



**CPAL**

Central Pennsylvania Alliance Laboratory

# Technical Bulletin

## No. 81

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### Flow Cytometry Immunophenotyping for Leukemia and Lymphoma (FCI)

**Starting Date:** May 10, 2010

**Clinical use:**

Immunophenotyping using flow cytometry is one of several methodologies used in the detection and characterization of hematopoietic neoplasms. Findings are evaluated in the context of several other ancillary tests in the evaluation of these tumors; these include morphology, immunocytochemistry, cytogenetics and molecular diagnostics.

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**Testing Schedule:** Mon-Fri, morning and afternoon; Saturday, morning

- ✓ **Important:** *Specimens drawn on Friday that would not arrive at CPAL with the Friday night courier should be sent to the reference lab used for ancillary studies.*

**Specimen:** requirements vary depending on tissue type

- Peripheral blood
  - Acceptable anticoagulants: EDTA, Sodium Heparin
  - Temperature requirements
    - Store at ambient temperature
    - Ship at ambient temperature

- Specimen age
  - Receipt at CPAL within (24) hours of collection is requested
  - Specimens received (24 – 48) hours post-collection will be accepted
  - Specimens older than (48) hours post-collection will **not** be processed
- Volume requirements
  - Minimum acceptable volume = 1 mL
- Rejection criteria: evidence of freezing
- PNH is a separate test for accessioning purposes, but is processed in the same fashion as FCI. There are several different requirements for PNH accessions:
  - Acceptable anticoagulant: EDTA only
  - Receipt within (24 hours) of collection is required
- Bone marrow aspirates
  - Acceptable anticoagulants: EDTA, Sodium Heparin
  - Temperature requirements
    - Store at ambient temperature
    - Ship at ambient temperature
  - Specimen age
    - Receipt at CPAL within (24) hours of collection is requested
    - Specimens received (24 – 48) hours post-collection will be accepted
    - Specimens older than (48) hours post-collection will **not** be processed
  - Volume requirements
    - Minimum acceptable volume = 1 mL
  - Rejection criteria
    - evidence of freezing
- Surgical Biopsies
  - Suspension medium: RPMI
  - Temperature requirements
    - Store at 2-8 degrees centigrade; do not freeze
    - Ship at 2-8 degrees centigrade; do not freeze
  - Specimen age
    - Receipt at CPAL within (24) hours of collection is requested
    - Specimens received (24 – 48) hours post-collection will be accepted
    - Specimens older than (48) hours post-collection will **not** be processed
  - Volume requirements
    - 1-5 tissue chunks with size approximating 0.5 square-centimeters
    - Suspend tissue in 10x volume of RPMI
  - Rejection criteria
    - evidence of freezing
    - evidence of fixation
- FNA Biopsies
  - Suspension medium: RPMI
  - Temperature requirements
    - Store at 2-8 degrees centigrade; do not freeze
    - Ship at 2-8 degrees centigrade; do not freeze

- Specimen age
  - Receipt at CPAL within 24 hours of collection is requested
  - Specimens received (24 – 48) hours post-collection will be accepted
  - Specimens older than 48 hours post-collection will **not** be processed
  -
- Volume requirements
  - Suspend tissue in 1-5 mL of RPMI
- Rejection criteria
  - evidence of freezing
  - evidence of fixation
- Fluids (Pleural, Peritoneal, Pericardial)
  - Temperature requirements
    - Store at 2-8 degrees centigrade; do not freeze
    - Ship at 2-8 degrees centigrade; do not freeze
  - Specimen age
    - Receipt at CPAL within 24 hours of collection is requested
    - Specimens received (24 – 48) hours post-collection will be accepted
    - Specimens older than 48 hours post-collection will **not** be processed
  - Volume requirements
    - Minimum acceptable volume = 10 mL
      - Volume to send is dependent on cellularity
  - Rejection criteria
    - evidence of freezing
    - evidence of fixation
- CSF
  - Temperature requirements
    - Store at 2-8 degrees centigrade; do not freeze
    - Ship at 2-8 degrees centigrade; do not freeze
  - Specimen age
    - Specimens older than (24) hours post-collection will **not** be processed
  - Volume requirements
    - Minimum acceptable volume = 2 mL
      - Volume to send is dependent on cellularity
    - If ancillary testing is being ordered, do not send specimen to CPAL
- Bronchial lavage/wash/scrapings
  - **Not** processed by CPAL, send to reference lab

## Summary

- Cases that are currently sent to a reference lab such as USLabs or GenPath for flow cytometry will be sent to CPAL for the flow cytometry component and to the current reference lab for other testing that may include cytogenetics, FISH and/or molecular diagnostics. Therefore, specimens need to be split at the submitting institution with one aliquot being sent to CPAL and the other being sent to the reference lab. In the event of volume-limited specimens, the entire sample should be sent to the reference lab so that flow cytometry would be performed at that site.

- Sample submissions going to CPAL for flow cytometry should include a copy of the form that accompanies the submission to the reference lab. There is no form for CPAL submissions; orders for FCI are placed through LIS.
  - Note that PNH is ordered as a test distinct from FCI in LIS since it can only be run on peripheral blood (EDTA only).
- The strategy for testing is based on recommendations from the 2006 Bethesda International Flow Cytometry Consensus Conference. <sup>(1)</sup>
- The composition and extent of FCI testing is based on the ordering information transmitted through the LIS accession. Reflexive testing will be performed based on results of initial studies, at either the discretion of CPAL flow cytometry staff, or at the instruction of the submitting pathologist after their review of results.

NHL (incl CLL)	CD5-/CD10- dim-neg sIg T-cell aberration	Bkg	T1	B1	B2
		B3			
		C1			(reflexive)
		T2	T3		
PCD		Bkg	PCD1	PCD2	C1
Blasts	ALL AML M6/M7 aberrant antigen	Bkg	ALL	AML	MDS
		B2			
		T1			(reflexive)
		M6/M7			
		C2			
Cytopenia		Bkg	T1	B1	MDS
CLL RD		Bkg	B1	B2	
PCD RD		Bkg	PCD2	C1	

- FCI is performed using 6-color panels, utilizing monoclonal antibodies conjugated to fluorochromes listed below. The panel is organized in a number of tubes, each containing 6 reagents combined in a manner that systematically examines antigens expressed on leukocyte subsets including:
  - Lymphocytes: T-, B-, NK-
  - Plasma cells
  - Monocytes
  - Granulocytes
  - Progenitor cells: lymphoid, myeloid

<u>Tube</u>	<u>FITC</u>	<u>PE</u>	<u>PerCP-Cy5.5</u>	<u>PE-Cy7</u>	<u>APC</u>	<u>APC-H7</u>
<b>Bkg</b>			ViaProbe			CD45
<b>T1</b>	CD8	CD7	CD16	CD3	CD4	CD45
<b>T2</b>	CD57	CD2	CD16	CD3	CD56	CD45
<b>T3</b>	TCR-AB	TCR-GD	CD8	CD3	CD4	CD45
<b>B1</b>	CD5	lambda	CD19	CD20	kappa	CD45
<b>B2</b>	CD5	CD23	CD19	CD38	CD10	CD45
<b>B3</b>	FMC7 or CD103	CD22	CD19	CD25	CD11c	CD45
<b>PCD1</b>	CD138	lambda	CD19	CD38	kappa	CD45
<b>PCD2</b>	CD138	CD117	CD19	CD38	CD56	CD45
<b>ALL</b>	HLA-DR	CD34	CD19	CD20	CD10	CD45
<b>AML</b>	CD64	CD117	CD16	CD33	CD14	CD45
<b>MDS</b>	HLA-DR	CD11b	CD16	CD13	CD56	CD45
<b>M6/M7</b>	CD61	CD235a	CD42a	CD38	CD71	CD45
<b>C1</b>	CD5 or CD138	©lambda	CD19	CD38	©kappa	CD45
<b>C2</b>	©MPO	©Tdt	CD19	CD3	©CD79a	CD45

- In most cases, results should be available within 24 hours of receipt of the specimen at CPAL. If there will be a delay beyond this point, CPAL will call a designated individual to apprise them of this situation.
- Results are conveyed to the submitting institution on two platforms:
  - LIS
    - List of markers used and the result for each in qualitative terms
      - Positive
      - Negative
      - Partial (heterogeneous expression)
      - Positive-bright (higher intensity than would be seen in normal tissue)
      - Positive-dim (lower intensity than would be seen in normal tissue)
    - CPT codes to be used by the institution to generate patient charges
      - Technical component (Part A)
        - 88184 (Flow cytometry, cell surface, cytoplasmic, or nuclear marker, technical component only; first marker)
        - 88185 (Flow cytometry, cell surface, cytoplasmic, or nuclear marker, technical component only; each additional marker)
      - Professional component (Part B)
        - 88187 (Flow cytometry, 2-8 markers)
        - 88188 (Flow cytometry, 9-15 markers)
        - 88189 (Flow cytometry, 16 or more markers)
    - PDF documents of flow cytometry histograms/plots, placed on the CPAL website (<http://www.cpallab.com/private/logon.asp>) for review by pathologists
  - Report is generated by submitting pathologists.

- Pathologist may instruct reflexive flow cytometry testing at CPAL, and/or reflexive ancillary testing at the reference lab after review of flow cytometry results.
- Patient charges are generated by the submitting hospital, not CPAL.

## Validation Studies

The validation process for FCI testing began in January 2010 and was completed in April 2010. The substrate material for the validation studies were samples that were provided by the laboratories from several member hospitals (York, LGH, ECH, Pinnacle). These samples were aliquots of samples that had been submitted to the reference labs (US Labs, GenPath) utilized by each institution for immunophenotyping studies. The steps in the validation were as follows:

Phase I studies utilizing 43 samples submitted between Jan 26, 2010 and Feb 25, 2010. The samples were comprised of (3) peripheral bloods, (33) bone marrow aspirates and (2) surgical biopsies.

- Construct 6-color immunophenotyping panels, with their constituent antibody cocktails, using the following resources
  - Recommendations from the Consensus Document <sup>(1)</sup> for this testing
  - Consultation with the reagent sales representative (Timothy Stewart) and application specialist (Judy McLeod) from the vendor supplying the reagents and instrumentation (Becton-Dickinson Immunocytometry Systems, BDIS).
  - Consultation with hematopathologists from York Hospital who are members of the CPAL Flow Cytometry Affinity Group (Dr. Ander Pindzola and Dr. Matthew Georgy).
- Test the reactivity of constituents of the reagent cocktails using the following steps:
  - Confirm that the performance characteristics of individual components within cocktails are the give results consistent with known reference ranges for these reagents. This substrate is control material (Multicheck, BDIS) from the vendor that is currently used for other flow cytometry testing (Lymphocyte Subsets) performed at CPAL. The process was the same as that is used to comply with a question from the CAP checklist for flow cytometry (FLO.23737).
  - Confirm that performance of reagents within a cocktail is the same as seen with the reagent alone. This step was accomplished using *Fluorescence-Minus-One* testing <sup>(2)</sup> whereby replicates of each cocktail mixture minus one of the constituents is compared to the complete mixture, to identify the “negative” distribution for each constituent, and, to qualitatively assess the adequacy of the compensation matrices.

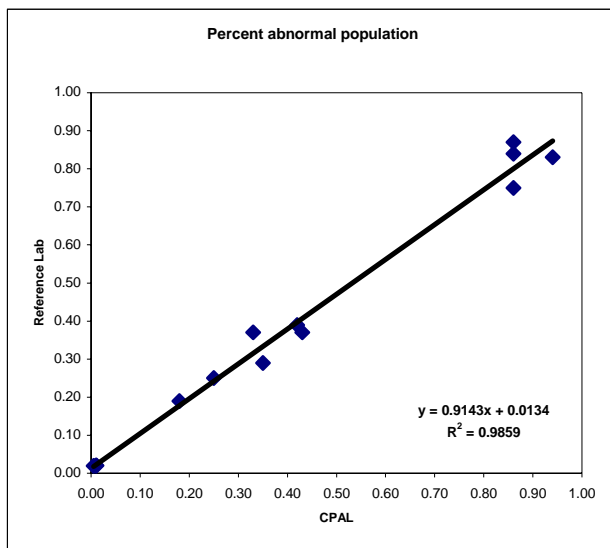
Phase II studies utilizing samples submitted between Feb 25, 2010 and April 19, 2010. The samples were comprised of (4) peripheral bloods, (29) bone marrow aspirates and (17) surgical biopsies.

- Perform FCI on samples and submit analyses completed by CPAL staff to the York hematopathologists noted above for their interpretation, and subsequent comparison with the results obtained by the reference labs noted above.
- A total of **50** samples were processed during this phase of the study.
- The diagnoses arrived at by the York Hospital hematopathologists were compared to those rendered by the reference lab to which the specimen had been submitted.

- The diagnoses were compared to calculate the % *Concordance* using a contingency table:

		CPAL	
		Malignant	Benign
Reference lab	Malignant	13	0
	Benign	0	37
% Concordance=		100%	

- As noted in the contingency table, 26% of the submitted samples showed evidence of hematopoietic malignancy. The proportion of each sample that contained neoplastic cells was compared between the reference labs and CPAL and excellent correlation was observed



- Disease entities identified during this phase of the process included:
  - Chronic lymphoproliferative disease (B-cells)
    - CLL/SLL
    - CD5(-)/CD10(-)
  - Chronic lymphoproliferative disease (T-cells)
  - Plasma cell dyscrasia
  - Acute lymphocytic leukemia

- Acute myelogenous leukemia
- Paroxysmal nocturnal hemoglobinuria

Phase III studies utilizing proficiency surveys from the College of American Pathologists

- FL3CD-A (February 2010): CD-ROM containing two cases of leukemia/ lymphoma with clinical histories, digital images, and ungated list mode files; allows users to examine gating strategies and interpret antibody staining patterns.
  - educational challenge submitted to CAP
    - Result: Both cases were successfully identified with the consensus diagnosis; one a chronic myelogenous leukemia, the other a myelodysplastic syndrome.
- FL3-A (March 2010) - Two 4.5-mL whole blood specimens and/or cell lines simulating leukemia/ lymphoma; color photographs of tissue sections, bone marrow, and/or peripheral blood smears with clinical histories.
  - standard challenge, evaluated for internal assessment of proficiency, not submitted to CAP
    - Result: First case successfully identified with the consensus diagnosis (acute B-cell lymphocytic leukemia). Consensus was not reached on the second case (acute myeloid leukemia, favor M3). An acceptable alternate diagnosis (acute myeloid leukemia, favor M4/M5) was rendered by CPAL.

For detailed analysis of the validation studies, or to discuss any aspect of FCI testing performed at CPAL, please call the laboratory (see contact information listed above).

**References**

- 1) [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.
- 2) [Baumgarth N](#), [Roederer M](#). A practical approach to multicolor flow cytometry for immunophenotyping. [J Immunol Methods.](#) 2000 Sep 21;243(1-2):77-97.