EuroFlow™ Screening Tubes: Standardizing Analysis of Hematological Malignancies via Flow Cytometry

Bethany S. Bell, PhD | ALPCO
EuroFlow™ Support Team | Cytognos

Abstract

Many of today's consensus leukemia and lymphoma antibody panels have not been validated, but rather consist of markers based on expert opinions. As a result, screening and diagnosis of hematological malignancies has not been standardized, hindering reproducibility in multicenter studies. In 2005, an independent scientific group, the EuroFlow™ Consortium, set out to innovate and standardize flow cytometric immunophenotyping.[1] Their efforts resulted in the development and validation of ≥8 color antibody panels for efficient and reliable screening of hematological malignancies. The lyophilized EuroFlow™ antibody panels are part of a 'start to finish' protocol whereby the flow cytometrist has access to standard operating procedures (SOPs) for instrumentation setup, sample preparation, staining and data acquisition, leading to improved accuracy and reproducibility across centers. In addition, Infinicyt™ software is available for intuitive integration and multidimensional analysis of resulting flow cytometry data.

Potential Assay Complications

Current testing of hematological malignancies by flow cytometry requires a skilled cytometrist, consistent instrument set up and calibration techniques, sample preparation, precise combinations of antibodies and validation of antibody/fluorochrome cocktails, accurate compensation, and a data analysis program that results in minimal subjectivity with

Potential Assay Complications *Continued*

maximal accordance during analysis. The complexity of classifying hematological malignancies calls for a simplified and standardized approach to minimize several of the following complications encountered when processing samples:

Inconsistent Instrument Setup & Calibration

To ensure accurate data acquisition and reporting, it is recommended to calibrate a flow cytometer daily. This requires each laboratory to individually establish and train their technicians on a cytometer setup protocol, leading to potential variability across centers and difficulty collating interpretable study conclusions.

Poorly Designed Compensation Controls

Improper compensation or lack of compensation control set up can yield false positives and inaccurate data reporting. This can occur when shortcuts are taken, control files are used from a previous lot of reagent, or when compensation controls are unavailable at the time of data acquisition.

Potential for High Variation Across Antibody & Fluorochrome Cocktails

When creating multicolor panels, it is common to use dim fluorochromes for detection of antigens expressed at high levels and bright fluorochromes for detection of antigens expressed at low levels. Therefore, technicians must repeatedly titer and test reagents to optimize their antibody/fluorochrome combination, then preferably document and validate their recipe to ensure cocktailing precision with future testing. Even when concocting laboratory validated combinations based on expert suggestions, there is still the potential for inter-laboratory cocktail variation, thus creating difficulty when trying to compare results across centers.

Complex Data Analysis

After multiple days of instrument set up, compensation, sample preparation and cellular staining, comes data acquisition and analysis. The generation of highly complex, multicolor data challenges current analysis tools and subjectivity could lead to variability in data reporting. Additionally, the ability to compare results across centers becomes cumbersome when labs are not working from the same protocols or using the same antibody combinations.

Complex Testing Simplified

A more specific example of the issues that surround testing of hematological malignancies involves development, validation and execution of intricate multi-tube antibody combinations. Today, when there is a high suspicion of acute leukemia, a technician has to prepare up to 16 different 8-color tubes with ≥8 antibodies in each tube. Following data acquisition, the technician must analyze the highly complex data to determine which form of leukemia is present: BCP-ALL, T-ALL or AML/MDS. It is nearly the same scenario when there is a suspicion of lymphoma—a large number of tubes must be prepared containing a precise amount of several antibodies per tube in order to accurately determine which form of lymphoma (B-CLPD, T-CLPD or NK-CLPD) is present.

In response to the clear need for a more efficient and standardized approach to classifying hematological malignancies, the EuroFlow™ Consortium spent several years validating multicolor antibody combinations. The resulting EuroFlow™ product line of pre-selected and lyophilized antibody panels offer a comprehensive method for proficient screening of suspect leukemia, myeloma and lymphoma samples. A cytometrist also has access to online SOPs, available at http://euroflow.org/usr/pub/pub.php, for guidance on instrument set up, calibration, compensation, sample preparation and sample staining to maintain consistency across testing, while the affiliated Infinicyt™ program allows for advanced data acquisition and analysis.

EuroFlow™ Antibody Panels

The EuroFlow™ product line provides solutions to many of the common issues impacting the formation of ideal antibody cocktail preparations, including:

Standardized & Optimized Reagents

All of the EuroFlow™ panels were tested against reference databases of normal and malignant cells from healthy subjects and WHO-based disease entities. The EuroFlow™ studies resulted in 8-color antibody panels optimally suited for immunophenotypic screening of hematological malignancies. The development of the EuroFlow™ screening tubes was also paralleled by a set of SOPs to assure full technical standardization with 3-laser flow cytometry instrument settings and laboratory protocols.

Efficiency with Pre-Mixed Antibody Cocktails

The EuroFlow™ screening tubes are provided as lyophilized mixtures of optimized combinations for each type of malignancy, where the technician simply reconstitutes the cocktail and proceeds directly to staining samples.

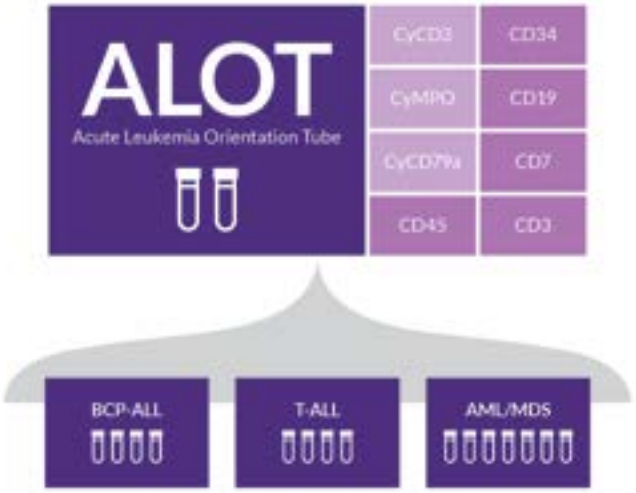
EuroFlow™ Antibody Panels *Continued*

Reproducible & Accurate Results

The lyophilized standardization of the antibody mixtures streamlines the instrument and assay training process, facilitates inter- and intra-laboratory reproducibility and eliminates uncertainty around which antibody/fluorochrome combinations are being used by other centers. Additionally, the Infinicyt™ data analysis software can objectively compare immunophenotypic profiles for a sample or group of samples. As a result, one can easily identify the phenotypic differences and similarities between cell populations, allowing for faster time to results and consistency of data reporting across centers.

Acute Leukemia Orientation Tube (ALOT)

The ALOT kit contains a combination of 8 antibodies designed for initial assessment of the nature of immature populations of hematopoietic cells in acute leukemia samples to allow appropriate orientation towards a full BCP-ALL, T-ALL and/or AML/MDS characterization panel.



Technicians can compensate using the reagents provided with the ALOT screening kit without needing to cocktail antibodies or validate fluorochrome combinations. Simply reconstitute the two vials included in the kit, one containing 5 extracellular antibodies and one containing 3 intracellular/cytoplasmic antibodies, then stain the samples and acquire data for analysis. Results obtained with the ALOT screening tube will help guide the user toward the appropriate next step in testing, leading to a complete diagnostic workup using their previously validated antibody combinations.

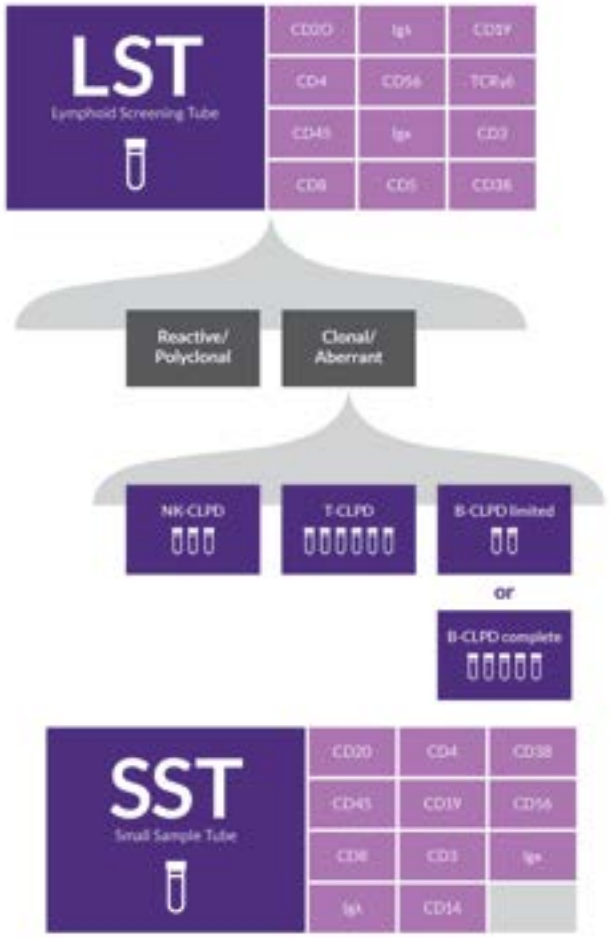
Lymphoid Screening & Small Sample Tubes (LST & SST)

The LST kit is a complete 8-color screening tube for lymphocytosis studies. This 12-marker combination of antibodies is aimed at detecting phenotypically aberrant populations of mature B-, T- and NK-cells including lymphocytosis, lymph node enlargement, splenomegaly, monoclonal serum components and unexplained cytopenias. The SST panel is an enhanced version of the LST panel with 11 conjugated antibodies optimized for processing low

Lymphoid Screening & Small Sample Tubes (LST & SST) *Continued*

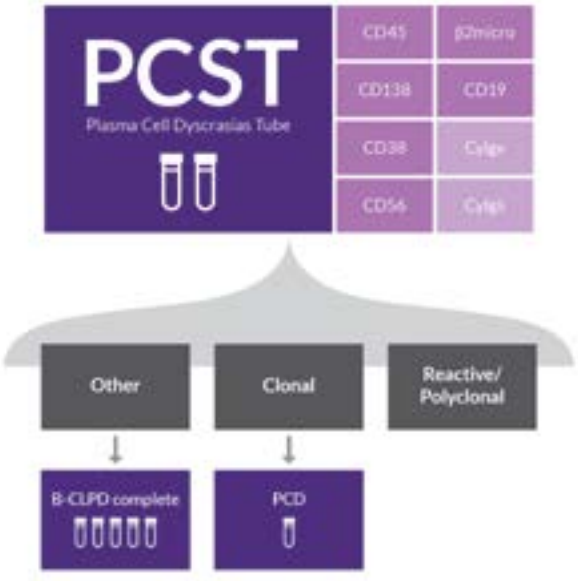
cellularity samples such as cerebrospinal fluid, vitreous humour, and fine needle aspirates.

Typical workflow for a suspected lymphoma sample may involve the analysis of up to 16 different 8-color tubes with ≥8 antibodies in each tube, which is not only time consuming but leaves room for high variation across samples. Instead, the LST and SST panels offer a straightforward screening method with reconstitution of one premixed antibody cocktail for staining samples and orientation towards further testing with previously validated B-CLPD, T-CLPD, NK-CLPD or PCD characterization tubes for a complete diagnostic assessment.



Plasma Cell Dyscrasias Screening Tube (PCST)

The PCST kit contains 8 conjugated antibodies designed for identification, discrimination and enumeration of coexisting normal/reactive and aberrant plasma cell populations. It is recommended for the initial screening of Plasma Cell Dyscrasias (PCD) to differentiate between monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM).



Plasma Cell Dyscrasias Screening Tube (PCST) *Continued*

Indications for PCD are bone pain, recurrent infections, anemia and/or lytic lesions.[2,3] PCD is apparent with the presence of an abnormal free light chain (FLC) ratio where the FLC ratio is determined using quantitative nephelometry or immunofixation electrophoresis (IFE).[4] Next, the plasma cells are classified as normal/abnormal using immunophenotyping by flow cytometry and then tested for the presence of common MM markers. To determine the tumor burden, the sample is subjected to beta-2 microglobulin testing by quantitative immunoturbidimetry. Some instances may occur where B-CLPD presents as MM but differentiation can be obtained through analysis of CD19 expression because lymphomas express this marker but myelomas rarely do.[2,3]

By replacing current testing standards for MM and MGUS with the PCST assay, only one technique is needed to determine the FLC ratio, state of the plasma cells, likelihood of presence of MM or B-CLPD and tumor burden. Official diagnosis can then be obtained by investigating bone lesion presence using previously validated techniques.

Infinicyt™ Software

The final component to the EuroFlow™ product line is the powerful and intuitive multi-dimensional data analysis software, Infinicyt™. Infinicyt™ provides a list of automated flow cytometry data analysis tools for research of various diseases. Key features of the Infinicyt™ software include:

- **APS:** Automatic n-dimensional separation of clusters
- **File Merge:** Analysis of multiple parameters simultaneously
- **Reference Image:** Compare new samples with previous events
- **Compass:** Identification of similarities and differences between samples
- **Maturation Tool:** Advanced evaluation of maturation patterns
- **Batch Analysis:** Automated gating and analysis strategies

For more information on Infinicyt™, please contact ALPCO for a copy of the Infinicyt™ white paper or visit http://www.alpco.com/products/Infinicyt.aspx to learn more. A free 30-day trial version of the program is also available for download at www.infinicyt.com.

Simple, Accurate & Standardized Screening

After several years of developing, testing and validating multicolor combinations, the EuroFlow™ screening panels released by the EuroFlow™ Consortium allow for simplified, accurate and standardized processing of samples. By integrating initial sample screening with ALOT, LST, SST and/or PCST into the workflow, laboratories can reduce time spent testing

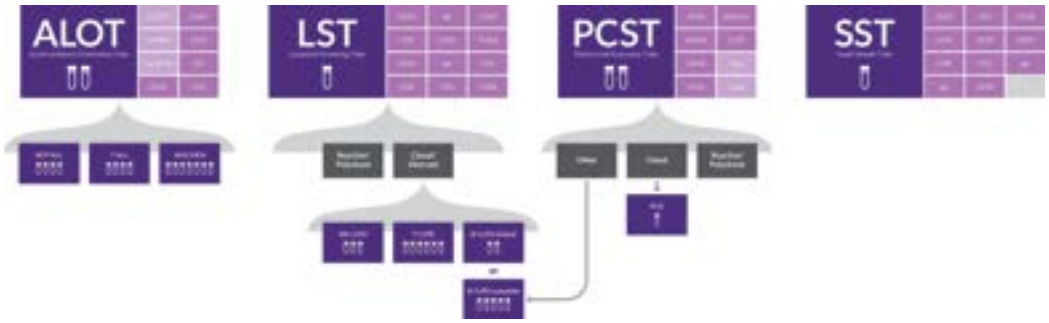
Simple, Accurate & Standardized Screening *Continued*

for all suspected disease possibilities and utilize the screening results to narrow subsequent testing toward confirmation of a specific disease state.

The EuroFlow™ ALOT, LST, PCST and SST assays are CE marked and available for Research Use Only in the USA. Diagnostic confirmation should be completed post EuroFlow™ screening via previously validated in-house techniques.

Summary

The EuroFlow™ panels were rigorously tested and optimized on 2031 informative samples following EuroFlow™ SOPs for sample preparation and utilizing novel Infinicyt™ software tools for data acquisition and analysis. This study showed the EuroFlow™ antibody panels to be highly efficient for sample screening and results indicated the combined use of EuroFlow™ panels and SOPs to be considered one of the most extensively validated approaches for standardizing multidimensional flow classification of hematological malignancies.



To learn more about the EuroFlow™ antibody panels or to discuss evaluation opportunities please contact **Bethany S. Bell** at:

Phone: 800-592-5726 ext 260
Email: bbell@alpco.com

Works Cited

1. Van Dongen JJM, et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia. 2012; 26:1908–1975.

2. ARUP Consult, Topics. Plasma Cell Dyscrasias. 2006. Accessed 10NOV13. <http://www.arupconsult.com/Topics/PlasmaCellDyscrasias.html>

3. ARUP Consult, Algorithms. Plasma Cell Dyscrasias. 2006. Accessed 10NOV13. <http://www.arupconsult.com/Algorithms/PlasmaCellDyscrasias.pdf>

4. Kyle, RA and Rajkumar, SV. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. Leukemia. 2009; 23 (1) : 3-9.

Additional References

5. Van Dongen JJM, et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia. 2012; 26:1908–1975.

6. Van Dongen JJM, Orfao A. EuroFlow: Resetting leukemia and lymphoma immunophenotyping. Basis for Companion Diagnostics in personalized medicine. Leukemia. 2012; 26: 1899–1907.

7. Kalina T, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. Leukemia. 2012; 26:1986–2010.

8. Pedreira CE, et al. Generation of flow cytometry data files with a potentially infinite number of dimensions. Cytometry, Part A. 2008; 73A: 834–846.

9. Pedreira CE, et al. A probabilistic approach for the evaluation of minimal residual disease by multiparameter flow cytometry in leukemic B-cell chronic lymphoproliferative disorders. Cytometry, Part A. 2008; 73A: 1141–1150.

10. Costa ES, et al. Harmonization of light scatter and fluorescence flow cytometry profiles obtained after staining peripheral blood leucocytes for cell surface-only versus intracellular antigens with the Fix & Perm™ reagents. Cytometry, Part B. 2010; 78B: 11–20.

Additional publications related to EuroFlow™ Consortium and EuroFlow™ product line can be found here:
http://www.euroflow.org/publications.php
http://www.infinicyt.com/index.php/publications