og CD61 er konjugeret til det samme fluorochrom

(xiv) HLADR, CD45, CD41, CD25, CD34, CD117, CD42b og CD9 eller

(xv) HLADR, CD45, TdT, CD56, CD34, CD117, CD22 og CD19.

3. Reagenssammensætning eller sæt af reagenssammensætninger ifølge krav 1 eller 2, hvor hver reagenssammensætning omfatter antistoffer konjugeret til pacific blue (PacB) eller Horizon V450, pacific orange (PacO) eller AMCA, fluoresceinisothiocyanat (FITC) eller Alexa488, phycoerythrin (PE), peridininchlorophylprotein/cyanin 5.5 (PerCP-Cy5.5), PerCP eller PE-

TexasRed, phycoerythrin/cyanin7 (PE-Cy7), allophycocyanin (APC) eller Alexa647, og allophycocyanin/H7 (APC-H7), APC-Cy7, Alexa680 eller Alexa700.

4. Sæt af reagenssammensætninger ifølge krav 3, der omfatter mindst reagenssammensætningen som anført i rør #20 i tabel 2, sammen med mindst én reagenssammensætning som anført i en hvilken som helst af tabel 3-5.

5. Sæt af reagenssammensætninger ifølge krav 3 eller 4, der omfatter mindst reagenssammensætningen som anført i rør #20 i tabel 2, reagenssammensætningen som anført i rør #1 i tabel 1, og reagenssammensætningerne som anført i rør #29, 30, 31 og 32 i tabel 5.

6. Diagnostisk kit til strømningscytometrisk immunfænotypebestemmelse af leukocytter, der omfatter et sæt af mindst to reagenssammensætninger ifølge krav 2-5, eventuelt sammen med instruktioner om anvendelse, buffer og/eller kontrolprøver.

7. Diagnostisk kit ifølge krav 6 til identifikation og karakterisering af myeloide celler, der omfatter et sæt af reagenssammensætninger anført i krav 2 under (ix) til og med (xv).

8. Fremgangsmåde til strømningscytometrisk immunfænotypebestemmelse af leukocytter, der omfatter følgende trin

(a) tilvejebringelse af en biologisk prøve, der omfatter leukocytter;

(b) etablering af kontakt mellem en første alikvot af prøven og en første reagenssammensætning fra et sæt ifølge et hvilket som helst af krav 2 til 5 og etablering af kontakt mellem mindst en anden alikvot af prøven og en yderligere reagenssammensætning fra sættet;

(c) analyse af leukocytter i alikvoterne i et strømningscytometer; og

(d) lagring og evaluering af de opnåede data.

9. Fremgangsmåde ifølge krav 8, hvor prøven er perifert blod, knoglemarv, vævsprøve, såsom lymfeknuder, adenoid, milt, eller lever, eller anden type af kropsvæske, såsom cerebrospinalfluid, glaslegemefluid, synovialfluid, pleuraexsudater eller ascites.

DRAWINGS

Figure 1

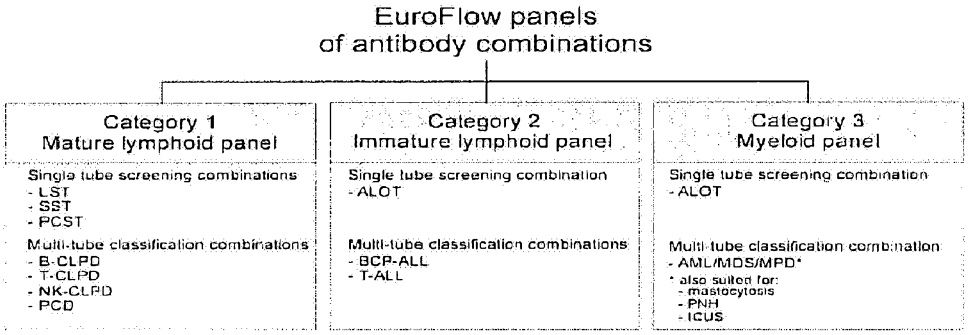


Figure 2

