NEW YORK STATE DEPARTMENT OF HEALTH
CLINICAL LABORATORY EVALUATION PROGRAM

COMMENTS and RESPONSES to PROPOSED CELLULAR IMMUNOLOGY – MALIGNANT IMMUNOPHENOTYPING STANDARDS

The Proposed Standards in the areas of Cellular Immunology – Malignant Immunophenotyping were circulated for comment on March 7, 2014. The announcement and copies of the proposed standards with a crosswalk were sent to NYS-permitted facilities that held or were in application for a permit (facilities). This distribution was by e-mail to the facility and laboratory contact person’s e-mail address. The documents were also posted to the CLEP website.

The comment period ended April 25, 2014. There were 10 commenters from regulated parties and coalitions with 10 comments. Modifications and clarifications to the Cellular Immunology – Malignant Immunophenotyping Standards have been made based on the comments received.

Since there were no substantial changes, the standards are considered to be generally accepted, with a few minor changes described below, and will be adopted with an effective date of July 14, 2014.
Proposed Standard

Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 1: Client Instructions for Specimen Collection and Transport

The laboratory shall provide specimen collection and transport instructions to clients indicating:

a) the anticoagulant(s), saline, or medium that must be used for each type of specimen to be collected;

b) the requirement for specimen collection date and time must be documented with each specimen;

c) blood and bone marrow samples must be maintained between 18 and 25 degrees Celsius, while tissue and other biological fluids must be maintained between 2 and 8 degrees Celsius;

d) the maximum time between collection and receipt so that the laboratory can test within required assay time frames; and,

e) any other information considered significant for specimen analysis.

d) Assay time frames are the interval from the time of collection to the time of processing, when the specimen is stained, lysed, and fixed. If the specimen is not fixed during the staining process, then the specimen age end point would be time of data acquisition on the flow cytometer.

Proposed Guidance

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Comment 1:

“Blood and bone marrow samples must be maintained between 18 and 25 degrees Celsius, while tissue and other biological fluids must be maintained between 2 and 8 degrees Celsius.” Comment: There is no definitive consensus but according to ICCS guidelines, 18-25 degrees Celsius is acceptable for all. Overnight refrigeration may improve sample stability for overnight storage particularly when granulocytes are considered in the differential.

Am J Clin Pathol. 1999 Nov; 112(5):687-95. (Granulocyte preservation for 24 hour storage)

Comment 2:

There are logistic concerns regarding part c of the proposed standard below. We can make changes to the directory of services regarding requirements and have concerns regarding meeting this requirement since we do not send shipping containers to our clients.

**RESPONSE:** The standard has been adjust to convey that client instruction should delineate for specimens that will be tested within 8 hours or less, handling and transportation must be in a manner to assist in maintenance of the specimens between 18 and 25 degrees Celsius. For specimens that testing is expected to occur in greater than 8 hr, the instructions should delineate that handling and transportation must be in a manner to assist in maintenance of the specimens between 2 and 8 degrees Celsius.
Proposed Standard | Proposed Guidance
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**Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 2: Specimen Age and Integrity**
Leukemia/lymphoma specimens:
a) shall be tested within 48 hours of collection unless the specimen is deemed irreplaceable and specimen re-collection would be harmful to the patient; and
b) shall be visually inspected for evidence of deterioration or unacceptable conditions upon receipt. Specimens, which are fixed, frozen, warmer than 37 degree Celsius or, in the case of peripheral blood or bone marrow, clotted or hemolyzed, shall be rejected.

Leukemia/Lymphoma specimens should be handled and transported in a manner that maintains optimal condition and expedites testing. Some specimen types and/or malignancies are more fragile and should be tested earlier.

**Comment 1:**
“b) peripheral blood or bone marrow, clotted or hemolyzed, shall be rejected.”— Bone marrow sample maybe difficult to aspirate & may have clots & be hemolyzed. Since these samples are often irreplaceable rejection would be not be an option. Analysis by flow cytometry involves a lyse step so this is not an issue for evaluation.

**RESPONSE:** Clots will not allow characterization of the whole specimen. A RBC lyse product will not break down the clot, therefore the captured cellular constituents will not be release and will not be immunophenotyped. A specimen that has been hemolyzed calls into question the quality of all cellular components of the specimen, including leukocytes, if the erythrocytes have been lysed. Either condition places any result obtained as questionable, the specimen should be rejected.

Bone marrow aspiration, while difficult for the patient, can be redone. The flow cytometry lab should consider the extent of either condition (clot or hemolysis), and how it may have compromised the analysis. If the decision outcome is the specimen is irreplaceable, the results must be reported with full disclosure.
Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 5: Antibody Lot Assessments

For each new lot of immunophenotyping antibody reagent, the laboratory must adjust the expected mean fluorescent intensity (MFI) values for each population analyzed when the MFI changes by more than 20 percent.

Lot checks should be conducted using a normal control blood to make the additional comparison against the laboratory’s determination of expected normal expression levels.

Saturation should be determined by titering the antibody to obtain the best signal-to-noise (S/N) ratio for cellular analysis. When MFI differences are over 20 percent, the antibody reagent should be re-titered to check S/N and investigated if the change is related to the lot’s fluorochrome to protein (F/P) ratio.

Comment 1:
Currently, antibody lot comparisons at our laboratory are performed on normal blood specimens, abnormal specimens, or assayed control material such as Streck CD-Chx CD 117. Acceptable results are % positive + 3 for the same fluorochrome. We utilize the manufacturer recommended volumes of antibodies from Becton Dickson, Beckman Coulter, and Dako. These antibodies are pre-titered and therefore of a known concentration.

Cytometer set-up includes monthly compensation that includes generic and specific antibodies. Therefore, monitoring MFI values for antibody lot comparison and the tittering of antibodies are not needed.

**RESPONSE:** The conjugation process for each new lot may have caused lot-to-lot differences in the fluorochrome to protein ratio (F/P) thereby influencing the individual biomarker mean fluorescent intensity (MFI). Manufacturers titer antibodies individually, and don’t dictate tube panel arrangements or assay procedures. Steric hindrance, pH, and charge may influence the final outcome within each stained tube.
Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 6: Antibody Fluorochrome Stability

Instructions for the protection of staining reagents from light shall be written in the SOP and include room lighting conditions during the staining process and storage of the stained tube until data acquisition on the flow cytometer. When reagents with different stabilities are combined, the shortest stability length shall be used for the combined reagent.

The stability length of each reagent shall be:

a) the manufacturer’s recommendations under the prescribed room lighting condition; or

b) determined by validation studies if not defined by manufacturer or when light conditions do not conform to manufacturer’s recommendations.

The validation shall determine the maximum incubation or storage length under specific lighting conditions. The maximum allowable difference from time zero, within the gated population, is a percent change of plus or minus three and a change in the mean fluorescent intensity (MFI) value of 20% or less using specimens from both normal and abnormal individuals.

Some tandem fluorochrome conjugates have short stability periods (e.g. six hours). The stability length is measured from aliquoting the reagent into the staining tube to data acquisition on the flow cytometer. Fluorochromes are sensitive to photobleaching (room lighting conditions) and/or undergo emission spectra changes by prolong exposure to paraformaldehyde (formaldehyde).

Comment 1:
Validating each antibody vial would be very time consuming and costly.

RESPONSE: If using the manufacturer’s recommendations for antibody use, validation would not be required. If the lab is working in a more lighted condition compared to the manufacturer’s recommendation of dim lighting, especially for fluorochromes susceptible to photobleaching, a comparison between the two conditions should be made, to assure that the lab’s lighted condition has not modified the biomarker expression in both the percentage value (± 3) and the MFI (≤20% change in channel value) compared to use in the recommended lighted condition. Inserts and manufacturer web pages offer guidance for their products use including information on fluorochrome behavior and recommendations for use.
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<td><strong>Cellular Immunology – Malignant Leukocyte Immunophenotyping</strong> Sustaining Standard of Practice 7: Daily Calibration of the Flow Cytometer</td>
<td>The manufacturer’s recommended procedures should be followed.</td>
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<td>On each day of use, after maintenance procedures, and after the resolution of any instrumentation failures, the following checks shall be performed on the flow cytometer and documented:</td>
<td>b) Electronic compensation can be first adjusted with individually fluorescent-labeled beads. Fine tune adjustments should be completed using cells stained with mutually exclusive antibodies brightly labeled with fluorescent dyes.</td>
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<tr>
<td>a) calibration with stable beads labeled with fluorochromes;</td>
<td>c) Each laboratory should establish acceptable separation between fluorescent peaks of different intensities. Monitoring of acceptable differences between beads with different fluorochrome intensities ensures that the laser and photomultiplier tubes (PMT’s) are functioning in a consistent manner.</td>
</tr>
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<td>b) compensation for color spectral overlap for each fluorescent dye that is used for testing;</td>
<td>d) The instrument is monitored using a stable fluorescent bead by either using a fixed voltage and measuring the variability of fluorescent peak channel or adjusting voltages to place the fluorescent bead signal at the same peak channel and recording the voltage variability.</td>
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<td>c) determination of adequate fluorescent resolution so that there is a measurable difference between the autofluorescent/non-specific peak and a dimly positive fluorescent peak for each fluorescent parameter used for testing; and,</td>
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<td>d) standardization to ensure that performance is consistent from day to day.</td>
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**Comment 1:**

“On each day of use ... perform compensation for color spectral overlap of each fluorescent dye that is used for testing.”  

**Comment:** Compensation in this context is determined solely by optical properties of the instrument and these are monitored by routine QC as recommended by the manufacturer. We have established that our instruments are optically stable therefore the same compensation matrix is used for months. The compensation matrix is monitored every day with every sample to assure correct compensation; this verification of optical stability is insufficient per the ICCS recommendations which represent expert consensus and the proposed new CLSI guidelines.

Cytometry Part B, September 2013, page 304: Verification and Monitoring Compensation “Most common practice for laboratories in performing and monitoring compensation are divided into two parts. One is a long procedure using the instrument’s recommended compensation matrix set for the laboratory and the other is a short verification procedure performed more routinely in between the full calibration procedures.... Verification in between the long procedures can be achieved by checking a well characterized specimen on each instrument in the laboratory. In addition to assessing staining characteristics or expected staining patterns, it should be verified that each tube within the panel for this particular sample does not present over-compensation or under-compensation. This is normally a quality control procedure performed for each sample in most laboratories. If no problems are noted, the compensation daily monitoring is performed this way.”

**RESPONSE:** If the lab is following routine QC recommendations outlined by the manufacturer for calibration, the standard components should have been obtained using the instrument’s calibration products and software. The lab may use standard settings but this would require monitoring instrument’s calibration results for drift and/or change that might occur as the flow cytometer’s optics age (progression to laser or PMT failure) or shift in optical alignment. Recommended scheduled preventative maintenance is also critical to monitor and correct. Verification by a stained normal specimen (or stained normal cells within a malignant specimen) should always be used as a final check before proceeding to the requested testing.
Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 8: Flow Cytometer Linearity

On a monthly basis, laboratories performing malignant leukocyte immunophenotyping shall assess and document their flow cytometer(s) for fluorescence linearity.

The assessment of flow cytometers for linearity shall include:

a) linearity at the settings used for clinical measurement using multi-level fluorescent beads. Correlation coefficient analysis of the Mean Fluorescent Intensity (MFI) versus fluorescent molecules per bead must be equal to or greater than 0.98 (1.0 is the ideal);

b) fluorescence sensitivity and resolution at settings used for clinical measurement; and,

c) evaluation of any photomultiplier tubes (PMT) changes.

The intensity of cellular antigens (markers) can assist in assessing the characteristics of the aberrant population(s).

For accurate quantification of any marker by flow cytometry, it is necessary to ensure fluorescence linearity for all fluorochromes routinely used by the laboratory.

b) Monitoring assesses the PMT’s range of measurement related to the marker intensity (antigen density on or in the cell) and the ability to resolve populations of different intensities.

c) Monitoring provides photomultiplier tubes (PMT) performance history and large shifts or fluctuations indicate that maintenance may be required.

Comment 1:
As per the manufacture’s guidelines (Beckman Coulter) the Flow Cytometer (Gallios) does not require routine verification of Linearity as the instrument uses digital signal processing and does not use logarithmic amplifiers.

RESPONSE: We consider any flow cytometer requires a linearity assessment for each PMT used for clinical analysis on a monthly basis. Our justifications for this requirement are outlined in this standard’s guidance.
Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 9: Normal Control Requirements

A freshly prepared whole blood specimen from a healthy donor shall be tested as a normal control at least monthly for malignant leukocyte immunophenotyping and results shall be documented. The normal control shall be used to evaluate:

a) normal staining expression (percentage and intensity) of the biomarkers on all leukocyte populations; and

b) appropriate flow cytometer(s) settings (PMT voltages, color compensation, etc) to achieve optimal resolution of leukocyte subpopulations and biomarker fluorescent quality and resolution.

Cell lines should also be used for the assessment of antibody reagents that are not positive on normal leukocyte populations.

The whole blood normal control for each assay should be collected and stored under conditions as similar as possible to the whole blood test specimen.

The normal control specimen should be used for the lot check process prior to finishing the current reagent lot to evaluate for lot differences in the laboratory derived normal expression definitions.

**Comment 1:**
They are **limited Cell lines** available from companies for the assessment of antibody reagents that are not positive on normal leukocytes.

**RESPONSE 1:** We are not sure what the questions is? Cell line use is a recommendation and may not be available to all testing labs and may not be obtainable for all markers in question. More importantly, a biomarker that is acknowledged to be negative on normal leukocytes should be expressed as negative on the lab’s normal specimens at validation and then for the monthly normal control assessment for marker definitions and lot checks.

**Comment 2:**
We recommend changing subpart a from requiring “all leukocyte populations” to “the leukocyte population being tested”: a) normal staining expression (percentage and intensity) of the biomarkers on all leukocyte populations the leukocyte population being tested; and....

**RESPONSE 2:** Malignant leukocyte immunophenotyping examines aberrant leukocyte populations, therefore this would require the examination of every biomarker used within testing tube panels to be characterize on each normal leukocyte population to define what is normal on each leukocyte population to allow the determination of abnormal aberrant expression.
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<td><strong>Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 10: Positive-Negative Bio-marker Determination</strong></td>
<td>a) Biomarkers within the patient’s testing panel that have concise negative and positive staining patterns may be used to define negative staining in other panel tubes that may contain more diffuse staining when analyzing within the same gated population with antibodies that are isotype-matched unless the antibody of interest is polyclonal. The isotype control antibodies are the negative control to detect non-specific antibody binding.</td>
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<td>To assess biomarker expression demarcation as positive or negative:</td>
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<td>a) negative staining cells within the same gated population shall be used if the antibodies are isotype-matched;</td>
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<td>b) when analyzing cellular antigens of dim fluorescent intensity, isotype control antibodies shall be used to assist the setting of cursors that distinguish negative from positive staining cells. These negative controls must be isotype-matched at similar concentrations and fluorochrome to protein (F/P) ratios as the test antibody.</td>
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<td>Comment 1: According to the Flow Cytometer consensus Isotype controls are not necessary to assess Bio marker determination. Internal control can be acceptable too.</td>
<td><strong>RESPONSE:</strong> The standard and guidance so state.</td>
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<td><strong>Cellular Immunology – Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 14: Result Review</strong></td>
<td>Information used to generate results may include, but is not limited to, raw data, worksheets, instrument readings, and personal observations. Minimally, the flow cytometric print-outs should be reviewed for accuracy in population gating and biomarker analysis.</td>
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<td>Results for each test and information used to generate those results shall be reviewed by an individual holding a certificate of qualification (CQ) in Cellular Immunology – Malignant Leukocyte Immunophenotyping prior to release of test results.</td>
<td>Temporary absence, as defined in Part 58-1 of 10NYCRR, is less than 21 days in duration and includes the inability to access and review the stated above information.</td>
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<td>When a CQ holder is not available:</td>
<td>A qualified hematopathologist is licensed physician who is board certified for this subspecialty by the American Board of Pathology.</td>
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<td>a) a person qualified as a Cellular Immunology-trained supervisor or hematopathologist may review and document results during a temporary absence of the CQ holder;</td>
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<td>b) this process shall be in accordance with a protocol approved by the CQ holder prior to his/her absence;</td>
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<td>c) any report issued without review by the CQ holder must denote that it has not been reviewed by the CQ holder prior to reporting; and</td>
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<td>d) supervisor or hematopathologist reviewed results shall be reviewed by the CQ holder upon his or her return in a timely manner, not to exceed the length of the absence, and this review shall be documented.</td>
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**Comment 1:**
To require a declaimer that the CQ holder has not reviewed is totally impractical and not necessary. Flow Cytometry supervisors are highly skilled and perfectly capable of spotting any issues with gating, etc. and they would not release the report if an uncorrectable problem was detected. If there is an issue, it is ALWAYS brought to my attention before my review. Secondly, to toggle a comment like this electronically would cause serious programing issues.

**RESPONSE:** The laboratory director and designated assistant directors are legally responsible for the testing that is conducted within the laboratory, therefore requiring their review and declaration that the testing is accurate and complete. Flow cytometric analysis of malignancy is highly specialized testing requiring high levels of educational training and experience that has been determined to sufficient by NYS. The requirement for a disclaimer has been removed from the standard.