CPAL
Central **P**ennsylvania **A**lliance **L**aboratory

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Improvements to Flow Cytometry Method of Immunophenotyping for Leukemia and Lymphoma (FCI)

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Effective Date: May 2, 2016

Testing Schedule: Monday through Friday, morning and afternoon. Saturday, morning only.

Specimen Types: No change. See Technical Bulletin No. 132 for details.

Clinical Use:
Flow cytometry immunophenotyping (FCI) is a testing method that allows multi-parametric analysis of cell-surface and intracellular antigens. FCI utilizes prepared single-cell suspensions, monoclonal antibodies conjugated to fluorescent dyes, and sophisticated instrumentation (flow cytometer) that provides a laser light source, a focused fluidics environment to carry the cell suspension through the light source, and an electronic signal detection and amplification system. As a result, FCI testing offers highly efficient and rapid evaluation of a submitted sample for a suspected hematologic malignancy.

The advantages of using FCI include 1) the ability to distinguish benign and neoplastic conditions, 2) the diagnosis and characterization of leukemias and lymphomas, 3) the capability to asses other neoplastic/pre-neoplastic disease states such as myelodysplastic syndrome and plasma cell dyscrasias, 4) the detection of residual disease in patients who have undergone a treatment regimen for a hematologic malignancy, and 5) provision of prognostic information for particular neoplastic states (i.e. CD38 expression in CLL patients).

Method Improvements:
Opportunities for improvement to FCI have been discussed with CPAL member pathologists, particularly to improve identification of plasma cell populations. As a result of these discussions, CPAL performed extensive experiments to determine the optimal conditions for this analysis, and

validated several changes to the current method as a result of these experiments. These changes include: a new intracellular staining kit, intracellular light chain, red cell lysis, and cell suspension fixation reagents and red blood cell lysis methods on the current 10-color Beckman Coulter Navios. The changes will be made to FCI only (High Sensitivity PNH testing is excluded from this change).

Validation Studies:
The validation process for reagent and red blood cell lysis method changes in FCI testing began in February 2016 and was completed in April 2016.

For detailed analysis of the validation studies, or to discuss any aspect of FCI testing performed at CPAL, please call the laboratory contacts listed on the first page or refer to www.cpallab.com/technotes for the validation document.

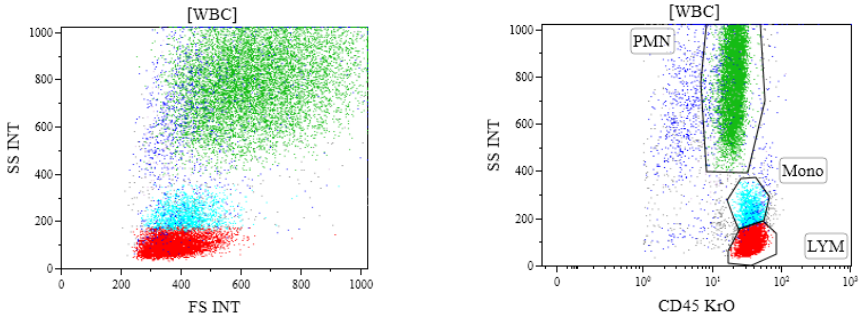
The following is a summary of the validated changes (Table 1) and their effects on the presentation of FCI data (Figures 1-4):

Table 1: Reagent/Method Change Summary

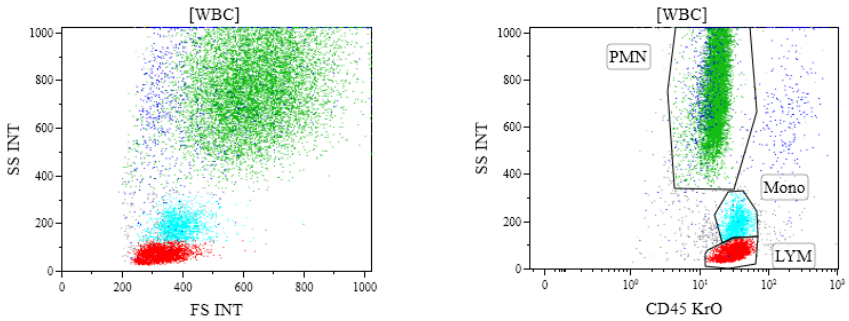
	Current Reagent/Method	**New Reagent/Method**	**Resultant Change in Data**
Red Blood Cell Lysis Reagent	Beckman Coulter IOTest 3 Lysing Reagent	BD Biosciences PharmLyse	Consistent RBC lysis in all data sets, improved WBC population resolution.
Red Blood Cell Lysis Methods	Tube Lysis (RBCs lysed in each panel tube after surface antigen staining)	Bulk lysis (RBCs lysed in a larger sample aliquot prior to surface antigen staining)	Consistent RBC lysis in all data sets, improved WBC population resolution.
Cytoplasmic kappa and lambda reagents, PCD tube only	Beckman Coulter FITC kappa, Beckman Coulter PE lambda	Dako FITC kappa, Dako PE lambda	Vast improvement in plasma cell light chain reactivity in both normal and neoplastic states
Intracellular Staining Kit	Beckman Coulter PerFix_nc	Dako IntraStain	Improvement in data presentation.
Sigma-Aldrich Mouse Serum FC-blocking reagent	Currently used	Discontinued use	Unexpected CD10 expression patterns resolved.

Effects of Changes:

Figure 1: Improved white blood cell scatter characteristics and separation with change in red blood cell lysis reagents and methods.

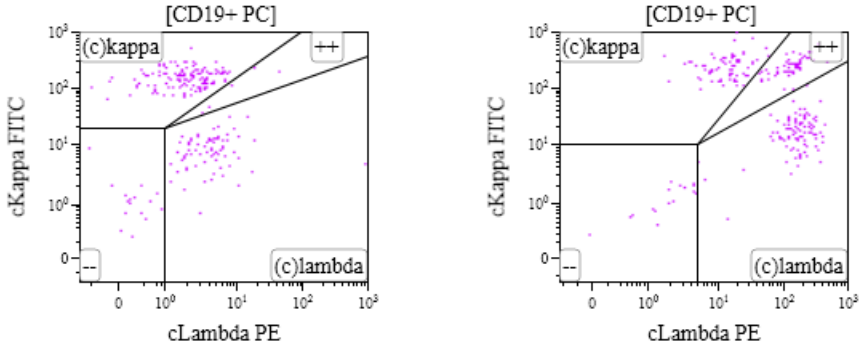


Normal Peripheral Blood, Beckman Coulter Lysing and Fixation reagents, Tube Lysis

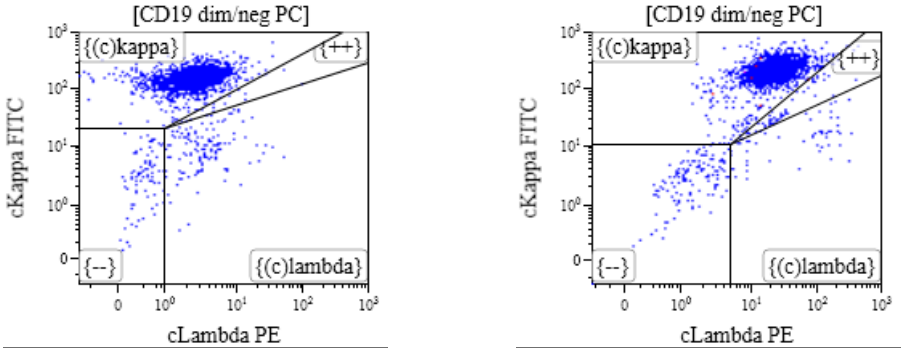


Normal Peripheral Blood, BD Biosciences Lysing and Fixation Reagents, Bulk Lysis

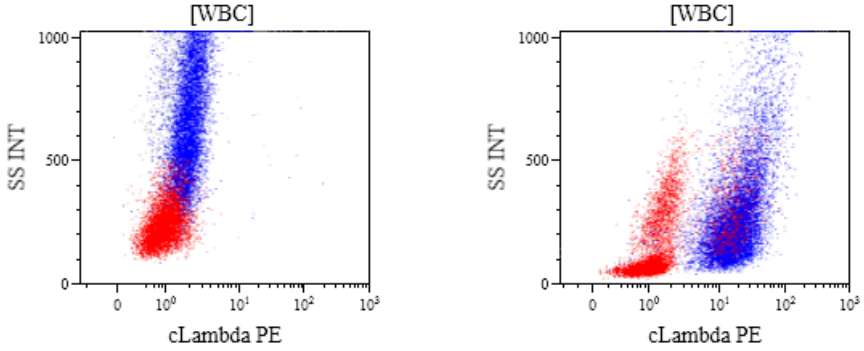
Figure 2: Improved intracellular light chain expression with another manufacturer's reagents. Bone marrow samples and Streck CD-Chex CD103 Plus control cells were used to compare Beckman Coulter and Dako reagent reactivity of intracellular kappa and lambda light chains in the PCD tube.



Polyclonal marrow plasma cells. Beckman Coulter reagents (left) and Dako reagents (right).

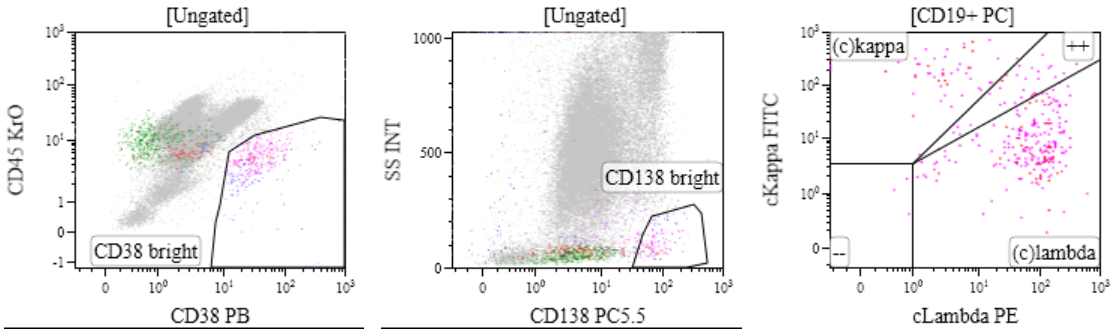


Kappa restricted marrow plasma cells. Beckman Coulter reagents (left) and Dako reagents (right).

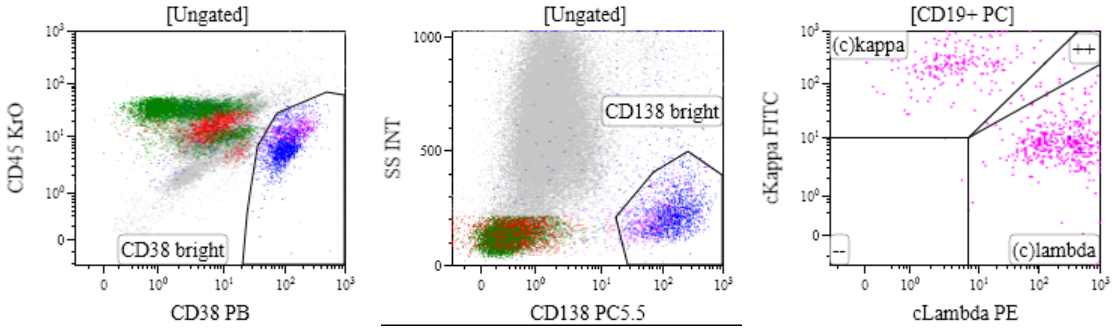


Lambda restricted Streck CD-Chex CD103 Plus cells. Beckman Coulter reagents (left) and Dako reagents (right).

Figure 3: Evaluated intracellular staining kits using a bulk lysed sample. Cellular event and fluorochrome reactivity with the Beckman Coulter PerFix nc Kit compared to Dako IntraStain Kit.

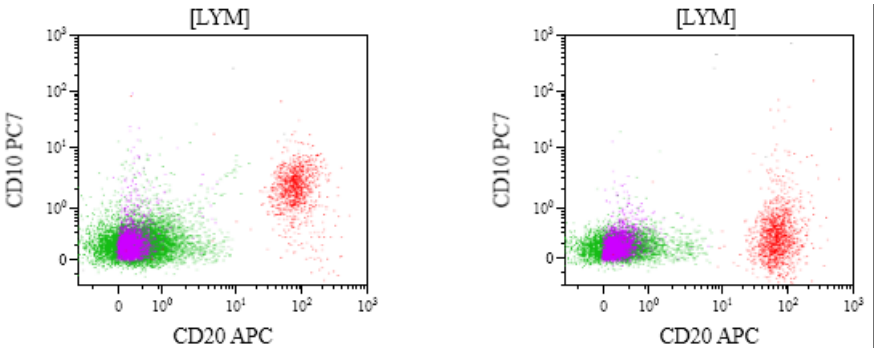


Polyclonal marrow plasma cells. Bulk lysed sample stained using PerFix nc Kit.

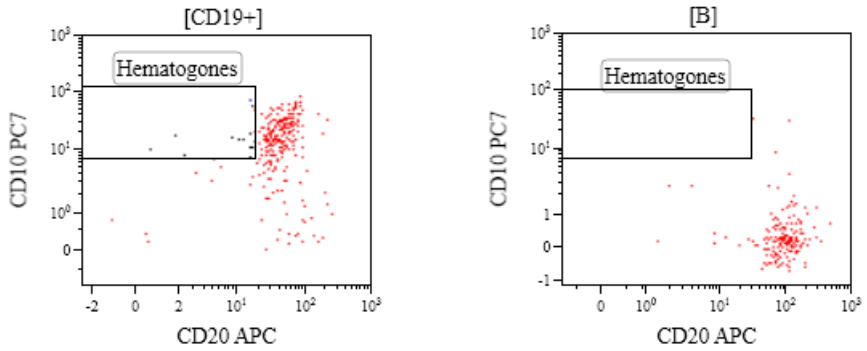


Polyclonal marrow plasma cells. Bulk lysed sample stained using Dako IntraStain Kit.

Figure 4: Confirmed that Sigma-Aldrich Mouse serum was causing unexpected CD10 expression patterns.



CD10 expression pattern on a peripheral blood sample, with (left) and without (right) mouse serum.



CD10 expression pattern on a bone marrow sample, with (left) and without (right) mouse serum.

Method Comparison:
The above changes were tested in parallel with the current method using 19 samples submitted between February 2016 and April 2016. The samples were comprised of 6 peripheral bloods, 10 bone marrow aspirates, and 3 surgical biopsies. Disease entities identified during this phase of the process included:

- Chronic lymphoproliferative disease (B-cells)
  - CLL/SLL
  - CD5(-)/CD10(-)
  - CD5(-)/CD10(+)
- Chronic lymphoproliferative disease (T-cells)
- Plasma cell dyscrasias
- Acute lymphocytic leukemia (simulated with control material)

The diagnoses determined by using the data from the new reagents and methods used on Navios 10-color panels were compared to those rendered by using the data from the currently validated reagents and methods. There was 100% concordance of diagnoses between the two methods.

References:

1) Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* : 111 (8); 2008.

2) 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.

3) BioLegend website, www.biolegend.com "Expression of Common Surface Molecules on Blood Cells" and "Fluorophore Brightness Index."

4) Beckman Coulter website, www.beckmancoulter.com , "Fluorophore Excitation and Emission Spectra" chart.

5) BD website, www.bdbiosciences.com , archived ppt presentation by Holden T. Maecker, "Design and Optimization of Multicolor Panels."