COMMENTS and RESPONSES to PROPOSED CELLULAR IMMUNOLOGY – NON-MALIGNANT IMMUNOPHENOTYPING STANDARDS

The Proposed Standards in the areas of Cellular Immunology – Non-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 23 comments. Modifications and clarifications to the Cellular Immunology – Non-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.
Cellular Immunology – Non-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) that must be used for each assay offered;
b) the maximum allowable transport time so that the laboratory can test within the required assay time frames;
c) the requirement for specimen collection date and time needs to be documented;
d) blood samples must be maintained between 18 and 25 degrees Celsius;
e) blood specimens that do not meet collection and transport criteria will be rejected; and
f) any other information considered significant for specimen analysis.

b) 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.

e) Blood specimens that do not meet collection and transport criteria for blood cannot be tested and reported with a disclaimer. These specimens are not irreplaceable. If other specimen types are to be tested, refer to the Client Instructions Standard in the Cellular Immunology-Malignant Leukocyte Immunophenotyping standards.

f) For example, transfusion history is needed for glycosylphosphatidylinositol (GPI)-anchored proteins for paroxysmal nocturnal hemoglobinuria (PNH) analysis.

Comment 1:
“The Laboratory shall provide specimen collection and transport instructions...blood samples must be maintained between 18 and 25 degree Celsius.” Comment: This condition is ideal but how do you assure these conditions during transport? Do you propose temperature monitoring for all forms of transportation – including NYS DOH PT samples?

RESPONSE 1: The standard wording has been adjusted to convey that client instruction should delineate specimen handling and transportation in a manner to assist in maintenance of the specimens between 18 and 25 degrees Celsius.

Comment 2:
“f) For example, transfusion history is needed for glycosylphosphatidylinositol (GPI)-anchored proteins for paroxysmal nocturnal hemoglobinuria (PNH) analysis.” Comment: We disagree that transfusion hx should be required or testing cannot be performed. Blood transfusions can possibly affect PNH clone size determination for red blood cell analysis, but would have no significant affect on WBC PNH analysis since few, if any, leukocytes remain in transfused blood. A statement on the reports should be sufficient to bring to the physicians’ attention that prior transfusions can have an effect on PNH results, but lack of provision of transfusion history should not be cause to prevent testing. To do so could compromise patient care.

RESPONSE 2: Standard 33 details the requirements of transfusion history and GPI deficiency reporting. The guidance in question of standard 1 is supportive of standard 33. Standard 33 states that the report must have a disclaimer when the transfusion history is not available. The guidance in standard 1 is an example of additional instructions that should be included in client instructions so that the testing lab can be fully informed to facilitate accurate testing and reporting.
CELLULAR IMMUNOLOGY – NON-MALIGNANT LEUKOCYTE IMMUNOPHENOTYPING SUSTAINING STANDARD OF PRACTICE 2: SPECIMEN AGE FOR WHOLE BLOOD

**Proposed Standard**

For non-malignant leukocyte immunophenotyping in whole blood, laboratories must:

a) process specimens within the manufacturer's recommendations for maximum specimen age;

b) when there are no manufacturer's age requirements or unless other time frames are noted in this document, process specimens from the time of collection within:

   i) 30 hours if using EDTA anticoagulant;

   ii) 48 hours if using ACD or heparin anticoagulant; or

   iii) establish the maximum acceptable age of the specimen by internal validation.

**Proposed Guidance**

The age of the specimen is calculated from the time the specimen is collected to the time the sample is fixed during the staining process, or a non-fixed sample has undergone data acquisition on the flow cytometer.

b) Validation studies must be submitted to NYS as required by the Validation Standards in Part 1 – General Systems. For additional information, see the Test Approval webpage for Cellular Immunology.

b) Viability analysis (CI-NML.S3) will be required if testing beyond the stated timeframes in i) and ii).

**Comment 1:**

"Viability analysis (CI-NML.S3) will be required if testing beyond the stated timeframes in i) and ii).”

**Comment:** What type of viability analysis? 7-AAD, PI staining, etc? Adding these analyses will delay testing and/or prevent multi-color analysis since the reagents used to analyze viability will use fluorescent channels already in use for labeled test reagents.

**RESPONSE:** Viability should be performed on an aliquot of blood before the requested testing starts in order to assess if the specimen is still in a condition to be tested and reported. If the specimen is already old, this would be quality assurance that the specimen is reportable before using valuable reagents on a specimen that may have increase non-specific staining due to cellular death, which also could have altered lymphoid subset proportions.
### Proposed Standard

**Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 5: 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 reagents.

The stability length of each reagent shall be:

a) the manufacturer’s recommendation 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.

i. 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 15% or less using specimens from both normal and abnormal individuals.

### Proposed Guidance

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:

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

i. 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 15% or less using specimens from both normal and abnormal individuals.”

**Comment:** Please clarify the underlined section. It does not make sense. Maximum allowable difference of what? Percent change of plus or minus three of what?

**RESPONSE:** Some fluorochromes undergo photobleaching whereas others are photostable; thus, assay development must take the differences amongst the fluorochromes into consideration. Dim or low light is recommended. Regarding to change in fluorescence over time, under the lighting conditions to be employed the samples should be tested immediately after staining and again after the longest time in which samples may be in the light prior to running on the flow cytometer. The difference should be less than 3 % of the original percentage of positive cells and less than a 15% change in the MFI value. Inserts and manufacturer web pages offer guidance for their products use including information on fluorochrome behavior and recommendations for use.
NEW YORK STATE DEPARTMENT OF HEALTH
CLINICAL LABORATORY EVALUATION PROGRAM

Proposed Standard

Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 6: Daily Calibration of the Flow Cytometer

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:

a) calibration with stable beads labeled with fluorochromes;
b) compensation for color spectral overlap for each fluorescent dye that is used for testing;
c) determination of adequate fluorescent resolution so that there is a measurable difference between the autofluorescence/non-specific peak and a dimly positive fluorescent peak for each fluorescent parameter used for testing; and,
d) standardization to ensure that performance is consistent from day to day.

Proposed Guidance

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.
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.
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.

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 sufficient 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 meet 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 or changes 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 the instrument’s optics. 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.
### Proposed Standard

**Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 10: Normal Reference Range Requirements**

Except for stem cell analysis, the laboratory shall verify or establish reference ranges for each leukocyte immunophenotyping marker.

The laboratory shall have laboratory-derived reference ranges for each assay; which are:

- divided into pediatric, adult, and geriatric ranges and determined using specimens that are within the range of patient ages of the specimens routinely tested by the laboratory;
- determined using a minimum of 25 known healthy donors for each reference range group (pediatric, adult and geriatric)
  - donor demographic records shall include age and sex;
  - these ranges shall be compared to published ranges to verify expected performance
- revised with each assay modification; and,
- included on the patient report along with the implementation date of the current reference range.

### Proposed Guidance

The Cellular Immunology Submission Guidelines for Assay Approval (CLEP webpage under Test Approval) provides guidance for specimen number and requirements. Assay modification and validation requires submission and approval by the Department of Health.

- There are FDA cleared or approved test systems that require the development of reference ranges by the laboratory.
- If normal subjects within the pediatric or geriatric population group are not available for reference range development, peer-reviewed published ranges from prominent, acknowledged sources will be accepted when the testing methodologies are similar or equivalent. Reference ranges should include race whenever possible.
- All reporting requirements (including normal values) noted in Section 58-1.11 of 10 NYCRR are applicable. The source of the ranges must be documented in the SOP and cited on reports.

### Comments

#### Comment 1:

“The laboratory shall verify or establish reference ranges for each assay.” **Comment:** 2008 Guidance stated “Ranges should be updated biannually and monitored for statistical changes.” This statement has been removed from 2014 Guidance. Is it still a requirement?

**RESPONSE 1:** The annual updating for Cellular Immunology testing has been eliminated. Normal reference ranges must be developed at validation and revised, if needed, after assay modification. The Cellular Immunology submission guidelines will be revised after these standards have been formally published.

#### Comment 2:

There are no established reference ranges for PNH cell populations in adult, geriatric, or pediatric patients, therefore, this standard should not apply to testing for this disorder. The values for each population are patient-specific, and vary over time as the disorder progresses. Please confirm that the State of NY agrees with this response.

**RESPONSE 2:** Reference ranges should be representative of the normal healthy population that includes markers defining GPI anchorage. PNH testing examines the deficiency of the expression of GPI anchored antigens or abnormal expression of these biomarkers.

#### Comment 3:

Obtaining appropriate specimens to meet this requirement is difficult with our current patient population. Does NYS have specimens that can be shared or recommended alternate approaches?
Reference range changes are reported on the patient report for a limited time; the wording of this standard implies an indefinite timeframe, please clarify NYS expectations.

**RESPONSE 3:** It is each lab’s responsibility to find age appropriate healthy donors to validate and define normal expression levels and conduct comparisons to published values for the verification of testing correctness during assay validation. After validation, these ranges should be used on the official reporting document as a means to reference normal using the lab’s testing procedure. For the more difficult age groups (pediatric and geriatric), labs are allowed to use published ranges from prominent journals providing testing is the same or similar. The source of the ranges must be cited in the SOP and on official patient reports. Laboratory developed ranges must acknowledge their date of implementation. This date should be maintained during the period of use. After assay modifications, reference ranges and their date of implementation must be revised. This requirement is critical for longitudinal care and tracking to assist interpretations of biological change of the patient vs a non-biological change due to assay modification.
Proposed Standard | Proposed Guidance
---|---
Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 12: Positive-Negative Bio-marker Determination For laboratory developed assays, the laboratory shall:

| a) | use negative staining cells within the same gated population, when using SS/CD45 gating, to determine the demarcation between positive and negative regions for isotype-matched antibodies; or, |
| b) | use isotype control antibodies for setting analysis cursors that distinguish negative from positive staining cells when using Forward Scatter/ Side Scatter gating techniques and/or analyzing cellular antigens of dim fluorescent intensity. These immunophenotyping negative controls (isotype controls) shall be isotype matched antibody at similar concentrations and fluorochrome to protein (F/P) ratios as the test antibody. |

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. The isotype control is the negative control to detect non-specific antibody binding.

Comment 1: “For laboratory developed assays, the laboratory shall: a) use negative staining cells within the same gated population, when using SS/CD45 gating, to determine the demarcation between positive and negative regions for isotype-matched antibodies; or, b) use isotype control antibodies for setting analysis cursors that distinguish negative from positive staining cells when using Forward Scatter/ Side Scatter gating techniques and/or analyzing cellular antigens of dim fluorescent intensity.” Comment: Use of isotype control antibodies in PNH testing have been shown to cause more nonspecific binding than marker-specific reagents in many instances, and one commonly used reagent for PNH testing, Fluorescent Aerolysin is not a monoclonal antibody so there is no isotype control. The better control to use for PNH testing is normal cells incubated in buffer.

RESPONSE: Please refer to the PNH specific standard for analysis requirements. The guidance in the PNH specific standard recommends the use of a control to define non-specific binding as the specimen ages. We have removed “isotype” and replaced with “antibody or non-antibody” to include FLAER use for this testing. As pointed out in the comment, staining granulocytes and monocytes have high background staining qualities (FcR variances) and are also more sensitive to cellular death as the specimen ages, therefore causing increased non-specific staining.
**NEW YORK STATE DEPARTMENT OF HEALTH**  
**CLINICAL LABORATORY EVALUATION PROGRAM**

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<td><strong>Sustaining Standard of Practice 14: Result Review</strong></td>
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<td>Results for each test and information used to generate those results shall be</td>
<td>Information used to generate results may include, but is not limited to, raw</td>
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<td>reviewed by an individual holding a certificate of qualification (CQ) in Cellular</td>
<td>data, worksheets, instrument readings, and personal observations. Minimally, the</td>
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<td>Immunology – Non-Malignant Leukocyte Immunophenotyping prior to reporting.</td>
<td>flow cytometric print-outs should be reviewed for accuracy in population gating</td>
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<td>When a CQ holder is not available:</td>
<td>and marker analysis.</td>
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<td>a) a person qualified as a Cellular Immunology-trained supervisor may review</td>
<td>Temporary absence, as defined in Part 58-1 of 10NYCRR, is less than 21 days</td>
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<td>and report results during a temporary absence of the CQ holder;</td>
<td>in duration and includes the inability to access and review the stated above</td>
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<td>b) the review must be in accordance to a protocol approved by the CQ holder</td>
<td>information.</td>
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<td>prior to his/her absence;</td>
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<td>c) reports shall be issued with a note indicating that it has not been</td>
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<td>reviewed by a person holding a CQ in Cellular Immunology – Non-Malignant</td>
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<td>Leukocyte Immunophenotyping prior to reporting; and</td>
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<td>d) supervisor reviewed results shall be reviewed by the CQ holder upon</td>
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<td>his or her return in a timely manner, not to exceed the length of the</td>
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<td>absence, and this review must be documented.</td>
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**Comment 1:**
To require a disclaimer 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.

**Comment 2:**
Review of all non-malignant immunophenotyping by CQ holder is not needed. Only those that are manually regated are reviewed by CQ holder other results are reviewed by supervisor or tech specialist. This would cause a huge delay in resulting & unnessary work for lab directors.

**Comment 3:**
A qualified cellular immunology trained supervisor level medical technologist (who is deemed qualified by the Laboratory Director and the CQ holder) should be allowed to verify patient results for Non-Malignant Leukocyte Immunophenotyping prior to a timely retrospective review by the CQ holder (without a disclaimer comment). Requiring the CQ holder to review all test results prior to sign out for such tests will unnecessarily delay test result verification (waiting for the CQ holder’s availability). Requiring a statement that it was "not reviewed by the CQ holder" if verified without review, will unnecessarily worry the provider about test validity. It also potentially will result in daily scores of tests (which were reviewed by CQ holder after verification) having a "reviewed by CQ holder" comment added to the report (generating amended/corrected reports). A qualified cellular immunology trained supervisor level medical technologist should be allowed to report these test results without prior CQ holder review (without a disclaimer comment).

**Comment 4:**
A Medical Technologist with skill and experience working in an immunophenotyping laboratory would be expected to have the knowledge to evaluate these results for their quality and should be able to review QC and release these results without requiring direct CQ Holder participation or the proposed comment. These
immunophenotyping results are numeric in character, and although their meaning in clinical context will require
the interpretation of the ordering physician, the results themselves do not require an interpretive statement, in
contrast to the interpretation associated with a Malignant Immunophenotype. If an appropriate SOP is followed,
a routine CD3/CD4/CD8/CD19/CD16+56 lymphocyte typing should fall within the scope of practice of a Medical
Technologist capable of performing high complexity testing. The addition of a note or comment would not add
to the value of the test and would lead to unnecessary concern on the part of the ordering physician.

**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 is highly specialized testing requiring high level of educational training and
experience that has been determined to sufficient by NYS. The requirement for a disclaimer has been removed from the
standard.
Proposed Standard

Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 16: Lymphocyte enumeration- Multi-color CD Subset Definitions

Laboratories shall report lymphoid subsets based on the markers available in the testing sample tube, as follows:

a) T cell subsets shall be assessed for single-positive, double-positive, and double-negative expression of CD4 and CD8 subsets of CD3 lymphocytes (T cells) when using four or more fluorochrome-antibody conjugates:

   - CD3⁺CD4⁺CD8⁻
   - CD3⁺CD4⁺CD8⁺
   - CD3⁺CD4⁻CD8⁺
   - CD3⁺CD4⁻CD8⁻

b) Both the natural killer (NK) cells (CD3⁻/CD56⁺ ± CD16⁺) and the NK-T cells (CD3⁺/CD56⁺ ± CD16⁺) shall be assessed.

Proposed Guidance

Lymphoid subsets have different functional capacities and each subset needs to be reported.

Comment 1:
The automated software does not do this. This means this would have to be a manual gate, negating the advantage of the automated software and greatly increasing workload and turn around times. Secondly, we have NEVER had a clinician ask for quantitation of these very minor double negative/positive subset populations. The same programing issues occurs for the NK and T-NK enumeration.

Comment 2:
“Laboratories shall report lymphoid subsets...report single positive, double positive, and double negative expression for CD4 and CD8 subsets of CD3 T cells. Also report the NKT cells.” Comment: Multitest, the FDA approved testing platform, does not have the capability to report double negative, double positive, and NKT. Please provide clarification, particularly with regard to how the additional subsets are clinically applicable.

Comment 3:
Our flow cytometers utilize Canto Clinical Software and Multiset software for Tcell subset testing. These programs do report CD3+CD4+CD8+ % and absolute values and we can add these values to our patient reports. However, CD3+CD4-CD8- and CD3+/CD56+CD16+ % and absolute values are not available. These determinations require importing data into Diva or Cellquest software and manual calculations to arrive at these values. The added data manipulation introduces the potential for error in results reporting. Therefore, we are not able to report these values. Our BD (Becton Dickson) applications representative was notified regarding these proposed standard and he forwarded this information to BD software engineering.

Comment 4:
In our laboratory service, the near inclusive clinical function for measurement of non-malignant lymphocyte subsets is infectious disease monitoring of HIV infected patients. CD4 lymphocyte testing via flow cytometry is an essential part of current HIV care, and this parameter functions to both clinically stage and to help manage the patient. Typically once a patient is diagnosed and confirmed to have HIV, only CD4 counts are ordered or needed (see: UptoDate, Techniques and interpretation of measurement of the CD4 count in HIV-infected patients, accessed April 22, 2014.) The exact role of both the two subsets proposed – DN and DP T cells
as it relates to clinical HIV monitoring or the underlying immunology is not clear (see: UptoDate, Immunology of HIV-1 infection, accessed April 23, 2014.). These DN T cells have been described in patients infected with HIV, and more commonly in patients with autoimmune diseases but given the multiple function role of these cells, in HIV patients there is no concise guidelines how to use this information in clinical medicine. Higher levels of DN and DP T cells are seen some laboratory animal models systems and in several species of non-human primates. My basic science colleagues ask about T regulatory cell measurements; however, no physician has ever inquired about either DP or DN T cells, much less asked for quantitation of these cells in actual patient specimens. I also consulted with several of my contemporaries who also regularly interface with clinicians in regards to immunology commented to me that they also have no recollection of requests for measurement of DN and DP T-cell lymphocyte subsets. Currently we follow the guidance given in the 2008 standard C137 (see page 180 of the 2008 standard) and offer both a HIV induced immunodeficiency and general immunodeficiency panels. Changing over to the proposed 2014 standard will result in greater monetary costs to the laboratory which would be ethically difficult to pass on to the patient. The current version of the software that we use for assay automation does not allow for the DN T cell subset to be measured. If this rule were to be enacted it would require major changes in how we process these specimens including potentially application of doublet discrimination to resolve the DP population. We would be required to run these samples using a manual method that currently doesn’t have associated automation. Secondly, we would have to manually analyze these specimens using a more complex analysis algorithm unavailable with our semi autonomous computer program that processes the majority of our samples. Currently one technologist spends about 2 hours a day for this one assay and this change would add between 4 to 6 additional hours to our analytical time on a daily basis. Currently because of tight budgetary restrictions that are in place locally, we have between one and two technologists in the laboratory on a daily basis and this additional requirement would add to an already heavy workload. Adding additional laboratory personnel for supplemental measurements that don’t appear to have current direct patient benefit would be a hard sell to our administration. The lack of a concise statement addressing allowance for abbreviated panels given in Cellular Immunology Standards 35-37 from 2008 deeply concerns me. The 2008/20014 cross walk document refers you to a document entitled “Cellular Immunology Validation Submission Guidelines.” Following this link ends at a document entitled “Cellular Immunology Checklist – 02/2011”. The dilemma is that “Cellular Immunology Checklist – 02/2011” is completely silent on this issue of abbreviated panels. Would we be required to only offer complete panels as suggested by the proposed 2014 standards or would we be able to offer abbreviated monitoring panels as given in the 2008 standard? If complete panels were the only way to stay compliant, locally the extra costs for performing a complete panel coupled with the fact that this additional data that is not currently needed for patient care in 2014 would be unacceptable. This oversight needs to be clearly addressed in the final 2014 Standard. In summary, I believe that given all of the uncertainties concerning DN and DP utility, lack of current clinical application and additional increased costs to the laboratory that we will likely not be able to pass on, it is premature to mandate these measurements. Further clarification concerning abbreviated panels is also needed.

**RESPONSE:** Some client and physicians requesting T cell subset analysis may not realize that the CD3\(^+\)CD4\(^+\) population reported by some software combines the CD4\(^+\)CD8\(^-\) T cells (DP T cells) with the CD3\(^+\)CD4\(^+\) (SP) population. Automated software from some manufacturers does provide quadrant values for the requested full subset analysis, but fails to report on the software’s physician report.

Some labs may NEVER have had a clinician request analysis of the DP or CD4-CD8\(^-\) (DN) T cell populations but some have had clinician requests, especially for the DN T cells which relates to autoimmune lymphoproliferative syndrome (ALPS) or other immune-associated health problems. Likewise, NKT cells have been related to health issues and the number of NKT cells is available without using extra reagents.
The FDA ruling is “to identify and determine the percentages and absolute counts of T, B, and natural killer (NK) cells as well as the CD4 and CD8 subpopulations of T cells in peripheral blood.” The CD4^+CD8^- T cell subset is not a CD4^-CD8^- or CD4 CD8^- T cell subset. When the FDA clinical application information discussed CD3^+CD4^+ T cells, it is not clear if they meant SP CD4^+ T cells and/or DP CD4^+ T cells; however, some CD3^-CD4^-CD8^- cells would not possess mature T cell activity. The DN T cell population can be quantified without any extra reagents.

It is unclear why FDA left off analysis of NKT cells since this information is already available and easily obtainable. NKT cells can influence certain disease conditions.

The standard has been modified to allow “specimens tested for CD4 quantification for only the purpose of HIV treatment, do not require the double negative determination.”

Removal of procedural and validation requirements from the laboratory standards document was requested. The submission guidelines will be revised in the near future and will provide detail of these requirements.

Standard 16 states “Laboratories shall report lymphoid subsets based on the markers available in the testing sample tube,” this doesn’t imply that a full panel must be used. The standard only indicates that subsets must be appropriately assessed and defined by the markers contained in each tube analyzed.
Proposed Standard | Proposed Guidance
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**Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 17: Lymphocyte enumeration- Quality Control using CD3 Tube Replicate**
The CD3 replicate shall be monitored when the analysis panel contains more than one stained tube.
a) The CD3 percentage values shall not exceed a difference of 3 between the highest and lowest CD3 value within the patient’s stained panel tubes; and
b) The CD3 absolute values shall not differ by greater than 10 percent among the CD3 absolute values within the patient’s stained panel tubes.

| The use of different fluorochromes or different monoclonal antibodies for CD3 could affect this determination. |
| When CD3 values do not replicate, the laboratory should document that the specimen was repeated and/or restained. |

**Comment 1:**
“Quality control using CD3 tube replicate...The CD3 absolute values shall not differ by greater than 10 percent among the CD3 absolute values within the patient’s stained panel tubes.” **Comment:** We already check consistency by assuring that the CD3 percent values do not exceed a difference of 3 percent between highest and lowest CD3 values. Checking absolute values would not provide useful clinical information specifically when CD3 values are low; the absolute values will differ by more than 10 percent at such levels.

**RESPONSE:** The CD3 replicate for absolute values would be a check of pipetting accuracy specific to each patient specimen. The CD3 percentage replicate only verifies that the lymphocyte region is appropriate for all panel tubes and the specimen within the stained tube panel didn’t undergo any individual tube change during the staining process. Absolute value generation based on bead events is critically dependent of volume accuracy. A large difference would note a probable pipetting error or suspension issues within one or more of the panel tubes for that specimen. Both would lead to inaccurate cell count reporting. We have modified the standard to allow specimens with very low CD3 counts to have a difference of 20 percent or less within the stained specimen panel.
Proposed Standard

Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of 19: Lymphocyte enumeration– Quality Control using T-Sum Determination

The T-sum shall be monitored as follows:

a) for three color analysis: The sum of CD3+CD4+ and CD3+CD8+ must be within 10 percent of the total CD3 mean.

b) for four or more color analysis: The summation of the single positive T cells (CD3+CD4+CD8- and CD3+CD4-CD8+ cells), the double positive T cells (CD3+CD4+CD8+) and the double negative T cells (CD3+CD4+CD8+) shall not exceed a difference of 3 of the total CD3 percentage mean.

Proposed Guidance

If a greater difference is found, the laboratory should repeat the analysis, including restaining, to confirm that no preparation problems occurred.

a) Three color analysis: A greater variance is acceptable in patients with an increased population of CD4+CD8-CD3+ cells (e.g., delta/gamma T cells).

b) As required in these standards (Cellular Immunology Non-Malignant Leukocyte Immunophenotyping Standard 16),

i. T cell subsets need to be assessed for single-positive, double-positive, and double-negative expression of CD4 and CD8 subsets of CD3 lymphocytes.

ii. Double positive (DP) CD4+CD8+ T lymphocytes should not be included in the single positive T cell populations and will affect the T-sum determination if this DP T cell subset has not been resolved.

Comment 1:

Our flow cytometers utilize Canto Clinical Software and Multiset software for T cell subset testing. These programs do report CD3+CD4+CD8+ % and absolute values and we can add these values to our patient reports. However, CD3+CD4-CD8- and CD3+/CD56+CD16+ % and absolute values are not available. These determinations require importing data into Diva or Cellquest software and manual calculations to arrive at these values. The added data manipulation introduces the potential for error in results reporting. Therefore, we are not able to report these values. Our BD (Becton Dickson) applications representative was notified regarding these proposed standard and he forwarded this information to BD software engineering.

Comment 2:

In our laboratory service, the near inclusive clinical function for measurement of non-malignant lymphocyte subsets is infectious disease monitoring of HIV infected patients. CD4 lymphocyte testing via flow cytometry is an essential part of current HIV care, and this parameter functions to both clinically stage and to help manage the patient. Typically once a patient is diagnosed and confirmed to have HIV, only CD4 counts are ordered or needed (see: UptoDate, Techniques and interpretation of measurement of the CD4 count in HIV-infected patients, accessed April 22, 2014.) The exact role of both the two subsets proposed – DN and DP T cells as it relates to clinical HIV monitoring or the underlying immunology is not clear (see: UptoDate, Immunology of HIV-1 infection, accessed April 23, 2014.). These DN T cells have been described in patients infected with HIV, and more commonly in patients with autoimmune diseases but given the multiple function role of these cells, in HIV patients there is no concise guidelines how to use this information in clinical medicine. Higher levels of DN and DP T cells are seen some laboratory animal models systems and in several species of non-human primates. My basic science colleagues ask about T regulatory cell measurements; however, no physician has ever inquired about either DP or DN T cells, much less asked for quantitation of these cells in actual patient specimens. I also consulted with several of my contemporaries who also regularly interface with clinicians in regards to immunology commented to me that they also have no recollection of requests for measurement of DN and DP T-cell lymphocyte subsets. Currently we follow the guidance given in the 2008 standard C137 (see page 180 of the 2008 standard) and offer both a HIV induced immunodeficiency and general immunodeficiency panels. Changing over to the proposed 2014 standard will result in greater monetary costs to the laboratory which would be ethically difficult to pass on to the patient. The current version of the software that we use for assay automation does not allow for the DN T cell subset to be measured. If this rule were to be enacted it would
require major changes in how we process these specimens including potentially application of doublet discrimination to resolve the DP population. We would be required to run these samples using a manual method that currently doesn’t have associated automation. Secondly, we would have to manually analyze these specimens using a more complex analysis algorithm unavailable with our semi autonomous computer program that processes the majority of our samples. Currently one technologist spends about 2 hours a day for this one assay and this change would add between 4 to 6 additional hours to our analytical time on a daily basis. Currently because of tight budgetary restrictions that are in place locally, we have between one and two technologists in the laboratory on a daily basis and this additional requirement would add to an already heavy workload. Adding additional laboratory personnel for supplemental measurements that don’t appear to have current direct patient benefit would be a hard sell to our administration. The lack of a concise statement addressing allowance for abbreviated panels given in Cellular Immunology Standards 35-37 from 2008 deeply concerns me. The 2008/20014 cross walk document refers you to a document entitled “Cellular Immunology Validation Submission Guidelines.” Following this link ends at a document entitled “Cellular Immunology Checklist – 02/2011”. The dilemma is that “Cellular Immunology Checklist – 02/2011” is completely silent on this issue of abbreviated panels. Would we be required to only offer complete panels as suggested by the proposed 2014 standards or would we be able to offer abbreviated monitoring panels as given in the 2008 standard? If complete panels were the only way to stay compliant, locally the extra costs for performing a complete panel coupled with the fact that this additional data that is not currently needed for patient care in 2014 would be unacceptable. This oversight needs to be clearly addressed in the final 2014 Standard. In summary, I believe that given all of the uncertainties concerning DN and DP utility, lack of current clinical application and additional increased costs to the laboratory that we will likely not be able to pass on, it is premature to mandate these measurements. Further clarification concerning abbreviated panels is also needed.

**RESPONSE:** The standard has been modified to allow “specimens tested for CD4 quantification for only the purpose of HIV treatment, do not require the double negative determination.”

Removal of procedural and validation requirements from the laboratory standards document was requested. The submission guidelines will be revised in the near future and will provide detail of these requirements.

Standard 16 states “Laboratories shall report lymphoid subsets based on the markers available in the testing sample tube,” doesn’t imply that a full panel must be used. The standard only dignifies that subsets must be appropriately assessed and defined by the markers contained in each tube analyzed.
Proposed Standard

Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 21: CD34 Stem Cell Enumeration - Specimen Age

For CD34 stem cell enumeration, laboratories shall process specimens within the following age limits:

a) the manufacturer’s recommendations for maximum specimen age cut-offs for the specimen type and the assay testing system used; or
b) for laboratory developed assays,
   i) four hours for peripheral blood and leukapheresis products;
   ii) six hours for bone marrow;
   iii) 48 hours for cord blood;
   iv) one hour for frozen/thawed specimens; or
   v) at an age validated by the laboratory that demonstrates that results at time zero (within zero to four hours of collection) and at the maximum time do not exceed a difference of 3 percent using specimens from both normal and abnormal individuals.

Proposed Guidance

The age of a specimen is the interval from the time of collection to data acquisition. Data acquisition is the process of acquiring event information, including light scatter and fluorescent parameters, from the stained specimen tube on the flow cytometer.

The specimen needs to be handled and transported in a manner that maintains an optimal viable condition and expedites testing.

Comment 1:
“For CD34 stem cell enumeration, process peripheral blood and leukapheresis products within 4 hours.”

Response: The standard has been modified.
**Proposed Standard**

**Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 22: CD34 Stem Cell Enumeration-Apheresis Specimen Requirements**

On apheresis specimens, the laboratory shall quantify the number of cells prior to staining. If a dilution is necessary to be within the test system's target cell range

- a) the dilution buffer shall contain a support protein to reduce or eliminate the “vanishing bead” phenomena, when using single platform methodologies; and,
- b) the dilution factor shall be documented and used for cell count calculations.

**Proposed Guidance**

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| Comment 1: |

In this standard, for specimens that require dilution, “the dilution buffer shall contain a support protein to reduce or eliminate the vanishing bead phenomena, when using single platform methodologies.” We have conducted several studies comparing the use of FACSFlow with and without the addition of protein, and found no significant difference in the results obtained. Furthermore, as a comparison study was performed using 40 stem cell samples. They were tested, undiluted on a hematology analyzer and diluted with FACSFlow, without protein, on the Flow Cytometer. A high correlation was found between the WBC count from the hematology analyzer and the total CD45 count. We don’t believe the omission of protein in the diluent affected the results. We’d be happy to provide this data if necessary. We requests that wording be added to this standard to allow for a laboratory to validate an alternated dilution protocol.

**RESPONSE:** We are not sure how you are using FACSFlow; this product is marketed as the flow cytometer’s sheath fluid. The flow cytometer fluidics operate using laminar flow that involves a sample stream within the sheath fluid stream; these streams have minimal, if any, intermixing, therefore adding a protein to the sheath fluid would be unproductive. This standard is relative to an apheresis specimen that would not contain patient’s serum because of the process by which it was acquired and the use of plastic beads for absolute value generation in the flow cytometer. The reference paper used for this standard was Cytometry 43:154-160 (2001). This paper notes that the specimen staining buffer for apheresis specimens should contain a support protein to diminish the effect of vortexing the plastic tube containing the single platform beads previous to loading on the flow cytometer for analysis. It is surmised, without a support protein in the staining buffer, a static charge develops during the vortexing process that pulls the plastic beads out of suspension toward close proximity with the plastic staining tube. This would lead to absolute value inaccuracies. Your experiment didn’t evaluate the root cause to the problem noted in this reference paper.
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<td>Both specimens (normal and patient) should be collected within four hours of each</td>
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<tr>
<td><strong>Standard of Practice 29: Analysis of GPI anchored proteins for PNH Diagnosis</strong></td>
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<td><strong>– Analysis Requirements</strong></td>
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<td>A normal blood shall be stained and analyzed concurrently with patient specimen</td>
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<td>to define normal expression in the analysis of GPI anchored antigens.</td>
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Comment 1:
“A normal blood shall be stained and analyzed concurrently with patient specimen to define normal expression in the analysis of GPI anchored proteins. Both specimens (normal and patient) should be collected within four hours of each other since antigens are naturally shed post-collection.” Comment: Please provide clarification for this proposed standard. It is nearly impossible to assure a normal specimen is collected within four hours of the patient specimen. ICCS standards do not require concurrent samples. We validated stability of 72 hours.

Comment 2:
“A normal blood shall be stained and analyzed concurrently with patient specimen to define normal expression in the analysis of GPI anchored antigens.

Both specimens (normal and patient) should be collected within four hours of each other since antigens are naturally shed post-collection. The degree of antigen expression determines the severity of the disease. Variable composite phenotype expressions may be present for each cell lineage within an abnormal specimen. Use of isotype controls is also recommended to define non-specific binding as the specimen ages post collection.” Comment: Underlined section: The requirement to collect both the normal and patient samples within 4 hours of each other will require the hospital clients to collect both samples and ship them together. We feel this is an excessive burden to place on the client, requires them to have a group of “normal” volunteers available, and will likely be impractical and increase costs of testing to patients. NY has supplied no data to validate that these samples must be collected within 4 hours of each other. We suggest the normal control sample can be collected at the testing sight, should be stored under appropriate conditions, and the age should be within the acceptable 48 hour time frame.

RESPONSE: The initially used GPI markers (CD55 and CD59) had been noted in many reference papers to shed naturally post collection. This has not been commented upon in later references for the additional GPI markers currently used by many labs, but this assay examines the granulocyte population which does undergo cell death in a more rapid manner than other leukocyte populations. The RBC evaluation could be affected by transfusion or hemolysis, while monocytes are typically very low in number. Therefore the accurate evaluation of granulocytes is critical and their viability will affect this analysis. The normal control blood is the only control specimen available for this assay and is used to determine normal expression levels. Both the control specimen and the patient specimen should be in the same condition which directs the need to have both specimens of similar age post collection.
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| Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 32: Analysis of GPI anchored proteins for PNH Diagnosis - Requirements for High Sensitivity Analysis | High sensitivity analysis for GPI-anchorage (PNH) shall be used for the detection of specimens containing less than 1 to 0.01 percent of events demonstrating anchorage deficiency within a gated population. High sensitivity analysis for GPI-anchorage (PNH) immunophenotyping shall:  
   a) collect a minimum of 250,000 events for each population analyzed;  
   b) use two parameter density plots of both GPI markers to determine the double negative events to define complete deficiency (Type III) and partial deficiency (Type II) to be defined by the events of intermediate brightness for both markers |
|  | Precautions should be taken to reduce cell carryover between analyzed marker tubes during rare event analysis. |

**Comment 1:**  
“Collect a minimum of 250,000 events for each population analyzed.” **Comment:** This high number of events is possible for RBC and Granulocytes but not for Monocytes.

**RESPONSE 1:** *The monocyte guidance from the routine analysis will be repeated in the high sensitivity standard.*

**Comment 2:**  
“High sensitivity analysis for GPI-anchorage (PNH) shall be used for the detection of specimens containing less than 1 to 0.01 percent of events demonstrating anchorage deficiency within a gated population. High sensitivity analysis for GPI anchorage (PNH) immunophenotyping shall:  
   a) collect a minimum of 250,000 events for each population analyzed;”  
**Comment:** This should only be required if after collection of 50,000 or more events, # GPI-negative events are observed (see M. Borowitz, et al. Cytometry Part B 2010;78B:220). “...at 50,000 events this probability becomes >99% (that sensitivity for PNH cells is ≤ 0.01%). Thus, one could screen with lower numbers of cells and reflex to collecting more events only in cases in which one or more putative PNH cells was seen.” The vast majority of samples submitted for PNH testing have <0.01% PNH cells so to collect 250,000 events in all of these cases is in our view excessive, not necessary, and probably not practical for many cases, because many suspected PNH patients are leukopenic, and this prevents the ability to obtain 250,000 events, even after staining the entire buffy coat from a blood sample. In these instances, it should be acceptable to state this fact with a disclaimer that “sufficient total events could not be collected, and repeat testing when the granulocyte count is higher should be considered.” Even in normal subjects it is virtually impossible to obtain 250K monocyte events so we request that this requirement should not apply to that cell population either.

**RESPONSE 2:** *The referenced paragraph in this comment discusses a screening process to determine if more events are required to be collected. In Table 2, from the same identified paper, notes that for high sensitivity analysis of 0.01% requires “at least 250,000 events of specific cell type collected.” Again the monocyte guidance from the routine analysis standard will be repeated in the high sensitivity standard.*
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<td><strong>Cellular Immunology – Non-Malignant Leukocyte Immunophenotyping Sustaining Standard of Practice 33: Analysis of GPI anchored proteins for PNH Diagnosis - Review Criteria and Reporting</strong></td>
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<tr>
<td>The laboratory shall review the patient’s history of any recent blood transfusions when assessing GPI anchored antigens. If laboratory cannot obtain transfusion history, a disclaimer must be on the report.</td>
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<td>The total PNH content per cell population shall be reported while acknowledging complete (type III) and partial (type II) deficiency components.</td>
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<td>Disease confirmation shall require an expression deficiency of at least two different monoclonal antibodies directed against two different GPI-anchored antigens assessed on a minimum of two different cell lineages (e.g., RBC and neutrophils).</td>
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<td>Transfused donor cells will dilute the patient’s blood phenotype composition.</td>
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**Comment 1:**
Reporting and clinical significance of Type II PNH cells is very controversial. The PNH community has determined that reporting of TYPE II cells can actually be confusing to clinicians. We suggest that NY investigate this reporting requirement by contacting various labs and revisit this requirement. A clarification of this standard should be made to include FLAER since some labs will test neutrophils with FLAER, which is a very good reagent for PNH testing, but is not a monoclonal antibody.

**RESPONSE:** The guidelines reference on PNH testing (M. Borowitz, et al. Cytometry Part B 2010; 78B:211-230) contradicts your statement. This reference states “The proportion of abnormal cells (i.e., the size of the PNH clone) in each lineage tested should be reported, including information on Type II cells if present, without overly complex numerical description of individual antibody results.” Another more recent PNH reference (D.R. Sutherland, et al. Cytometry Part B 2012; 82(4):195-208) also notes “However for reporting purposes it is important to include both Type II (when present) and Type III granulocytes and monocytes in the total PNH clone size reported. While the clinical significance of finding Type II WBC phenotypes is unknown at this time, it is recommended that their presence be reported alongside the total clone size as the clinical significance may be established at a later point.”