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Title: Assay Development and Validation of T, B, and NK Lymphocyte Subset Enumeration

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INTRODUCTION

Lymphocyte subset enumeration is one of the most common assays performed in clinical flow cytometry laboratories. The assay is used in monitoring the absolute counts of lymphocyte subsets in human immunodeficiency virus (HIV) infected individuals and other immunodeficiency states, and in the follow-up of certain autoimmune diseases, or for assessing the immune response to immunotherapy. There are a few lymphocyte subset enumeration kits on the market, some of which have been approved by the FDA as in vitro diagnostic (IVD) devices. Although IVD kits may appear to be the best option since their performance characteristics have already been validated, such kits have inherent limitations.

Pros and cons of currently available IVD lymphocyte subset (TBNK) immunophenotyping kits:

1. **Beckman Coulter's** current IVD kit for the FC500 and Navios/Navios EX flow cytometers is based on 4 detectors and was designed for use on older 4- or 5-color instrument platforms (CYTO-STAT tetraCHROME, Product Nos. 6607013 and 6607073). The major limitations of this panel include: a) suboptimal separation of CD56+ NK cells from the CD56-negative cells (largely the result of the omission of CD16 which can aid in a more accurate assessment of the NK cells), b) the inability to modify gating logic or plots, c) the need for two tubes (one for T cells and the other for B and NK cells), and the substantial cost. This IVD kit could be improved by optimizing detector use and voltage settings to achieve better separation between antigen-negative cells and antigen-positive cells and by the addition of CD16. It is worth noting that Beckman's AQUIOS Tetra-2+ Panel (IVD kit developed for the AQUIOS cytometer) does contain CD16 and CD56 together. Other improvements include the addition of other antibodies such as CD20 (which is often ordered as part of this panel), the addition of CD14 (to exclude monocytes and improve the accuracy of the lymphocyte panel), modifying the gating, and combining all of the desired antibodies into a single tube which is possible on any 6+ color cytometer.
2. **Becton Dickinson's Multitest™ 6-color TBNK:** BD's 6-color TBNK IVD assay is designed for BD's FACSLyric™ and BD FACSCanto™ II systems, with the data acquired and analyzed using BD FACSuite™ Clinical application/ BD FACSCanto™ Clinical software v2.4 or later. Similar to Beckman's tetraChrome kit, the IVD Multitest assay prevents the users from modifying the gating hierarchy. It also does not allow the repositioning of the lymph gate before the completion of acquisition. This creates problems when its behind-the-scenes

algorithm fails to gate on the correct population (seen often in “difficult” samples), tripping the stoppage criteria limit by unintentionally collecting 'other' cells. In such cases, the number of true lymphocytes acquired can be well below the required 2,500 events.

It is also worthwhile to note that kit manufacturers state in the kits’ Instruction For Use (i.e., Technical Datasheet) literature that one of the limitations of these kits is “absolute counts are not comparable between laboratories using different manufacturers’ equipment”.

GOALS OF THIS MODULE

As platforms change and more fluorescence detector options become available and clinical practice evolves, the addition of certain markers and adjustment of instrument settings may improve the performance of lymphocyte subset enumeration kits while still taking advantage of the extensive efforts already put into their validation. In addition, issues related to short specimen stability times, limited types of anticoagulants tested, limited number of events acquired, and the locked-down nature of gating logic in IVD devices often *forces* laboratories to create laboratory developed tests (LDT) for lymphocyte subset enumeration to overcome these limitations.

The goal of this module is to provide considerations for alternative options to the above-mentioned IVD kits. However, any change or modification to an existing IVD assay alters the status to an LDT assay. IVD tests are regulated by the FDA and validated by manufacturers prior to approval for marketing. In contrast, an LDT is designed and validated by a single laboratory and several steps should be followed: Designing the assay, writing the Standard Operating procedure (SOP), writing a validation plan, performing the validation based on current validation guidelines (CLSI H62), writing a validation summary report, developing QC monitors, implementing the assay and training the staff performing this new assay. This module provides suggestions and practical examples of how a laboratory might approach this complex undertaking.

GENERAL CONSIDERATION OF ASSAY DEVELOPMENT

A. Additional markers

In flow cytometry analysis, visualization of all events and adequate separation between positive and negative populations are crucial to both accuracy and precision, especially in complex patient samples (e.g., transplant patients with dim CD3 expression on T cells or dim CD19 expression on B cells). Including additional markers can often improve the separation of populations from different cell lineages and enable the identification of more granular subpopulations.

- The inclusion of CD14: There is inherent overlap between monocytes, and to a lesser extent, myeloid precursor cells and the lymphocyte population in the FSC vs. SSC and CD45 vs. SSC plots commonly used to gate on the $CD45^+$ lymphocyte population. Addition of CD14 aids in preventing the inadvertent inclusion of monocytes and myeloid precursor cells in this gate. An example is shown in Figure 1. A CD45 vs. CD14 plot can be used to exclude any $CD14^{dim/+}$ cells. Many of these cells are $CD3^-CD16^+$ and their exclusion improves not only the accuracy of the total CD45+ lymphocyte cell count, but also the $CD3^-$ population, and the NK cell population ($CD3^-CD16^+CD56^+$).

This results in an overall improvement of the Lymphosum QC parameter.

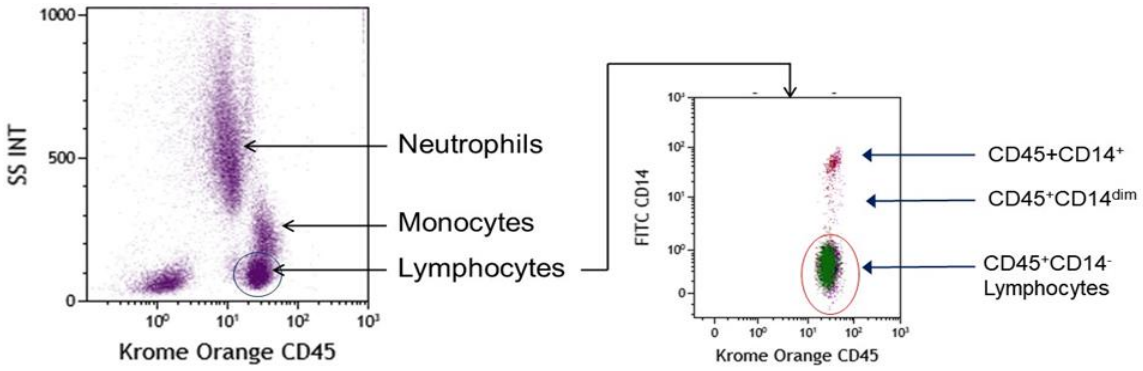


Figure 1: Inclusion of CD14 allows the separation of true lymphocytes (green dots) by refining their gate based on CD45+/CD14- expression. A small, but potentially significant, proportion of CD14+ and CD14dim monocytes (red dots) are excluded.

- Addition of CD16 improves identification of NK cells:

The NK cell population is comprised of 4 main subpopulations which are CD3 negative and have differential expression of both CD56 and CD16 (shown in Figure 2): $CD56^{hi}CD16^{lo/-}$, $CD56^{dim}CD16^{-}$, $CD56^{dim}CD16^{+}$ and $CD56^{-}CD16^{+}$. The $CD56^{dim}CD16^{+}$ and $CD56^{hi}CD16^{lo/-}$ subpopulations respectively account for around 90% and 5-10% of total NK cells in healthy individuals, although other subpopulations have been shown to expand in certain clinical settings. Therefore, the addition of CD16 improves identification of NK cells in two ways. First, the bright CD16 staining makes it easier to differentiate positive and negative populations, as compared to the dim CD56 staining alone which may result in NK cells being included in the negative gate in error. Second, it allows for the inclusion of the $CD56^{-}CD16^{bright}$ subpopulation in the total NK cell count (see Figure 3).

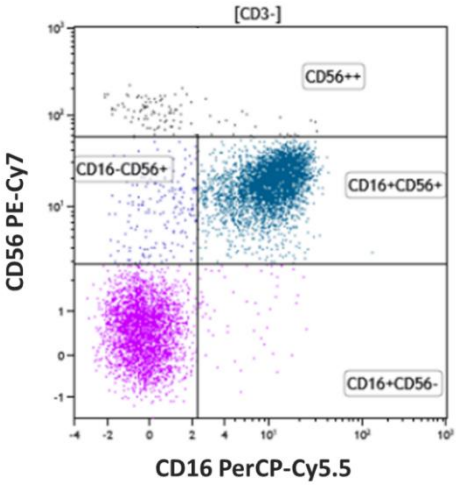


Figure 2: NK cell population subsets in a healthy individual based on CD56 and CD16 expression (gated on CD3 negative lymphocytes). The events in the lower left quadrant (in violet) are the B cells.

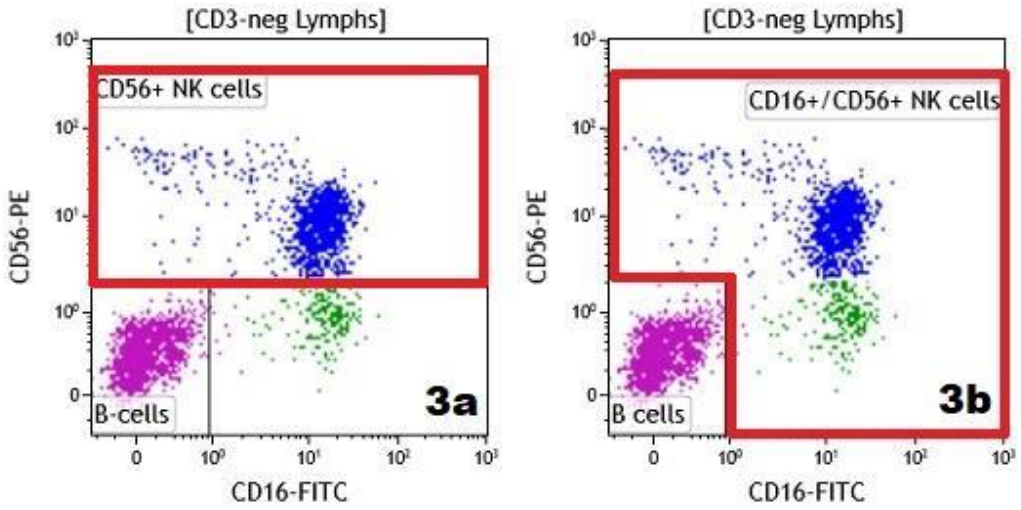


Figure 3: Comparison of NK cell population subsets using CD3 and CD56 only (3a) versus CD3, CD56 and CD16 (3b). The lymphocytes were gated on CD45vsSS first and CD14+ monocytes were excluded, with subsequent gating on CD3-neg lymphocytes (B-cells and NK cells). Figure 3a shows the quantification of NK cells (blue) with the CD56 only gating while Figure 3b shows the more including quantification of NK cells capturing the CD56+/CD16-neg NK cells (blue) as well as the CD56-neg/CD16+ NK cells (green).

B. Panel suggestions:

There are numerous ways to improve TBNK panels. The suggested panels listed below are just some of the options.

Panel Options	FITC	PE	ECD	PC5/ PC5.5	PC7	APC	APC700	APC750	PB	KrO	Application
LDT-1			CD8	beads		CD4	CD14		CD3	CD4 5	CD4/8 only panel
LDT-2	CD16	CD56	CD8	beads	CD19	CD4	CD14	CD20	CD3	CD4 5	Combined T, B, NK and CD14, CD20
Mod-tetra2	CD45	CD16 CD56	CD19	CD3							NK and B cells

Table 1: Options for 10C Navios and Navios EX Beckman Coulter cytometers. LDT-1 and LDT-2 panels are primarily addressed within this module. The Mod-tetra2 is a slight modification of an IVD, FDA-approved assay to help improve the resolution of NK cells when the signal from CD56 alone is weak/dim (e.g., Proficiency Testing samples).

Panel	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-R700	APC-H7	V450	V500C
TBNK	CD8	CD19	CD3	CD16+CD56			(CD14)	CD4	CD45
CD4			CD3				(CD14)	CD4	CD45
CD4/CD8	CD8		CD3				(CD14)	CD4	CD45
CD19/CD20		CD19			CD20		(CD14)		CD45
CD2/CD3			CD3		CD2		(CD14)		CD45

Table 2: Options for a "modular" lymphocyte subset enumeration assay on a 10+ color BD cytometer. Note: (CD14) is shown in parentheses to indicate that this is optional.

C. Acquisition settings:

The lab predetermines the number of events to acquire, which are required to achieve the desired lower limit of quantification (LLoQ) based on the type of specimens the lab is expecting to run and the context of use. For counting beads, a minimum of 1000 events need to be collected. Increasing the stoppage criteria

based on the number of lymphocyte events to 5,000 or 10,000 events will improve the assay's LLoQ and allow the reporting of low cell counts. However, this will also mean longer acquisition times. The assay setup needs to be established and validated in a way that works best for the lab balancing acquisition time constraints for throughput and what is needed for the clinical use (e.g., for patients receiving anti-CD20 therapy, it is important to be able to report accurately at the very low end of B cells).

D. Single platform vs dual platform

In 1996, the CDC published a recommendation article discussing the use of single platform (SP) technology. Based on the data used in that article, which was also presented as an abstract presented at the Clinical Applications of Cytometry 10th Annual Meeting, the authors conclude that the resulting outcomes associated with SP (and CD45 gating) include:

1. increased confidence in results.
2. more reproducible results.
3. increased ability to resolve discrepant problems.
4. decreased proportion of unacceptable specimens received for testing.
5. decreased proportion of specimens requiring reanalysis.
6. fewer incidents that could pose biohazard risks.

While the authors strongly recommend using SP technology to reduce the imprecision, the results from the 2023 FL-C lymphocyte immunophenotyping CAP survey show that 41.6% of participants continue to use dual platform (DP) methodology.

IMPORTANT CONSIDERATIONS FOR SAMPLE PREPARATION

A. Pipetting:

Accurate pipetting is critical when performing cell enumeration. Consensus recommendations is the use of reverse pipetting technique when pipetting the sample and the counting beads. For reverse pipetting, collect the specimen after pushing the pipette's button beyond the first stop. Release the button to draw excess sample into the tip. To dispense, press the button to the first stop to expel a precise volume of sample, leaving excess sample in the tip.

Figure 4: What constitutes a single inversion.

B. Sample mixing:

Thoroughly mixing specimens (and counting beads) prior to pipetting is important to ensure the sample transferred contains a homogenous mix. It is recommended to fully invert the blood specimen by 180 degrees at least 5 times just before pipetting it (see figure 4 which displays what constitutes as one full inversion). The CAP and major control material manufacturers do not recommend shaking the vial or using a mechanical mixer (vortex) - please refer to the technical data sheets. If a vortex is used to mix specimens, it should be gentle to prevent cellular damage from sheer forces.

C. Use of counting beads:

It should be noted that beads need to be mixed carefully to avoid generating an excess of bubbles, which affects absolute counts. For this reason, vigorous vortexing is not recommended. It is important to use accurate pipetting techniques, preferably with an automatic pipettor. Beads need to be added consistently right before acquisition of the sample. Beads (for Beckman Coulter platforms) are typically detected in an empty channel off the blue laser.

VALIDATION EXAMPLE OF A LYMPHOCYTE SUBSET ENUMERATION ASSAY AS A LABORATORY DEVELOPED TEST (LDT)

Guidelines for performing assay validation are available from the CLSI (H42 and H62 Guidelines) and through the ICCS Quality & Standards Modules (https://www.cytometry.org/web/quality.php). While these guidelines are available to use as references, the details of the validation plan are at the discretion of the laboratory's medical director. Listed below are suggestions based on these guidelines as well as examples of our laboratory's validation experiments.

A. Instrument optimization

The cytometer should be optimized using appropriate voltages and compensation to allow for clear separation between antigen-negative and antigen-positive populations (see https://www.cytometry.org/web/modules/module2.pdf for voltage adjustment and https://www.cytometry.org/web/modules/Module%208.pdf for compensation tips).

B. Assay development

Two assays were developed on a 10C Beckman Coulter (BC) Navios EX cytometer: LDT-1, a 9-color panel (which included CD14, CD20, and CD16 and CD56 on separate fluorochromes), and LDT-2, a limited 5-color panel (to focus on CD4 and CD8 cells). In both panels, counting beads were included and were detected in the PC5/PC5.5 channel. The antibodies were titrated for best signal to noise ratio (see https://www.cytometry.org/web/modules/Module%207.pdf) and cocktails were validated (see https://www.cytometry.org/web/modules/Module_16.pdf). The module will describe the assay development and validation of LDT-1 and LDT-2 (Table 1).

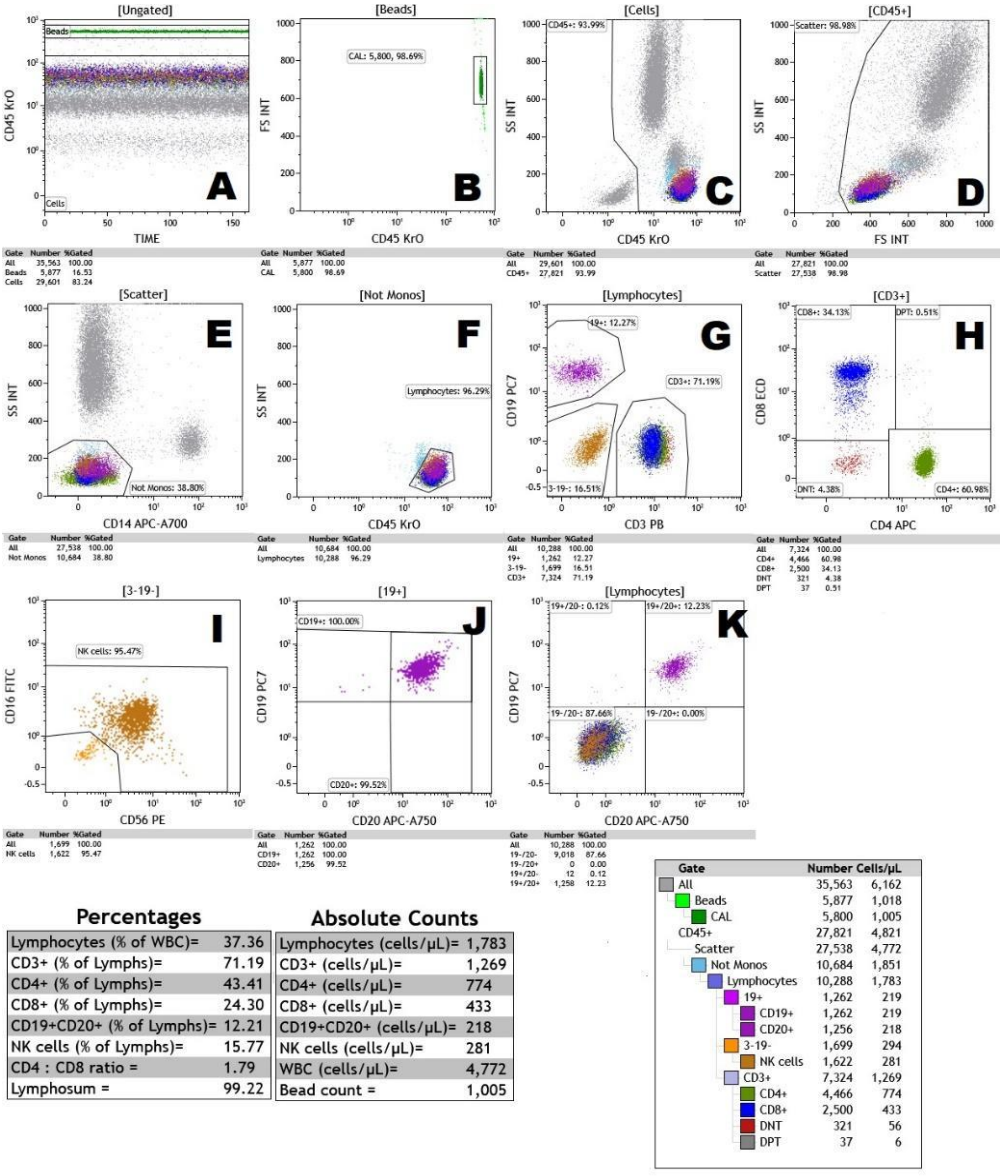
C. Sample Preparation

The laboratory should follow their standard operating procedure (SOP) with a stain-lyse-no wash approach. It is highly recommended to use the single platform method which includes the addition of count beads right before acquisition.

D. Gating strategy

The gating strategy recommended here is only one of many ways to identify lymphocyte subsets cleanly and consistently. However, different panels, analysis software, and preference will inevitably allow for alterations as long as the gating strategy makes biological sense.
The authors have also opted for the use of standard hierarchical gates and not include any Boolean gates. This decision was made to avoid the creation of gates that are built in the software's background and cannot be typically "seen" by the user. Figure 5 shows an example of the gating strategy adopted by the author.

- The initial gate must be set for the beads if counting beads are used. Ideally, this is drawn on a parameter plot that is not used elsewhere in the assay – to avoid catching cells in the beads' gate. The rest of the events in the plot can be gated on as "Cells". "Cells" are then displayed on 45/SSC plot to gate on "CD45+" events and exclude RBCs, platelets, etc. If CD14 is used in the assay, a "non mono" gate can be created to exclude CD14+ events, "non mono" is displayed on a 45/SSC plot again to create a "Lymph" gate on $CD45^{high}$, SSC^{low} lymphocytes.
- Subsequent gates for CD3+ (T cells), CD19+ (B cells), and CD3-CD19- events can be on the same plot or separated plots. CD3+ events are then displayed on CD4/CD8 plot to identify CD4+ (helper), CD8+ (cytotoxic), double-positive (DPT), and double-negative (DNT) T cells.
- NK cells from the CD3-CD19- gate can be refined by gating on $CD16^{+}$ and/or $CD56^{+}$ events.
- CD20 is displayed with CD19 to check the immunophenotype of the CD19+ events.



Percentages		Absolute Counts	
Lymphocytes (% of WBC)=	37.36	Lymphocytes (cells/µL)=	1,783
CD3+ (% of Lymphs)=	71.19	CD3+ (cells/µL)=	1,269
CD4+ (% of Lymphs)=	43.41	CD4+ (cells/µL)=	774
CD8+ (% of Lymphs)=	24.30	CD8+ (cells/µL)=	433
CD19+CD20+ (% of Lymphs)=	12.21	CD19+CD20+ (cells/µL)=	218
NK cells (% of Lymphs)=	15.77	NK cells (cells/µL)=	281
CD4 : CD8 ratio =	1.79	WBC (cells/µL)=	4,772
Lymphosum =	99.22	Bead count =	1,005

Gate	Number	Cells/µL
All	35,563	6,162
Beads	5,877	1,018
CAL	5,800	1,005
CD45+	27,821	4,821
Scatter	27,538	4,772
Not Monos	10,684	1,851
Lymphocytes	10,288	1,783
19+	1,262	219
CD19+	1,262	219
CD20+	1,256	218
3-19-	1,699	294
NK cells	1,622	281
CD3+	7,324	1,269
CD4+	4,466	774
CD8+	2,500	433
DNT	321	56
DPT	37	6

Figure 5: Gating strategy through Kaluza Software. The data in the plots are as follows:

A. CD45vsTIME with a gate for cells vs beads
B. FSvsPC5.5 with gate for beads
C. SSvsCD45 with gate for CD45+ events
D. SSvsFS with Debris exclusion
E. SSvsCD14 with gate for CD14-negative lymphocytes
F. SSvsCD45 with gate for lymphocytes excluding basophils
G. CD19vsCD3 with gates for CD19+ B-cells, CD3+ T-cells and CD3-neg/CD19-neg lymphocytes
H. CD4vsCD8 with regions for CD4+/CD3+ cells, CD8+/CD3+ cells, CD4/8 double-negative and CD4/8 double+ cells
I. CD16vsCD56 with gate for NK cells with are CD56+ and/or CD16+
J. CD19vsCD20 with gate for B-cells
K. Additional plot CD19/CD20 with quadrants, allowing for quantification of other B-cell subsets

E. Dilution Factor

If the specimen requires dilution (e.g., high WBC count, presence of interfering substances), a dilution factor (DF) needs to be entered at acquisition as a keyword in the listmode data (FCS) file. The DF can then be displayed in the report and used in the calculation of dilution-corrected absolute counts, as follows:

$$Absolute\ Counts\ (cells/\mu L) = \frac{(Number\ of\ Cells\ Counted\ \times Count\ Bead\ volume)}{(Number\ of\ Count\ Beads\ Counted\ \times Cell\ volume)} \times Bead\ concentration\ \times DF$$

F. Validation

1. <u>Accuracy (trueness):</u> This can be done through interlaboratory comparison or comparing results to a predicate methodology. If the lab already runs an IVD kit, the accuracy of the new assay can be validated by comparing results with those from the IVD kit. These samples should be composed of materials from both normal donors and patients with a range of abnormal subset percentages (e.g., low CD4/CD3, low B-cells etc.). Statistical analysis should include a comparison of all reported subset percentages as well as the absolute numbers and may be done through R^2 correlation or Bland-Altman Analysis (easier to see bias from mean).

   Example: Our lab decided on a comparison between BC's IVD Tetra 1 and Tetra2 and compared the results of all subsets and absolute numbers to the results obtained with the new panels, using 20 sample with a mixture of normal donors as well as patients with low subsets (especially for CD4 and CD19). We also used beads to determine the absolute numbers, using the single platform approach. The percentages and absolute numbers were compared and a CV of less than 10% was acceptable. An example of the comparison of absolute numbers of CD4 and CD8 (in use assay vs new 9C LDT assay) is shown in Figure 6.

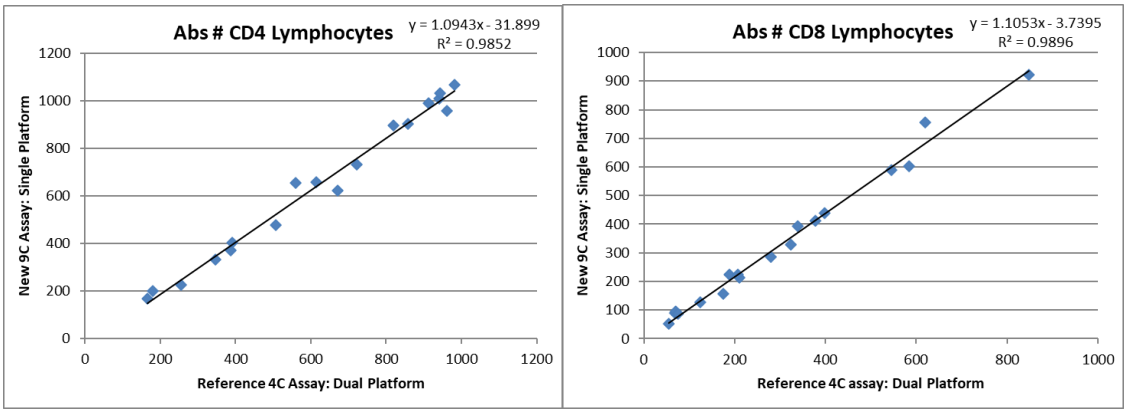


Figure 6: Comparison of absolute numbers of CD4 and CD8 lymphocytes: In use 4C Reference Assay (Dual Platform) versus new 9C LDT Assay (Single Platform)

2. Precision/reproducibility: For the purpose of intra-assay, inter-assay, inter-operator, and inter-instrument precision, a multi-factorial design should be developed to allow multiple factors to be evaluated in a single setting. It is suggested to use 3-6 samples with a mixture of normal and abnormal subsets for these experiments. The percentages and absolute numbers are compared and a CV of less than 10% is typically considered acceptable. Figure 7 shows a multi-factorial design for precision/reproducibility testing based on a lab setup of two cytometers and 4 medical laboratory scientists. The final decision on the design of these experiments is the responsibility of the laboratory medical director.

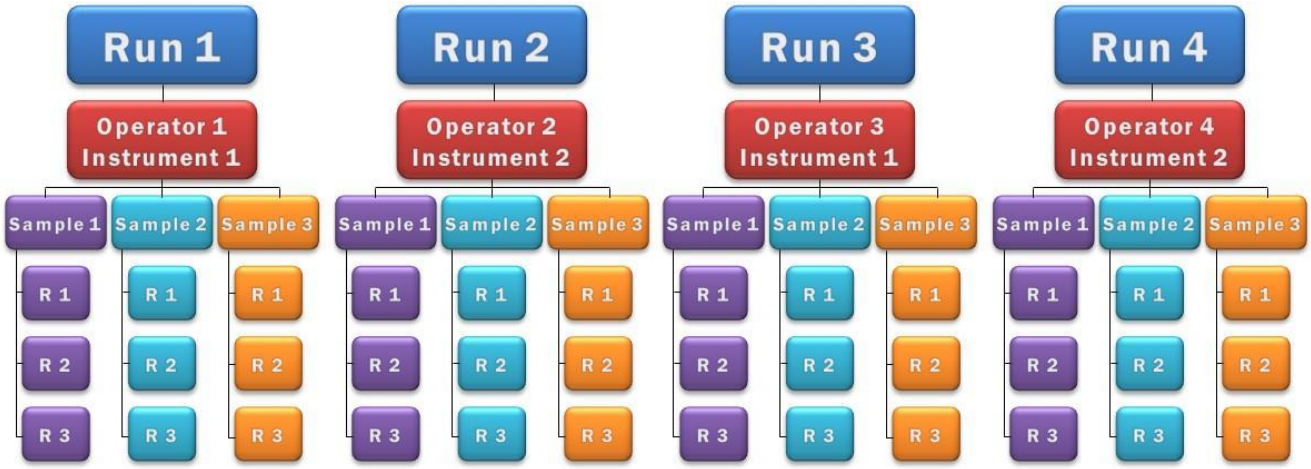


Figure 7: An example of a multi-factorial design for precision/reproducibility testing. The letter R in the distal spokes represent the "replicate" number. A "Run" constitutes the staining and processing of three samples by a single operator, followed by acquiring each sample three times on a single instrument.

Example of data presentation:

- Intra-assay precision: setting up (staining and processing) three samples, acquiring them 3 times on the same cytometer by the same tech, comparing the results and calculating the CV from the replicate acquisitions of samples 1, 2, and 3 within any/all of the runs. The data from each sample is displayed as shown in Table 3.

Replicate	%CD3	Abs. CD3	%CD4	Abs. CD4	%CD8	Abs. CD8	%CD19	Abs. CD19	%CD56+ 16	Abs. CD56+16	%CD20	Abs. CD20
R 1	76.0%	2,922	43.2%	1,641	33.0%	1,253	8.5%	324	15.1%	573	8.5%	324
R 2	76.1%	2,769	42.7%	1,541	33.1%	1,193	8.3%	301	15.3%	551	8.3%	301
R 3	76.2%	2,671	42.8%	1,491	33.4%	1,163	8.9%	309	14.9%	517	8.9%	309
Mean	76.1%	2,787	42.9%	1,558	33.2%	1,203	8.6%	311	15.1%	547	8.6%	311
SD	0.1	103	0.2	62	0.2	37	0.2	10	0.2	23	0.2	10
CV	0.1%	3.7%	0.5%	4.0%	0.5%	3.1%	2.9%	3.1%	1.1%	4.2%	2.9%	3.1%
Pass/Fail	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS

Table 3: Intra-assay precision data. R = replicate. SD = Standard Deviation. CV = Coefficient of Variation (%).

- Inter-assay: comparing the means and calculating the CV from samples 1, 2, and 3 between the four different runs (e.g., Run 1 and Run 2).
- Inter-operator: comparing the means and calculating the CV from samples 1, 2, and 3 between Run 1 and Run 3 and/or Run 2 and Run 4.
- Inter-instrument: comparing the means and calculating the CV from samples 1, 2, and 3 between Run 1 and Run 2 and/or Run 3 and Run 4.

3. Detection capability (analytical sensitivity):
   The strategies and number of replicates used to assess detection capability should be fit-for-purpose based on the intended use of the assay. Serial dilution of replicate samples, with linear regression analysis (R^2) and calculation of CVs within the replicates, is one such strategy that can be used. It is recommended to include samples with high subset percentages (and counts), whenever possible. It is also critical to use a diluent that does not create a matrix effect, such as 5% AB serum in PBS or RPMI without phenol red. PBS with 1% BSA may also be used.
   Example: Analytical Sensitivity for CD19+ B cells
   To determine the analytical sensitivity for quantifying CD19+ B cells, 5 patient samples with a range of B cell concentrations were used. Each was run in triplicate at 10-fold serial dilutions from neat to 100,000-fold. The average, standard deviation, and % CV were calculated for each set of triplicates. Expected results were plotted against the measured results and linear regression analysis performed. Acceptance criteria for % CV of the replicates were ≤ 10 for any values > 10 cells/µL and differences of ≤ 2 cells/µL for values ≤ 10 cells/µL. Acceptance criteria for the linear regression analysis were an $R^2 \geq 0.98$ with a slope (y) of 0.9-1.1.
   Results from a representative sample are shown in Tables 4 and 5. The results indicate that the assay's analytical sensitivity for CD19+ cell reporting is acceptable down to 0 cells/µL.

Dilution Factor	Replicate	CD19+ cells/µL	Average	Standard Deviation	% CV
Neat	1	2,212	2,168	43	2.0
Neat	2	2,126			
Neat	3	2,165			
10	1	182	179	7	3.7
10	2	183			
10	3	171			
100	1	19	19	0.9	5.0
100	2	20			

100	3	18			
1,000	1	2	2	0	0
1,000	2	2			
1,000	3	2			
10,000	1	0	0	0	NA
10,000	2	0			
10,000	3	0			
100,000	1	0	0	0	NA
100,000	2	0			
100,000	3	0			

Table 4: CD19+ cell counts from a 5-fold dilution experiment, performed in triplicate to establish the analytical sensitivity of the assay for CD19+ B cell detection.

Dilution Factor	Measured cells/µL	Expected cells/µL
Neat	2,168	2,168
10	179	217
100	19	22
1,000	2	2
10,000	0	0
100,000	0	0

Table 5: CD19+ cell counts measured cells/µL (average of triplicate) and expected cells/µL (calculated from neat) from a 5-fold dilution experiment, to establish the analytical sensitivity of the assay for CD19+ B cell detection.

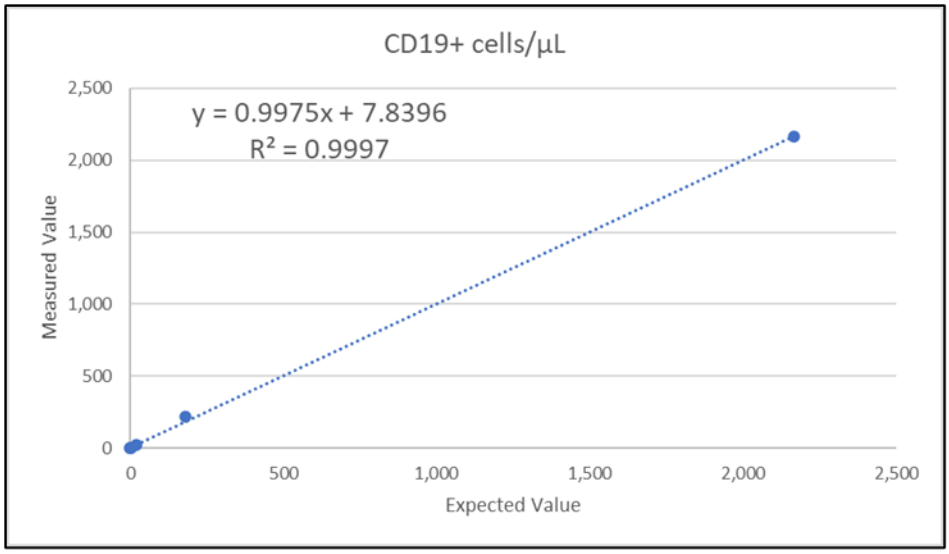


Figure 8: Scatterplot showing the measured CD19+ cell counts in cells/µL (average of triplicate) and expected cells/µL (calculated from neat) from a 5-fold dilution experiment.

4. Selectivity (analytical specificity): This often includes antibodies which the lab has already validated (usually within other panels) but the performance within the new panel configuration needs to be verified by comparing the staining pattern (Figure 9) as well as the subset percentages and absolute numbers to the predicate method/IVD panel (Table 6). It is also important to re-titrate the antibodies used if the original titration was

performed on cells that are prepared using a stain-lyse-wash protocol if the TBNK assay is to be run as a stain-lyse-no-wash protocol. Comparing the patterns of staining to what has been published in the literature is helpful in establishing selectivity.

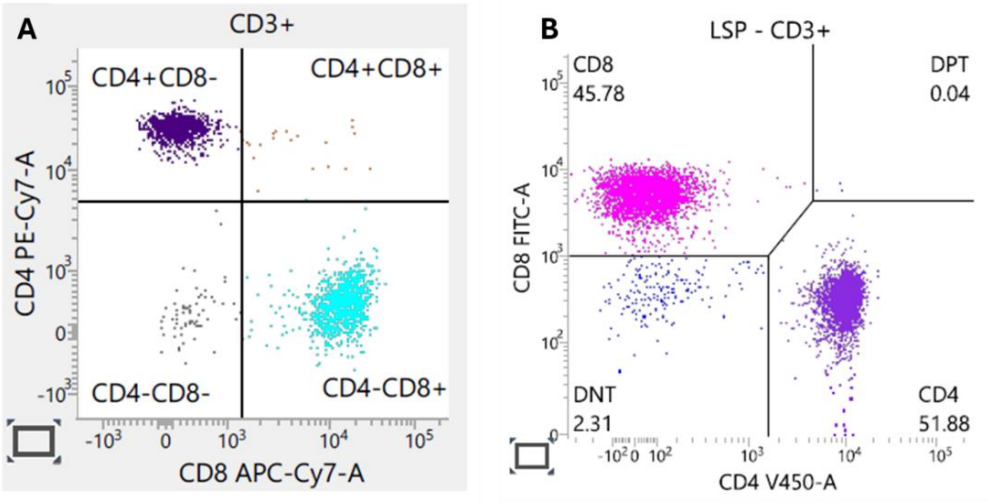


Figure 9: Establishing specificity - Comparison of the staining patterns of CD4 and CD8 antibodies from the IVD assay (A) with those obtained from the antibodies used in the LDT assay (B). *Please note that the axes are transposed.*

A		**IVD**	**LDT**	**CV%**
CD4 %	Sample # 1	23.24	23.53	0.62%
	Sample # 2	6.17	5.5	5.74%
	Sample # 3	14.94	16.92	6.21%
CD8%	Sample # 1	26.81	29.2	4.27%
	Sample # 2	85.18	83.81	0.81%
	Sample # 3	44.09	42.27	2.11%

B		**IVD**	**LDT**	**CV%**
CD4 (cell/µL)	Case # 1	195	164.1	8.60%
	Sample # 2	55	51.2	3.58%
	Sample # 3	64	71.2	5.33%
CD8% (cell/µL)	Sample # 1	225	203.7	4.97%
	Sample # 2	762	779.7	1.15%
	Sample # 3	190	177.7	3.35%

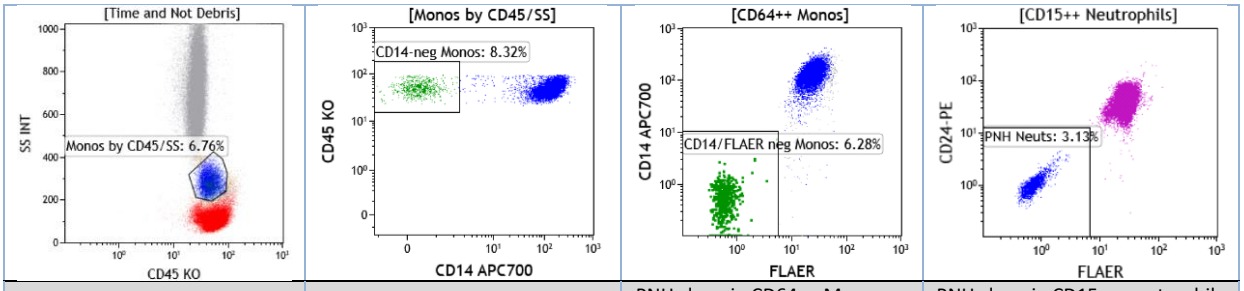
Table 6: Establishing specificity - Comparison of the percentages (A) and absolute counts (B) of CD4 and CD8 T cells obtained from the IVD assay with those obtained from the LDT assay from three samples. Coefficient of Variation (CV) percentages are also shown.

5. Sample and Reagent Stability: Sample stability should be validated (example below shows stability over 72 hours) and stability of the new reagent cocktails must also be validated (see https://www.cytometry.org/web/modules/Module_16.pdf). 5 donors with a mixture of normal and low absolute numbers were assessed on day 0 (draw date), day 1, day 2 and day 3 to compare percentages and absolute numbers.

6. Reference ranges: Establish new reference ranges and/or verify current ranges. Refer to CLSI EP28 document. For pediatric reference ranges, the study by Shearer et al, determined the lymphocyte subsets in a cross-sectional study of 807 children in the USA from birth through 18 years of age.

The advantages of this panel were:

1. Significant cost savings are made. Purchasing individual antibodies, rather than premade cocktails, is almost always more cost effective. Titrating antibodies to optimal stain index usually equates to more tests per vial thus reducing costs even further.
2. Making use of all detectors of the 10C Navios allowed more antibodies to be analyzed in the same tube - redundancy was kept to a minimum.
3. Addition of CD14 to improve the purity of lymphocyte gate.
4. Addition of CD16 for better identification and separation of NK cells from other subsets.
5. Inclusion of beads allowed for the single platform approach with absolute numbers of lymphocyte subsets.
6. The limited panel with a focus on CD4/CD8 uses the same antibodies as used in the 9C panel.
7. Addition of CD20 to allow for identification of CD20-neg/CD19+ cells or other subsets.
8. The combination of CD14 and CD16 may help in identifying the various subtypes of monocytes: Classical monocytes ($CD14^{+}CD16^{-}$) give rise to non-classical monocytes ($CD14^{dim}CD16^{+}$) through an intermediate step of $CD14^{+}CD16^{+}$ monocytes.
9. The use of the GPI-linked antibodies CD16 and especially CD14 allowed for possible identification of CD16-negative cells (eosinophils and/or PNH cells as well as CD14-negative monocytes. These findings would not be diagnostic by maybe suggestive of PNH and reflex testing for PNH may be helpful (see figure 10 below).



Monos gated on CD45/SS	CD14-neg population	PNH clone in CD64++ Monos based on PNH Panel	PNH clone in CD15++ neutrophils based on PNH Panel

Figure 10: Detection of PNH clone: Left 2 plots show CD14-negatve cluster based on the TBNK panel, right 2 plots show reflex PNH panel confirming the presence of a PNH clone in the WBCs and the RBCs

QUALITY CONTROL

- **Controls:** Current regulatory requirements from both CAP and the NYS DOH state that laboratories must use at least two different levels of positive controls for T, B, and NK lymphocyte subset enumeration assays, one of which is at or near a clinical decision-point. Historically, a control with a low CD4+ lymphocyte count of approximately 200 cells/µL has been used to fulfill the clinical decision level requirement and is the standard practice. There are multiple commercially available two-level control sets that meet these requirements. Control ranges must be either established or verified prior to use and procedures defined on corrective action to take if acceptability criteria are not met.
- **Quality assurance/Quality Monitors**: This should include standard QC for antibodies and cocktails, establishing tolerability (acceptability) criteria for reporting the results, competency assessment of all MLS performing the assay, and appropriate documentation and oversight.
    - **Tolerability criteria:** Examples include:
        - Not exceeding the limits of acceptability of lymphosum and %T-sum (see "Reporting Results")
        - Acquiring sufficient number of counting bead events (> 1,000)
        - Acquiring sufficient number of lymphocyte events (> 2,500)
        - Ability to objectively gate on lymphocytes and subsets
        - No significant discrepancy between the lymphocyte count obtained by the hematology analyzer and the single platform flow cytometric assay
    - If these laboratory-established tolerability criteria are not met, the standard operating procedure (SOP) should provide guidance on how to proceed and how to report the results. The lab can also establish criteria for requesting a pathologist to review unusual data (e.g., suspicion of blasts, suspicion of monoclonal B lymphocytosis, etc.) which allows the pathologist to request leukemia/lymphoma test added on.

REPORTING RESULTS

Results are displayed in the report as positive cells as a percentage of lymphocytes. If counting beads are used, or if hematology results are provided from another instrument or method, the report should also display the number of positive cells per microliter of blood (absolute count). An example of a lymphocyte subset enumeration report is shown in Table 7.

Typical QC messages that are added to the report include:

1. The calculated "lymphosum" (total lymphocyte subset percentage) which is derived from the addition of % CD3+ (T) lymphocytes + % CD19+ (B) lymphocytes + % CD56+/16+ (NK) lymphocytes. It is used as a "gate purity" quality control check for a specimen in terms of total lymphocyte percentage determined, which should be 95-105%. If lymphosum value is <95%, the results should be reviewed for proper gate placement. If this does not resolve the issue, prepared samples should be re-acquired and/or new samples should be prepared and analyzed.

2. The % T-sum, which is typically calculated as the summation of the single positive T cells (CD3+CD4+CD8- and CD3+CD4-CD8+ cells). The % T-sum value should equal the total percentage of CD3+ cells ±5%. However, this metric does not take into account the double negative (DNT) and double positive (DPT) T cells. The NYS DOH defines the % T-sum as the summation of the single positive T cells, plus DNT and DPT. It considers a difference of >3 of the total CD3 percentage mean as unacceptable. The laboratory should repeat the analysis, including re-staining, to confirm that no preparation problems occurred.

The reporting of low cell counts is based on the performance characteristics determined during assay development and validation. Values below the assay's LLOQ should be reported as "less than…". Optional "flags" may be built into the LIS system for pathologist review for reflex testing or the addition of interpretive comments.

Reference ranges (established or verified) should be included in the report. Age-specific refence ranges are very important in lymphocyte subset enumeration assays.

		Reference range
Total WBC	Cells/µL	
Lymphocytes	%	
Total lymphocyte count	Cells/µL	
CD3+ T cells	%	
	Cells/µL	
CD4+/CD3+ cells	%	
	Cells/µL	
CD8+/CD3+ cells	%	
	Cells/µL	
CD19+ B cells	%	
	Cells/µL	
CD56+/CD16+ NK cells	%	
	Cells/µL	

CD4/CD8 ratio =

QC messages:

Lymphosum = PASS/FAIL

T-sum % = PASS/FAIL

Comments:

Table 7: An example report for lymphocyte subset enumeration. Please note that if the WBC count, lymphocyte percentage, and count are derived from the single-platform flow cytometric assay, validation of these values must be performed in the same manner as all other reportable values.

IMPLEMENTATION

Upon completion of validation, there are several steps required prior to implementation of the assay in the laboratory which are discussed in Chapter 7 of the CLSI H62 Guidelines. The SOP should be updated to include any additional information determined during the validation, such as lower and upper limits for reporting or maximum allowable dilutions. QA and QC procedures specific to 1) assay performance and reporting of results, 2) monitoring of the environment, instrument, reagents, and assay, and 3) training and competency of performing employees should also be finalized. Training materials should be created. These typically include gating exercises, case studies, and troubleshooting examples. Once all associated SOPs have been locked down and training materials prepared, technologist training and competency assessment must then be performed and documented.

SUMMARY

The validation of a laboratory developed test (LDT) is a complex undertaking and should be performed based on current guidelines and collaboration between the laboratory staff and the Medical Director's input. The validation plan should include a reason for developing the assay (e.g., addition of CD14 and CD16 to improve the accuracy of lymphocyte subsets, or other improvement over the current assay) and should focus on the assay-specific components of the new assay. The (semi)- quantitative TBNK assay example shown in this module includes general validation components such as accuracy, precision, detection capability, selectivity, stability, and reference ranges, but also focuses on the more assay-specific aspects of lymphocyte subset enumeration including potential issues affecting the performance of the assay (e.g., using counting beads for the single-platform approach). Careful design of the validation experiments based on the context of use, in a fit-for-purpose manner, as well as implementing quality control monitors, are essential in order to address all aspects of its performance over the life of the assay.

REFERENCES

1. CLSI. Validation of Assays Performed by Flow Cytometry. 1st ed. CLSI guideline H62. Clinical and Laboratory Standards Institute; 2021.
2. CLSI. Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition. CLSI document EP28-A3c. Wayne, PA: Clinical and Laboratory Standards Institute; 2008
3. ICCS Q&S Modules, Glenview, IL. https://www.cytometry.org/web/quality.php
4. Poli A, Michel T, Thérésine M, Andrès E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology. 2009 Apr;126(4):458-65. doi: 10.1111/j.1365-2567.2008.03027.x. PMID: 19278419; PMCID: PMC2673358.
5. Guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus infection. MMWR. Recommendations and reports: Morbidity and mortality weekly report. Recommendations and reports vol. 41,RR-8 (1992): 1-17.
6. Rosner ER, Siragusa MT. Laboratory standardization in CD4 testing: results of a nationwide impact evaluation study [Abstract A1]. Clinical Applications of Cytometry 10th Annual Meeting, August 1995. Cytometry (Communications in Clinical Cytometry) 1996;26:78--97
7. CAP Master Flow Cytometry Checklist – accessed 08/24/2023.

8. Shearer WT, Rosenblatt HM, Gelman RS, et. al.; Pediatric AIDS Clinical Trials Group. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003 Nov;112(5):973-80.
9. NYS DOH Laboratory Standards: Cellular Immunology and Cytokines 08/01/2020

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