Flow Cytometry User's Guide

Updated September 6, 2021

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Version 03.27.2020 – Updates to Flow User's Guide:

- **Page 18:** Edited Flow regating software minimum requirements to Window Vista or higher. Updated NeoGenomics' FCS Express version to 6.0

Version 06.24.2020 – Updates to Flow User's Guide:

- **Page 17:** Updated the markers for the MRD panels

Version 07.08.2020 – Updates to Flow User's Guide:

- **Page 16:** Updated the Automatic Add-on Criteria, specifically for AML

Version 08.05.2020 – Updates to Flow User's Guide:

- **Page 4, 6-10, 12:** Updated gating images

Version 08.18.2020 – Updates to Flow User's Guide:

- **Page 11-12:** Updated the info under Monocytes and added Figure 10 for examples of gating images.

Version 11.10.2020 – Updates to Flow User's Guide:

- **Page 16:** Removed biomarkers CD28 and CD200 from Myeloma MRD Panel

Version 02.25.2021 – Updates to Flow User's Guide:

- **Page 17:** Updated automatic add-on criteria for B-cell tubes

Version 09.06.2021 – Updates to Flow User's Guide:

- **Page 15-16:** Updated the AML Add-on tube's markers

NeoGenomics Flow Medical Guidance

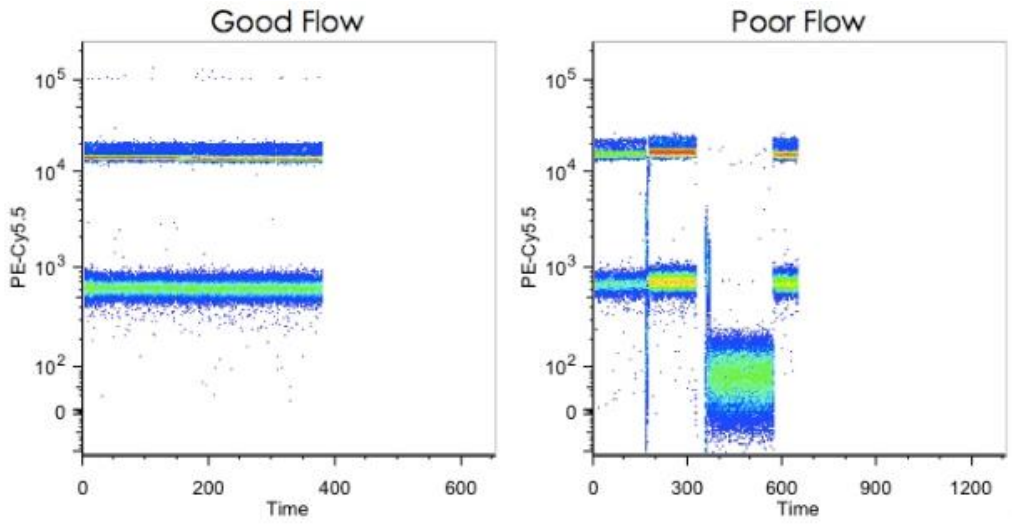
Flow cytometry is a method to evaluate the expression of surface and cytoplasmic antigens on individual cells using fluorescently labeled antibodies. NeoGenomics Laboratories uses a 10-color antibody panel for leukemia/lymphoma flow cytometry testing. 10-color analysis maximizes the amount of information that can be obtained from short samples as well as increasing the combinations of markers that can be evaluated on every case.

NeoGenomics Gating Strategy

In order to ensure the highest quality flow cytometry data, NeoGenomics performs a multi-fold gating strategy to reduce artifacts. This involves gating on time, single cells, and forward versus side scatter prior to looking at antibody staining patterns.

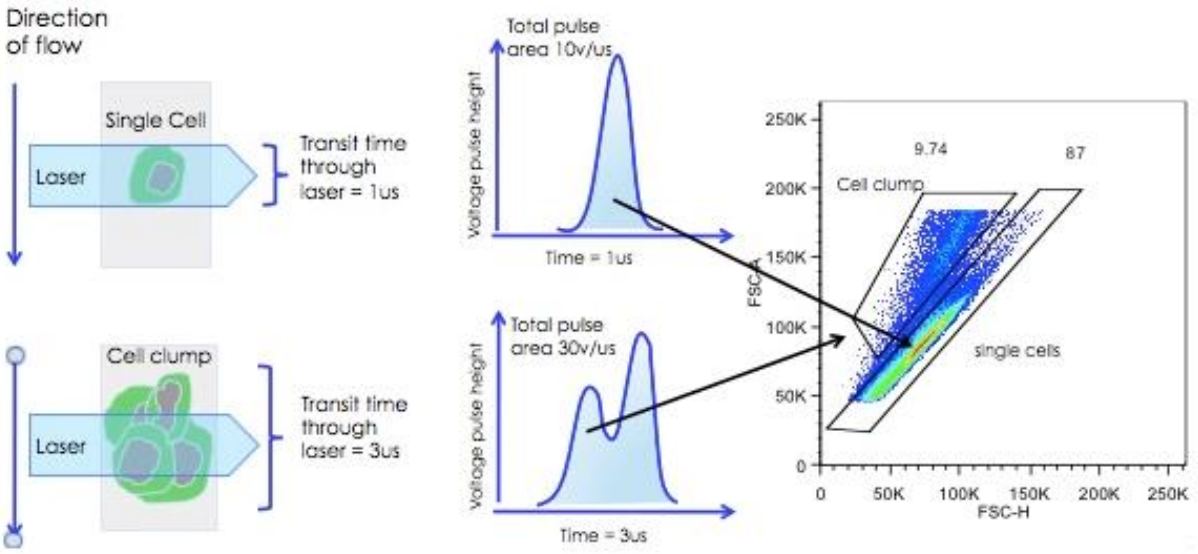
Time/Stable Flow Gate is a plot of time vs a scatter plot to determine how even the flow was during the run (**Figure 1**). Areas where there was poor flow can be excluded from areas of good flow by time gating, which will ensure a higher quality of data. Causes of poor flow include clogs, back pressure, air bubbles and tubes that run dry.

Figure 1: Time gate



Proper flow cytometry data analysis requires single cells (singlets) (**Figure 2**). When cell clumps pass through the laser intercept, they will take longer than single cells. This in turn affects the area of the signal. Using a pulse geometry gate (such as FSC-H x FSC-A), doublets can be easily eliminated. This reduces the possibility of false co-expression of antigens because two cells passed through the flow cytometer at the same time.

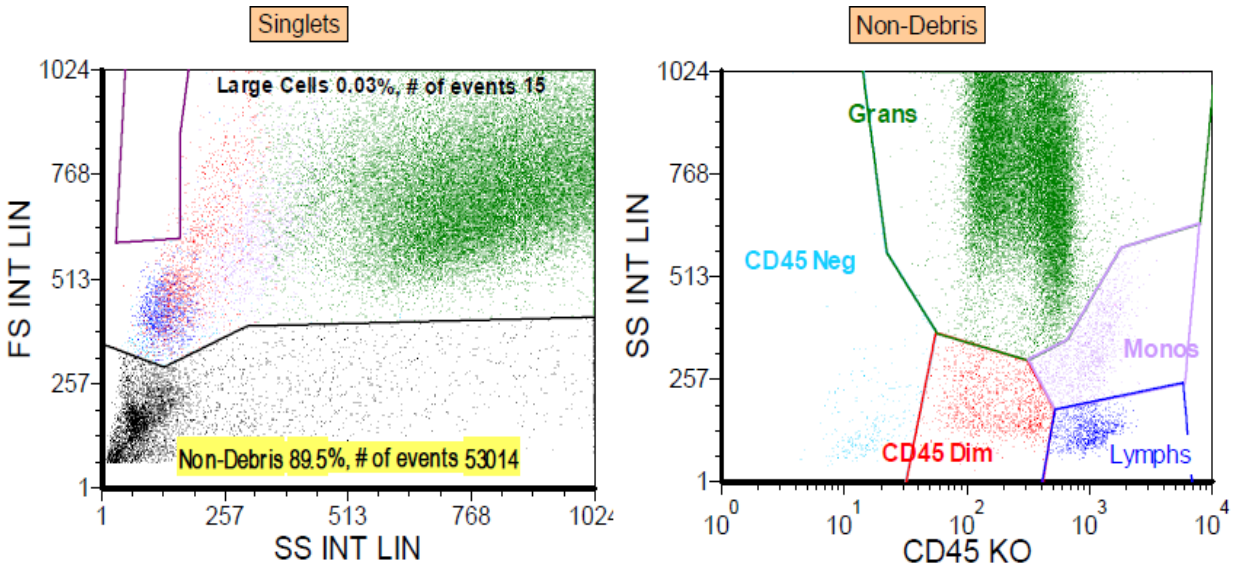
Figure 2: Singlet gate



Forward (FS) is a measure of cell size and side scatter (SS) is a measure of cell granularity. Degenerating cells and debris have lower forward and side scatter than viable cells. Therefore gating on forward versus side scatter allows debris to be removed from analysis.

Most of the flow cytometry data analysis is based on linear side scatter (SS) vs. CD45 gating. We have CD45 in every tube, thereby making back gating much more informative. All gates have their own particular color, making them easy to follow throughout the analysis **(Figure 3)**. In some plots, total CD19 or CD3 gating is applied.

Figure 3: Gating strategy



Population analysis: Population analysis is the analysis of combined immunophenotypic and optical properties of each population of lymphocytes, monocytes, CD45-dim cells, CD45-neg cells and granulocytes as well as other cellular populations, if necessary.

Gates

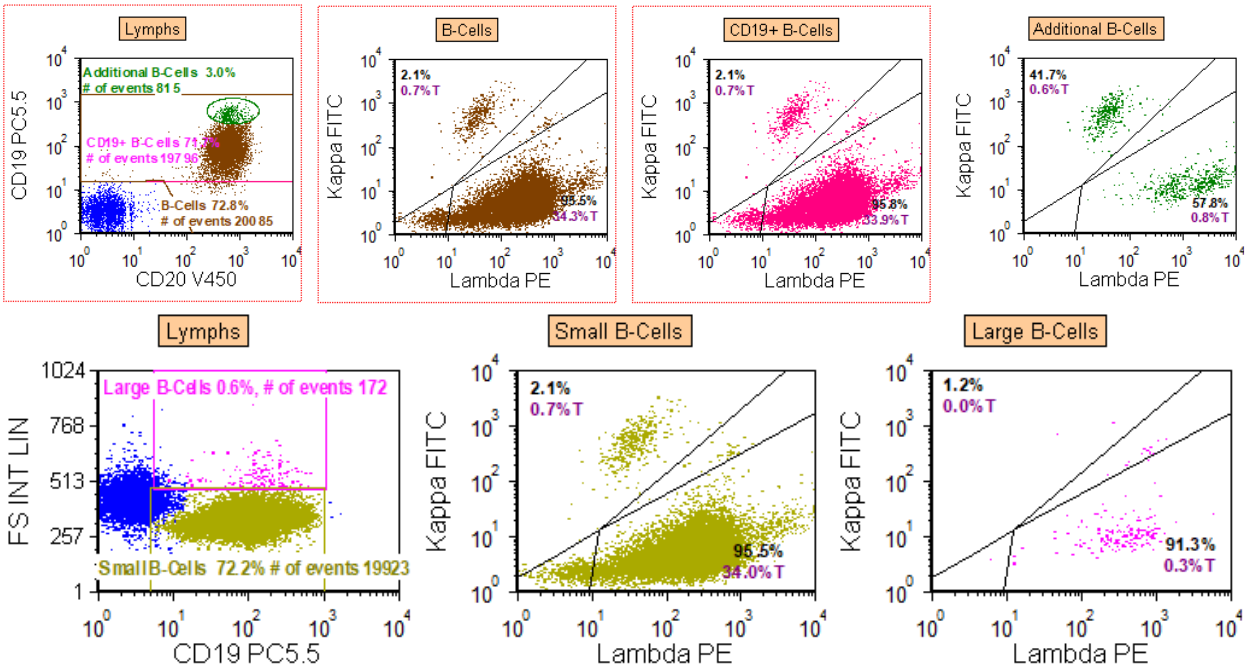
1. **Lymphocytes:** These events have a very intense CD45 expression and the least amount of SS. They are depicted in dark blue.
2. Monocytes: These events have an intense CD45 expression and slightly more SS than Lymphocytes. They are depicted in lavender.
3. **CD45 Dim** (Blast pocket): These events have a dim CD45 expression and varying SS properties. These events include normal myeloid & lymphoid precursors and basophils as well as blasts. They are depicted in red.
4. CD45 Neg: These events have a dim to negative CD45 expression and varying SS properties. These events include erythroid precursors, cell debris and few non-hematopoietic cells in normal samples as well as abnormal plasma cells, lymphoid blasts, and metastatic tumor cells in abnormal samples. They are depicted in light blue.
5. **Granulocytes**: These events have intermediate CD45 expression and the greatest SS of all events. They are depicted in green.
6. **Mononuclear**: The mononuclear gate consists of lymphocytes, monocytes, CD45 -dim and CD45-negative events. The benefits of the mononuclear gate include: assessing true size of abnormal populations that are not confined to a single region based on CD45 versus side scatter gating and the ability to use internal negative control populations for assessing true dim staining versus increased background staining. In addition, the mononuclear gate also allows simultaneously assessment of multiple populations at once due to display of color back-gates for each CD45 versus side scatter region.
7. **Plasma cells**: The plasma cells have variable CD45/SS properties and therefore cannot be easily detected by the CD45/SS gating strategy; therefore CD45/CD38 gating strategy is used. CD45-positive plasma cells are depicted in orange and CD45-negative plasma cells are depicted in green. Initial screening with CD38/CD56/CD19/CD45 in the standard panel allows for detection of increased or abnormal plasma cells. An add-on tube is available to assess clonality and includes CD138 and cytoplasmic kappa and lambda. Normal plasma cells are usually positive for CD19 and CD45, and negative for CD56, while abnormal plasma cells are often positive for CD56 and negative for CD19 and CD45. Plasma cells express bright CD38 and variable CD138 with rare exceptions. Note: Myeloma patients who have been treated with anti-CD38 monoclonal antibody therapy may not have detectable CD38 expression by flow cytometry, but these cells usually retain CD138 expression.

B-cell Gating

Several different gating strategies are employed to evaluate B-cells **(Figure 4)**.

1. B-cells gate: Highlighted in brown, based on the lymphocyte gate, encompasses lymphocytes expressing CD19 and/or CD20.
2. CD19-positive B-cells gate: Highlighted in pink, based on the B-cells gate, encompasses all B-cells expressing CD19.
3. Additional B-cells gate: Highlighted in green, based on the B-cells gate, used to highlight secondary B-cell populations falling within the B-cell gate.
4. CD5-positive B-cells gate: Highlighted in orange, based on the lymphocyte gate, encompasses lymphocytes co-expressing CD5 and CD19.
5. CD10-positive B-cells gate: Highlighted in dark purple, based on the lymphocyte gate, encompasses lymphocytes co-expressing CD10 and CD19.
6. CD11c-positive B-cells gate: Highlighted in pink, based on the mononuclear gate, encompasses all mononuclear events co-expressing CD11c and CD19.
7. CD10/CD19-positive Gate: Highlighted in red, based on the mononuclear gate, encompasses all mononuclear events co-expressing CD10 and CD19.
8. CD19-positive cells are gated based on cell size (forward scatter) and the kappa/lambda ratio for small and large CD19-positive cells is displayed.

Figure 4: B-cell gating strategies



T-cell Gating

Several gating strategies are employed to evaluate T-cells. First, total CD3-positive cells are gated on cell size (forward scatter) and the CD4:CD8 ratio is displayed on both small and large cells. Second, each quadrant of the CD4 vs CD8 plot showing total CD3-positive cells is highlighted in a different color. These colors are displayed on the plots showing patterns of expression for other T-cell markers **(Figure 5)**. This allows simultaneous evaluation of T-cell markers on each subset of CD3+ cells (CD4+, CD8+, CD4/CD8 double positive and CD4/CD8 double negative). **The T-cell markers are displayed on gates of total lymphocytes so that abnormal T-cells, which may lose CD3, will not be missed.**

Third, an additional page is added to show markers expressed on NK-cells (CD56+CD3-) and CD56+ T-cells (**Figure 6**). T-cell large granular lymphocytes (T-LGLs) usually have variable expression of CD56 and CD57. Absence of CD56 does not exclude the presence of T-LGLs. The T-Cell Receptor/LGL Add-On is available for further characterization of T-LGLs and NK-cells.

Figure 5: T-cell gating strategies

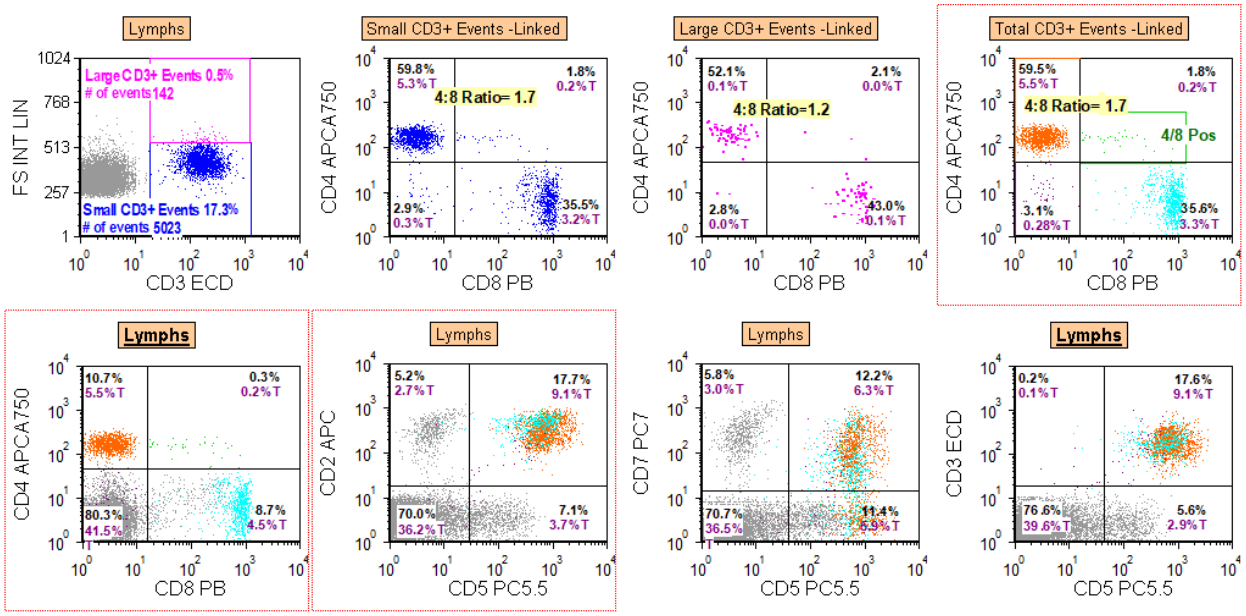
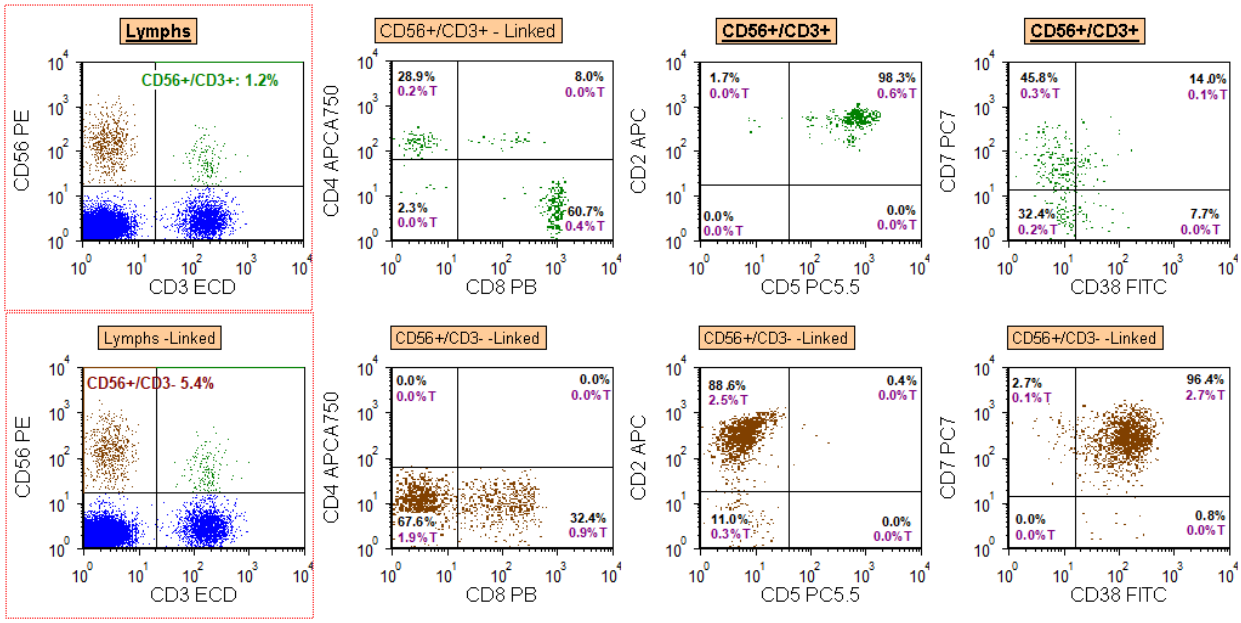


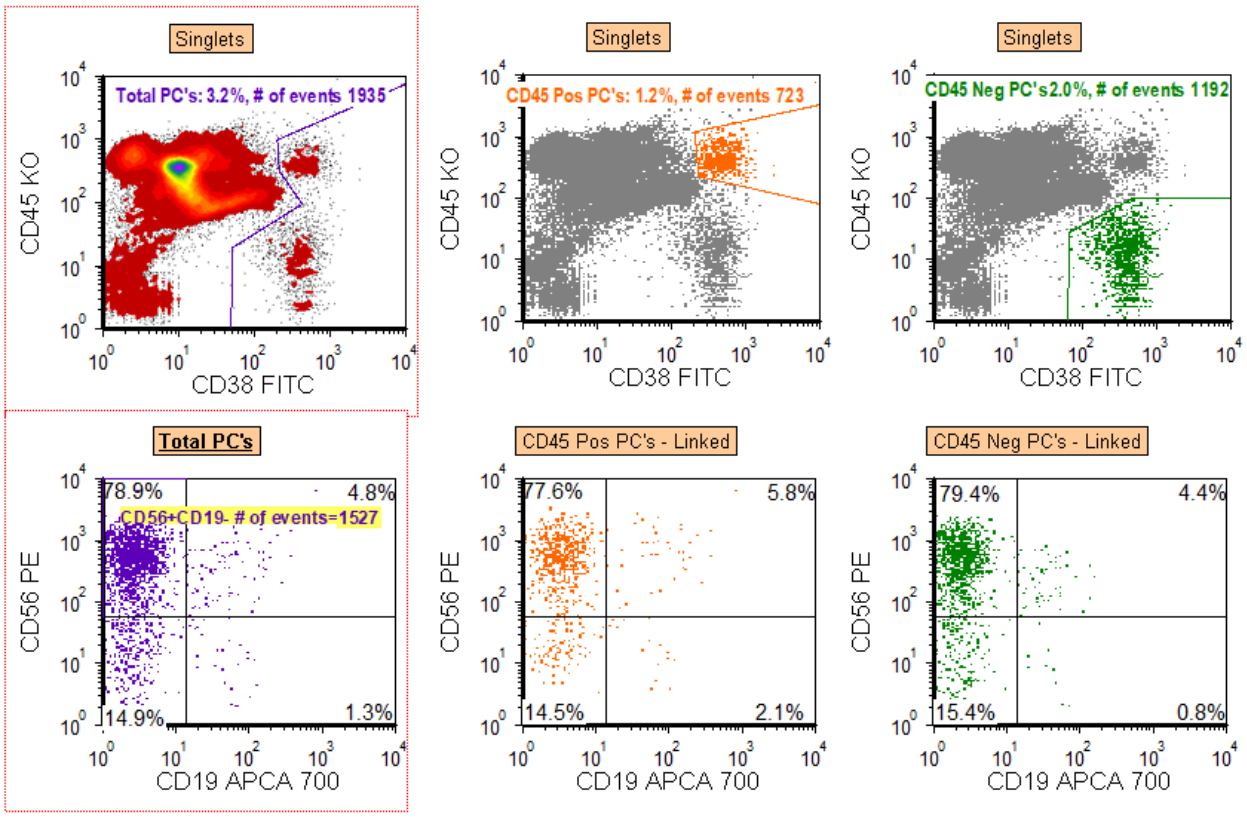
Figure 6: Sub-gating for CD56+CD3+ T-cells and NK-cells (CD56+CD3-)



Plasma Cell Gating

CD45/CD38 gating strategy is used to gate on plasma cells in the main panels **(Figure 7)**. In the plasma cell add on tube, CD138 gating is also employed.

Figure 7: Plasma cell gating strategy



Lymphocytes

1. A relative increase in lymphocytes raises concern for a lymphoproliferative disorder. Conversely, a relative decrease by itself has unknown clinical significance.

2. There are usually more T-cells than B-cells, otherwise a B-cell disorder is suspected.

3. A polyclonal kappa/lambda pattern makes a B-cell leukemia/lymphoma unlikely. However, small monoclonal populations in a polyclonal background may exist.

4. Features suggestive of a B-cell lymphoma/leukemia are:
    a. Kappa/lambda ratio > 3.0 or < 0.3. NOTE: Some normal reactive germinal center cells may have a kappa/lambda ratio as high as 14.4 or as low as 0.13 (rare reports in the literature).
    b. Abnormal Pan-B-cell marker expression, such as CD19+, CD20-neg/dim or CD19-/CD20+.
    c. Aberrant expression of CD5 or CD10 or bright CD11c. NOTE: CD23 expression can be seen in normal B-cells. CD10 expression can be seen in normal germinal center cells.
    d. Mature B-cells (CD45+++, CD20++) without (or with very dim) surface light chain expression.
    e. Large B-cells (FS higher than normal lymphocytes in the same sample).
    f. If the total of B-cells, T-cells and NK-cells does not account for the majority of the cells within the lymphocyte gate, then one should carefully review the flow data for a possible abnormal population, such as CD3-negative T-cells, CD19-negative B-cells, myeloid cells, or other population. Lymph Sum token in Lymphocyte

Differential section of the flow workbook will turn red if the total of CD19+ B-cells, CD3+ T-cells and CD56+CD3- NK-cells is less than or equal to 95%.

5. Clonal T-cell proliferations can be detected by flow with an add-on test called V-Beta T-Cell Clonality. (This test is only offered with interpretation.) Note: The clinical behavior of a clonal T-cell proliferation cannot be determined by flow cytometry alone. Features that are suspicious for a T-cell lymphoma/leukemia, and when the add-on is recommended include:
    a. A markedly elevated CD4/CD8 ratio >10:1 or a markedly inverted CD4/CD8 ratio <0.1,
    b. CD4/CD8 dual expression (except thymocytes) and CD4/CD8 double negative T-cells, or
    c. Variable loss of pan-T-cell markers, such as CD2, CD3, CD5 and CD7.

6. The possibility of a NK-cell lymphoma/leukemia may be raised by relative increase in NK-cells (sCD3-, CD7+, CD56+ lymphocytes) to >20% of all lymphocytes, but it cannot be confirmed by clinical flow cytometry at this time.

7. Add-on tests:
    a. Intracytoplasmic B-cell tube (ICB) can be requested when B-cells lack detectable surface light chain expression: cKappa, cLambda, CD23, CD19, CD11c, CD10, CD5, CD34, CD20, CD45.
    b. Hairy cell leukemia markers: CD22, CD25 and CD103 with Kappa, lambda, CD19, CD11c, CD20.
    c. CLL/Mantle Cell Companion tube: CD3, CD5, CD19, CD22, CD36, CD43, CD45, CD52, CD200, FMC-7; can be useful in discrimination of chronic lymphocytic leukemia/small lymphocytic lymphoma and mantle cell leukemia/lymphoma. FMC7 is usually lost in CLL, but is expressed in other lymphomas. Positivity for CD52 expression on lymphocytes indicates possible response to therapy with alemtuzumab, a recombinant anti-CD52 antibody. CD200 is a sensitive marker for CLL and is usually highly expressed, however it is not specific for CLL. Most mantle cell lymphomas have negative or weak staining for CD200.
    d. T-Cell Receptor/LGL Add-On: CD3, CD4, CD7, CD8, CD16, CD45, CD56, CD57, TCR-gamma/delta, TCR-alpha/beta.
    e. T-Cell Lymphoma Companion Panel: CD3, CD4, CD7, CD8, CD25, CD26, CD30, CD52, CD279 (PD-1), CD45. This panel assesses T-cells for the presence of targetable antigens to guide therapeutic decisions.
    f. Sezary T-cell Add-On Panel: CD3, CD4, CD5, CD7, CD8, CD19, CD26, CD43, CD45. This panel is designed to detect abnormal circulating T-cells seen in Sezary syndrome, a disseminated form of mycosis fungoides, a cutaneous T-cell lymphoma. CD26 is usually negative or weakly positive on Sezary T-cells.
    g. V-beta T-cell clonality is available to assess T-cell clonality and detects 24 different V-beta families (about 70% coverage of normal human TCR V-beta repertoire). (This test is only offered with interpretation.)
    h. CLL MRD assay is available for monitoring of minimal residual disease in patients with CLL. It follows the standardized protocol developed by the European Research Initiative in CLL (ERIC) and can detect MRD at the 0.01% level.

Granulocytes

1. Most mature granulocytes are CD10-positive. The CD10-positive/CD10-negative granulocyte ratio is the greatest in peripheral blood samples **(Figure 8)**. The vast majority of peripheral granulocytes are CD10+. A relative increase in CD10- peripheral granulocytes suggests left shift. The CD10-positive/CD10-negative ratio is around 0.2 (20%) for normal bone marrow samples **(Figure 9)**. A higher ratio in a bone marrow sample suggests hemodilution. A lower ratio suggests left-shifted myeloid maturation.

2. CD13 is a myeloid maker with biphasic expression. One peak is in immature granulocytes that are CD11b and CD16 negative or dim. Another is in mature granulocytes that are CD11b+ and CD16+.

3. Eosinophils are also CD16- granulocytes and have bright CD45 with high side scatter. Loss of CD16 can be seen in PNH cells, since CD16 is a GPI-anchored antigen on neutrophils (it is a transmembrane molecule on NK-cells).

4. The CD13 vs CD11b plot of granulocytes shows a "U" shaped distribution. A distorted "U" is often seen in MDS and MPN (dyssynchronous maturation) as well as other non-specific conditions. The CD13 vs CD16 plot of granulocytes shows "V"-shaped distribution. Dyssynchronous maturation also leads to a distorted "V" shape often seen in MDS or MPN.

5. Aberrant CD56 expression is seen in MDS or MPN as well as some reactive conditions.

Figure 8: Granulocyte pattern recognition in normal peripheral blood

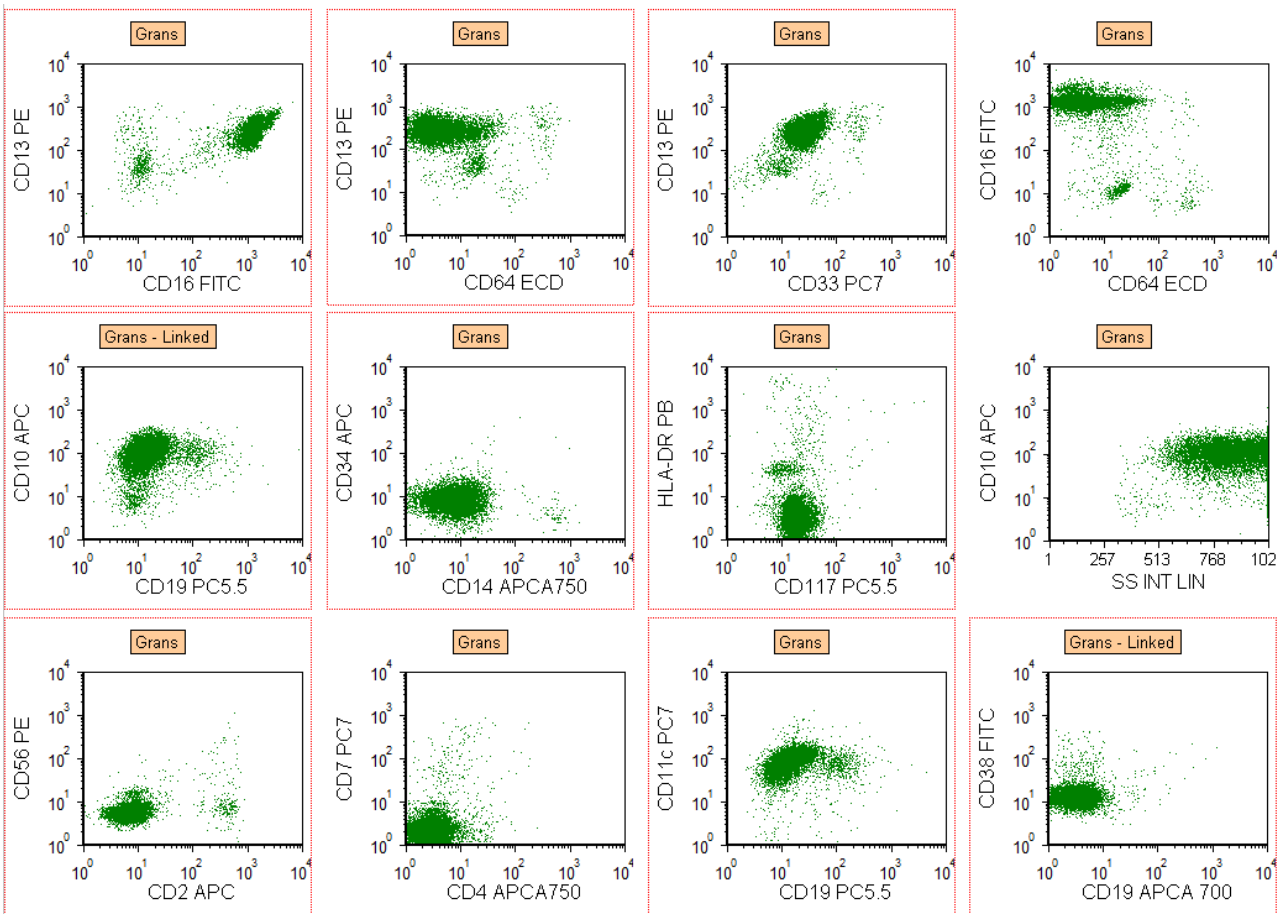
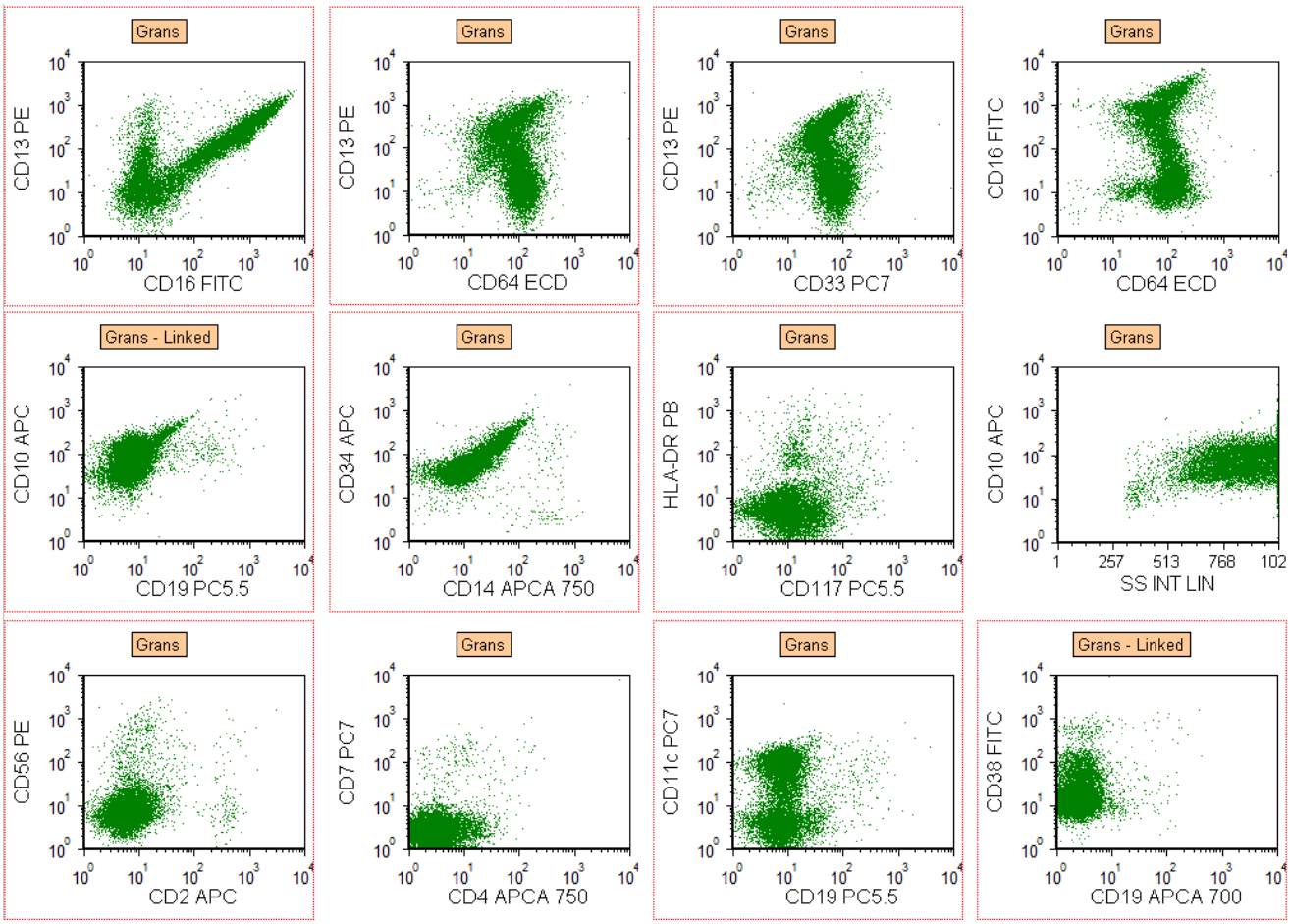


Figure 9: Granulocyte pattern recognition in normal bone marrow

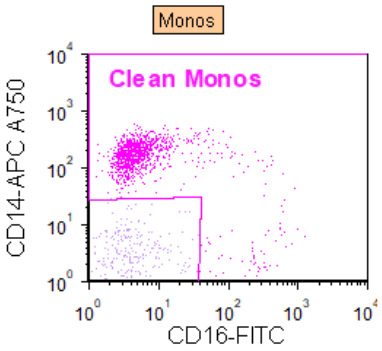


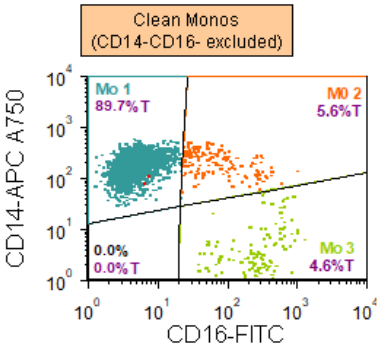
Monocytes

1. Peripheral blood monocytes account for approximately 10% of all circulating leukocytes and are traditionally divided into three phenotypically and functionally distinct populations based upon differences in expression of CD14 and CD16 encoding for the lipopolysaccharide receptor and the low affinity FC gamma receptor (FCGR3), respectively.
2. Classical ($CD14^{hi}CD16^{neg}$) monocytes account for 80–90% of human blood monocytes, intermediate ($CD14^{hi}CD16^{hi}$) monocytes comprise ~2–5% and the non-classical ($CD14^{low}CD16^{hi}$) monocytes account for the remaining 2–10% (PMID: 28596372).
3. Multiple diseases including chronic myelomonocytic leukemia (CMML), infections, autoimmunity, and chronic inflammation are associated with changes in monocyte subsets.
    a. An increase in the fraction of classical monocytes to >94.0% of total monocytes has been reported to help distinguish CMML from other causes of monocytosis (PMID: 25852055).
    b. Intermediate monocytes are more abundant in bacterial sepsis, dengue fever, Crohn's disease, cardiovascular disease and rheumatoid arthritis (PMID: 28596372).
    c. Non-classical monocytes are more prevalent in periodontitis and reduced in stroke (PMID: 28596372).
4. Normal monocytes also express CD64 and will show variably decreased CD14 expression with left-shifted maturation.
5. Monoblasts may be negative for CD14 and are often negative for CD34.
6. Aberrant CD56 expression can be seen in MDS or MPN, but also in reactive conditions (e.g. post chemotherapy).

7. When monocytes are gated based on CD45 vs SSC pattern, other cells may also be present in the gate, including, but not limited to, abnormal lymphocytes (e.g. hairy cell leukemia), dendritic cells and granulocytes with low side scatter.
    a. Before evaluating the proportions of various monocyte subsets, the CD14/CD16 double negative cells are excluded, yielding what is named the "Clean Monos" gate in the workbook **(Figure 10)**.
    b. Classical, intermediate and non-classical monocytes are referred to as Mo 1, Mo 2 and Mo 3, respectively, in the workbook **(Figure 10)**.

Figure 10. Example of Normal Peripheral Blood Monocytes





Population	% of Gated	% of Total
Clean Monos	83.1	4.7
Mo 1	89.7%	4.2%
Mo 2	5.6%	0.3%
Mo 3	4.6%	0.2%

8.

Basophils

1. Basophils express slightly less CD45 than lymphocytes, but more than blasts. Basophils can be separated from monocytes in the mononuclear gate based on expression of CD33 without HLA-DR.

2. Basophils express CD9, CD11b, CD13, CD33, CD36, CD38 (bright), CD123 (bright) and are negative for CD19, CD34, CD64, CD117, and HLA-DR.

Eosinophils

1. Eosinophils have high side scatter and express brighter CD45 than most granulocytes.

2. Eosinophils are distinguished from the rest of the granulocytes by lack of CD16 and CD10. They are positive for CD13 and CD33.

CD45 Dim Cells

1. Relative increase raises concern for acute leukemia or excess blasts.

2. Acute leukemia is usually straightforward and tentative morphologic and immunophenotypic classification can be achieved in the vast majority of cases. However, acute promyelocytic leukemia (APL) cannot be completely excluded. Not all APL cases have the typical immunophenotype characterized by lack of expression of both CD34 and HLA-DR. Stat FISH for t(15;17) is recommended on any case of suspected APL. Proper WHO classification requires completion of cytogenetics, FISH and/or molecular studies as well as morphological confirmation of blast count (the current gold standard).

3. Excess blasts may be seen in a regenerating marrow or in patients receiving growth factors. Abnormal myeloid blasts may be detected by their maturation pattern of HLA-DR /CD33 /CD34 /CD117 /CD45.

4. Excess precursor B-cells (CD19+, CD10+, CD45 dim), or so called hematogones, may be seen in young patients and other non-neoplastic conditions. The differentiation of hematogones from lymphoblasts can be challenging. CD10/CD20 plot may be helpful because hematogones show gradual gains of CD20 with decreasing CD10, while lymphoblasts form a tight cluster or have aberrant expression of myeloid markers, for example.
5. Add-on tests:
    a. B-ALL tube: nTdT, cMPO, cCD3, CD10, CD19, cCD22, CD34, CD45, cCD79a
    b. T-ALL tube: nTdT, cMPO, CD1a, cCD3, CD7, CD11b, CD19, CD43, CD45
    c. AML tube: nTdT, cMPO, cCD3, CD11b, cCD22, CD34, CD45, cCD79a, CD117, CD123
    d. Erythroid/Megakaryocyte tube: CD13, CD34, cCD41, CD45, cCD61, CD71, CD117, CD235a
    e. CD123 is now available in the AML-add-on tube and can be helpful in diagnosis of blastic plasmacytoid dendritic cell neoplasm, but can be seen in ALL and AML.
    f. CD1a is now available in the T-ALL tube for further characterization of T-lymphoblasts.
    g. B-ALL MRD assay is designed for monitoring of minimal residual disease and follows a standardized protocol with a sensitivity of 0.01%. It is useful to run at initial diagnosis to have a baseline phenotype for correlation with MRD results.

CD45 Neg Cells

1. Relative increase raises concern for suboptimal sample processing (unlysed erythroids), true erythrocytosis, metastatic cancer cells, plasma cell myeloma and acute leukemia (ALL, erythroid leukemia).

2. Dysplastic erythroid precursors may show loss of CD71 (transferrin receptor). Normal mature erythrocytes in the peripheral blood are negative for CD71.

3. Add-on tests:
    a. Erythroid/Megakaryocyte tube: CD13, CD34, cCD41, CD45, cCD61, CD71, CD117, CD235a

Plasma Cells

1. Plasma cells express bright CD38 and variable CD138 with rare exceptions.

2. Plasmacytosis (no numeric reference range for flow cytometry) raises concern for plasma cell dyscrasia, plasma cell myeloma (multiple myeloma) or monoclonal gammopathy of undetermined significance (MGUS).

3. Normal plasma cells are usually CD19+, CD45+, and CD56-, while abnormal plasma cells are usually CD19-, CD45-, and often CD56+. If an abnormal plasma cell population is predominant (>95%), a plasma cell neoplasm (multiple myeloma or plasmacytoma) is likely. If the abnormal plasma cell population is between 50% and 95% of all plasma cells, the diagnostic entity is either MGUS or a previously treated multiple myeloma in the appropriate clinical setting.

4. When variable plasma cell populations are present, a diagnosis of multiple myeloma is unlikely.

5. In any case suspicious for plasma cell dyscrasia, an add-on test for intracytoplasmic light chain markers is warranted. Again, a clonal population should be at least 95% of all plasma cells for a phenotypical diagnosis of plasma cell dyscrasia. NOTE: a discrepancy between flow cytometry and morphological examination may be encountered due to uneven distribution of sample cells between biopsy and aspirate or fragility of plasma cells during flow cytometry.

6. Add-on test:
a. Plasma Cell Tube: cKappa, cLambda, CD20, CD38, CD56, CD138, CD19, CD117, CD45

Flow Cytometry Panels

NeoGenomics Laboratories offers several panel options to suit our clients' needs (see **Table 1**). Our **Standard Panel** is recommended for all specimen types (PB, BM, tissues and fluids) and consists of 24 markers covering B-cell, T/NK-cell, plasma cell and myeloid neoplasms (**Tubes 1-3, see Table 1**). The **Extended Panel** (**tubes 1-4 in Table 1**) consists of 31 markers and is primarily recommended for BM or PB specimens as it includes markers for erythroid and megakaryocytic differentiation. The **T&B Tissue Panel** (**tubes 1-2, see Table 1**) consists of 17 markers for workup of T/NK- and B-cell lymphomas in tissues where there is no suspicion for myeloid sarcoma. For specimens with limited cellularity, the laboratory will perform a targeted antibody panel based on the reason for referral, supporting documents and any prior history in our LIS.

We also offer smaller follow-up flow panels, which are recommended only for patients with a previous diagnostic flow specimen performed at NeoGenomics (see **Table 2**). The **Follow-up Panels** include: AML (Myeloid tube + AML tube), B-ALL (B-cell tube + B-ALL tube), B-cell lymphoma (B-cell F/U tube), HCL (HCL tube), Plasma Cell (PC tube), T-ALL (T-cell tube + T-ALL tube), and T-cell lymphoma (T-cell tube). High sensitivity minimal residual disease flow panels are available for monitoring of B-ALL, CLL, and MM (see **Table 3**).

Table 1. Flow cytometry tube configuration

Tube #	Tube Name	Antibodies									
		FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PB/V450/BV421	KrO
1	T-Cell	CD38	CD56	CD3	CD5	CD7	CD2	CD19	CD4	CD8	CD45
2	B-Cell	Kappa	Lambda	CD23	CD19	CD11c	CD10	CD5	CD34	CD20	CD45
3	Myeloid	CD16	CD13	CD64	CD117	CD33	CD34	CD19	CD14	HLA-DR	CD45
4	Extended	FMC-7	CD235a	CD41	CD138	CD11b	CD34	CD19	CD71	CD15	CD45
Short Sample	Short Sample T/B	Kappa	Lambda	CD3	CD19	CD10	CD34	CD5	CD4	CD8	CD45
Add-On Tubes	AML	nTdT	cMPO	CD34	CD117	cCD22	cCD79a	cCD3	CD11b	CD123	CD45
	B-ALL	nTdT	cMPO	CD19	cCD22	CD10	cCD79a	CD34	cCD3	none	CD45
	T-ALL	nTdT	CD1a	cCD3	cMPO	CD7	none	CD19	CD43	CD11b	CD45
	CD52 Analysis	CD52	None	CD3	none	none	none	CD19	none	none	CD45
	CLL/MCL Companion	CD52	CD200	CD3	CD22	CD5	CD36	CD19	CD43	FMC-7	CD45
	Erythroid-Mega	CD71	CD13	cCD41	CD117	cCD61	none	CD34	CD235a	none	CD45
	Hairy Cell	Kappa	Lambda	CD19	CD22	CD11c	CD103	CD25	CD20	none	CD45
	ICB	cKappa	cLambda	CD23	CD19	CD11c	CD10	CD5	CD34	CD20	CD45
	Mast Cell	none	none	CD34	CD117	none	CD2	CD25	none	none	CD45
	Plasma Cell	cKappa	cLambda	CD20	CD38	CD56	CD138	CD19	CD117	CD45	none
	T-Cell Lymphoma Companion	CD52	CD26	CD3	CD279	CD7	CD30	CD25	CD4	CD8	CD45
	T-cell Receptor/ LGL	TCR α/β	TCR γ/δ	CD16	CD3	CD56	CD7	CD8	CD4	CD57	CD45
	Sezary T-cell Add-	CD43	CD26	CD3	CD19	CD7	none	CD5	CD4	CD8	CD45
PNH	WBC	FLAER	CD64	none	none	CD14	CD24	none	none	CD15	CD45
	RBC	CD235a	CD59	none	none	none	none	none	none	none	none
BAL 4/8	Qualitative 4/8 Ratio	none	none	CD3	none	none	none	none	CD4	CD8	CD45

Note: This table is a complete itemization of our current tube configuration. Combinations are subject to change as the NeoGenomics Medical Director deems it necessary. **V-beta T-cell Clonality** is available with interpretation only**. BAL 4/8** (CD4/CD8 for BAL) does not have a professional component. It is imperative on the submitted requisition that the patient's clinical history and the diagnosis under consideration are as accurate and precise as possible. Providing such information will give us a better idea whether to run a comprehensive panel or a sub-panel in a more targeted approach.

Table 2. Flow cytometry follow-up panels tube configuration

Panel (follow-up)	Tube Name	Antibodies									
		FITC	PE	ECD	PC5.5	PC7	APC	APCA 700	APCA 750	PB/ V450/ BV421	KrO
AML	Myeloid Tube #3	CD16	CD13	CD64	CD117	CD33	CD34	CD19	CD14	HLA-DR	CD45
	AML	nTdT	cMPO	CD34	CD117	cCD22	cCD79a	cCD3	CD11b	CD123	CD45
B-ALL	B-Cell Tube #2	Kappa	Lambda	CD23	CD19	CD11c	CD10	CD5	CD34	CD20	CD45
	B-ALL	nTdT	cMPO	CD19	cCD22	CD10	cCD79a	CD34	cCD3	none	CD45
B-cell Lymphoma	B-Cell F/U	Kappa	Lambda	CD23	CD19	CD11c	CD10	CD5	CD20	FMC-7	CD45
Hairy Cell	Hairy Cell	Kappa	Lambda	CD19	CD22	CD11c	CD103	CD25	CD20	none	CD45
Plasma Cell	Plasma Cell	cKappa	cLambda	CD20	CD38	CD56	CD138	CD19	CD117	CD45	none
T-ALL	T-Cell Tube #1	CD38	CD56	CD3	CD5	CD7	CD2	CD19	CD4	CD8	CD45
	T-ALL	nTdT	CD1a	cCD3	cMPO	CD7	none	CD19	CD43	CD11b	CD45
T-Cell Lymphoma	T-Cell Tube #1	CD38	CD56	CD3	CD5	CD7	CD2	CD19	CD4	CD8	CD45

Table 3. Flow cytometry minimal residual disease (MRD) panels tube configuration

Panel (MRD)	Tube Name	Antibodies							
		FITC	PE	PC5.5	PC7	APC	APCA 750	BV421	V500
B-ALL MRD	#1	CD38	CD19	7-AAD	CD34	CD22	CD10	CD123	CD45
	#2	CD58	CD19	7-AAD	CD34	CD13 + CD33	CD10	CD20	CD45
	#3	CD66c	CD19	7-AAD	CD34	CD9	CD10	CD38	CD45
Panel (MRD)	**Tube Name**	**FITC**	**PE**	**PC5.5**	**PC7**	**APC**	**APC-H7**	**BV421**	**V500**
CLL MRD	CLL MRD	CD81	CD79b	CD22	CD19	CD43	CD20	CD5	CD3
MM MRD	#1	cLambda	cKappa	CD117	CD56	CD138	CD19	CD38	CD45
	#2	CD81	CD27	none	none	CD138	CD20	CD38	CD45

Flow Cytometry Automatic Add-on Criteria

Add Plasma Cell tube:

- ≥1% possible phenotype PCs (CD56-CD19+)
- Distinct clustering of suspicious possible plasma cells with any % , CD19- and /or CD56+, and /or CD117+56-19-

Add AML tube:

- ≥20% suspicious of Blast population
  - CD34+,
  - And/or CD34-, CD117+/- blast with or without any other myeloid markers
  - Prior NeoGenomics history of AML of >6 months

Add B-ALL tube:

- ≥10% suspicious of Blast population
  - CD34+,
  - And/or CD10+/CD19+
  - Prior NeoGenomics history of B-ALL of >6 months

Add HCL tube:

- B-cell population at least 5% of total cells that have the following phenotype: CD5-, CD10-, CD11c+(mod-bright)

Add Mast Cell tube:

- A Mast Cell/Mastocytosis diagnosis or history AND > 0.1% mast cells

Repeat B-cell tube after warm wash:

- B-cell population at least 5% of total cells
  - Poor separation of Kappa/Lambda pattern
  - Unclear Kappa/Lambda pattern that could be cleaned up

Add B-cell tube with intracytoplasmic kappa/lambda:

- B-cell population at least 5% of total cells that has negative, very dim or unclear light chain (Kappa & Lambda) expression

Note: All other add-ons will be requested by the client or the pathologist signing out the case. Tech-Only clients may opt out of these add-on criteria by marking the appropriate box on the requisition.

IT Guidance

Reporting Cases

The NeoGenomics Laboratory information system is called NeoLINK (APvX). This system is used to send data files to clients and can be used by clients to report their cases. Through NeoLINK (APvX), clients will receive a .pdf file of the flow histograms (flow workbook), an image of the CD45 versus side scatter plot, the cell differential and percentages for each of the gated regions.

Personal Macros

Personal macros can now be created in NeoLINK (APvX) for Flow cytometry. To access macro database, click on Flow Macros button. For detailed instructions on how to create personal or practice specific macros, please refer to the NeoUniversity online on demand training video under NeoLINK Training at www.neogenomics.com.

Regating

If regating of the Flow cytometry data is needed for any reason, NeoGenomics provides several regating options based on desired level of interaction.

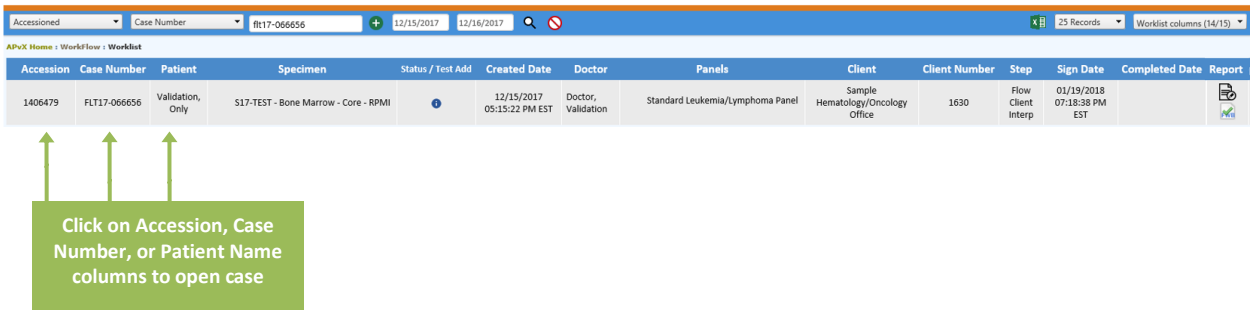
- Regate requests may be made via the Test Add On or Regate Request buttons,
- By contacting Client Services at 866-776-5907, Option 3, and/or
- If a client prefers to do his or her own regating, then publishing of flow cytometry data can be arranged by contacting his/her sales representative. Published flow data is accessed via the Self Regate File button. FCS Express Reader software is required to perform self-regating and is available for free download.

More information on regating is available in the NeoUniversity online on demand training video under NeoLINK Training at www.neogenomics.com.

Flow Regating Software (FCS Express Reader) Minimum Requirements

- Windows 7 or higher. FCS is not compatible with Windows Vista.
- Pentium III –class PC, 500 MHz
- 1 GB RAM
- 400 MB hard drive space
- Recommended system: 4 GB RAM, multi-core computer
- The manufacturer recommends downloading the version of the reader that corresponds to the version of the software used to publish the workbook. NeoGenomics is currently using FCS Express version 6.0.
- Visit the manufacturer's website for free download of FCS Express Reader: http://www.denovosoftware.com/site/Reader.shtml

Figure 10: Worklist



- **(Figure 10)** A sample NeoGenomics Laboratories NeoLINK (APvX) Worklist. The worklist includes all cases within a specific account and can be filtered or sorted as desired.
- Click on the accession number, patient name or case number to open the Flow case that is ready to be analyzed and completed.
- After clicking on the case to analyze, the Flow Input screen opens as shown in **(Figures 11-14)**.
- The Patient Summary tab displays the patient demographics at the top of the page, while the Prior/Concurrent Cases tab displays concurrent cases and any associated previous to which access is available **(Figure 11)**.

Working with Single or Multiple Workbooks

- From the worklist, navigate and review the .pdf scatter plots (by selecting the One Workbook Available/Multi Workbooks Available button) and preview / sign the final report **(Figures 12, 15)**.
- If there is only one workbook associated with a case, then it will open directly by clicking the One Workbook Available button.
- If add-on tubes are performed, then they will be uploaded in a separate workbook(s). The original workbook will be named with the case number (e.g. FLT20-012345) and add-on tubes will be named with the case number followed by a descriptor (e.g. FLT20-012345AML).
- If more than one workbook is available, then after clicking on the Multi Workbooks Available button, a list of the workbooks will appear in a new window.
- Click on the name of the workbook to open. More than one workbook can be opened at a time by holding down the CTRL key while clicking to highlight the file names.
- If a workbook is re-gated, amended or corrected, then the file name and top of the workbook .pdf will be labeled as such.
- The workbook can be minimized while the Flow input screen is open so that the scatter plots are available while completing the final report.

NOTE: Multiple workbooks within the same case number will be incorporated into the same report.

Features of Professional Input Screen

Figure 11: Report input screen: patient info versus prior/concurrent case view

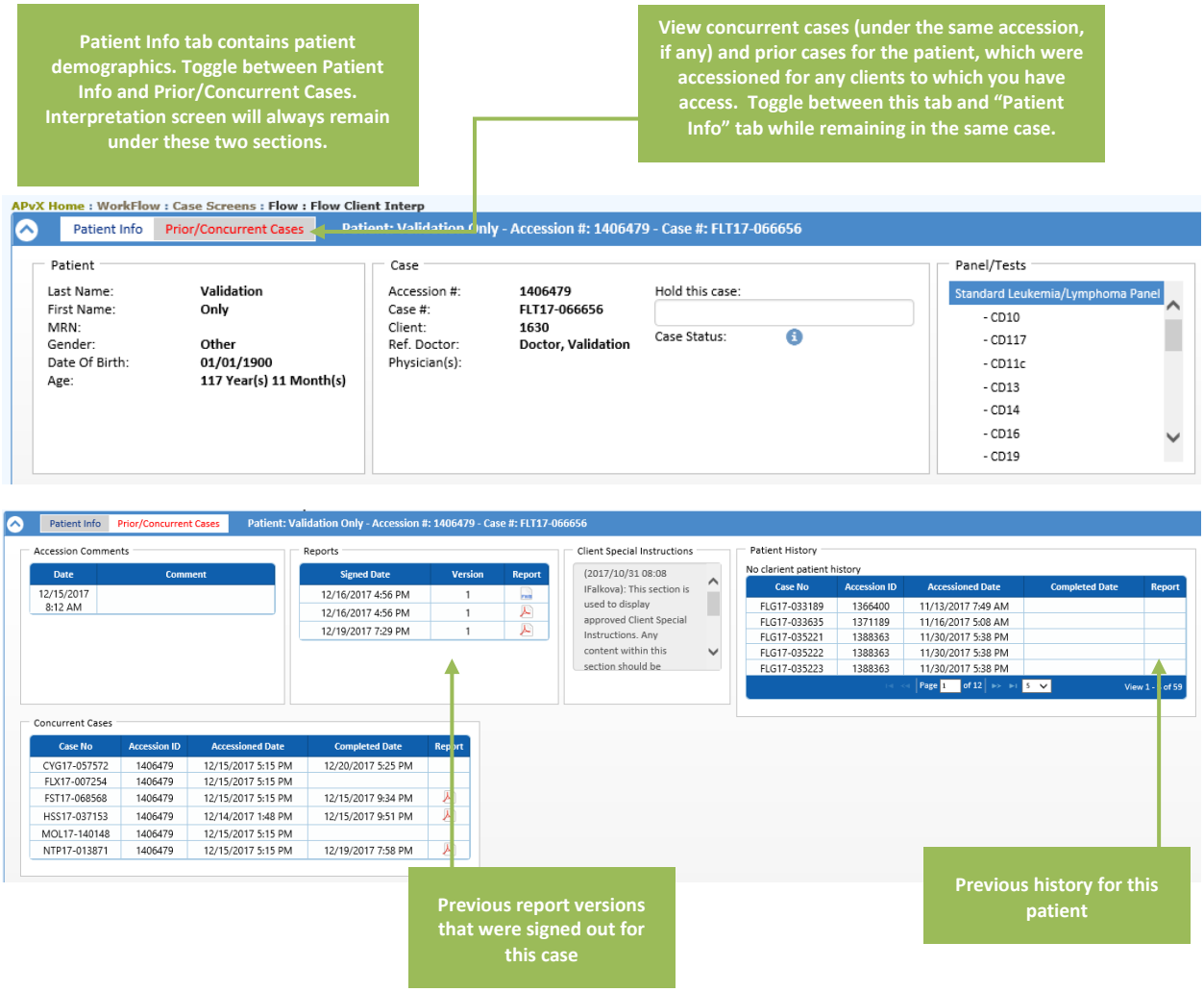


Figure 12: Report input screen

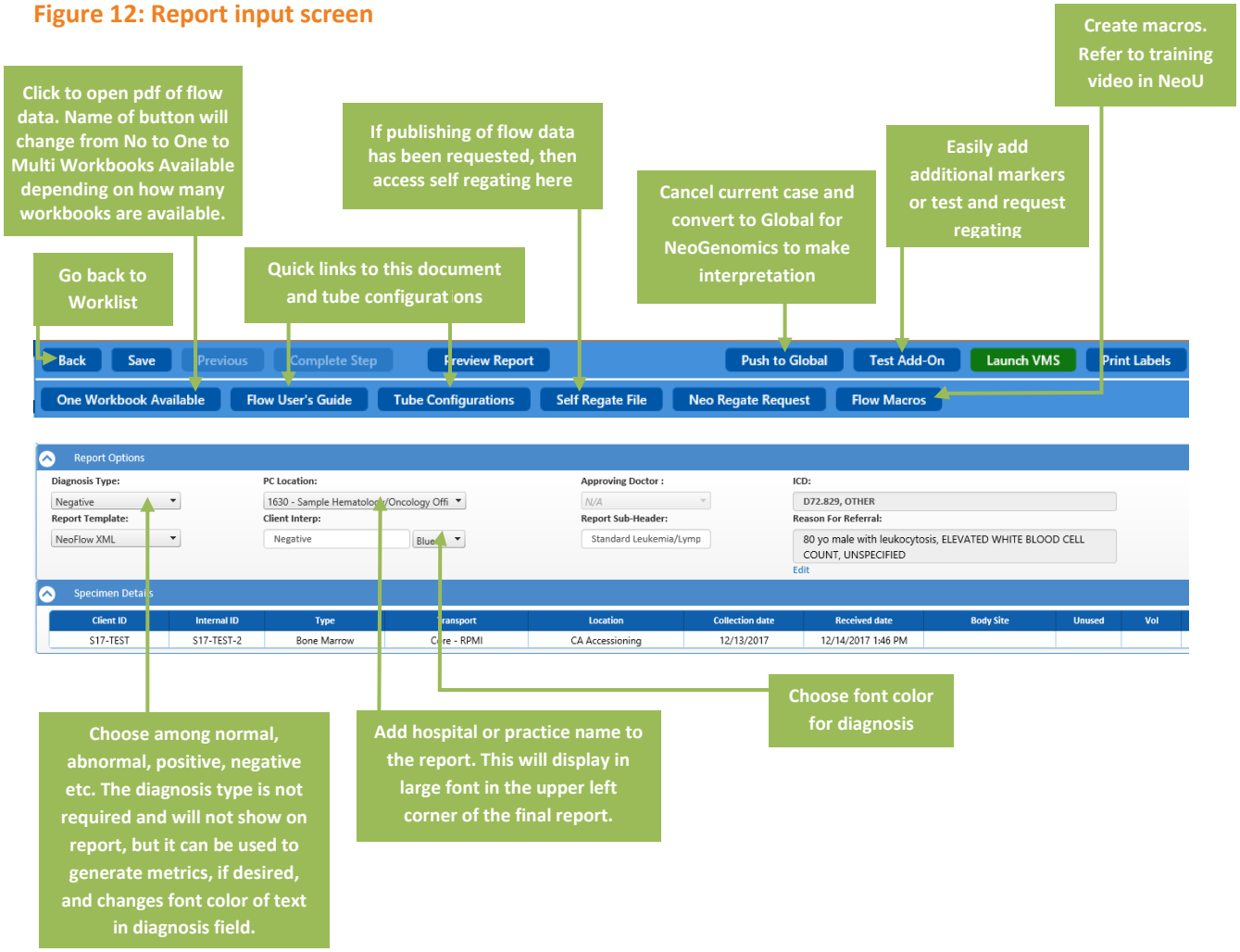


Figure 13: Report input screen continued

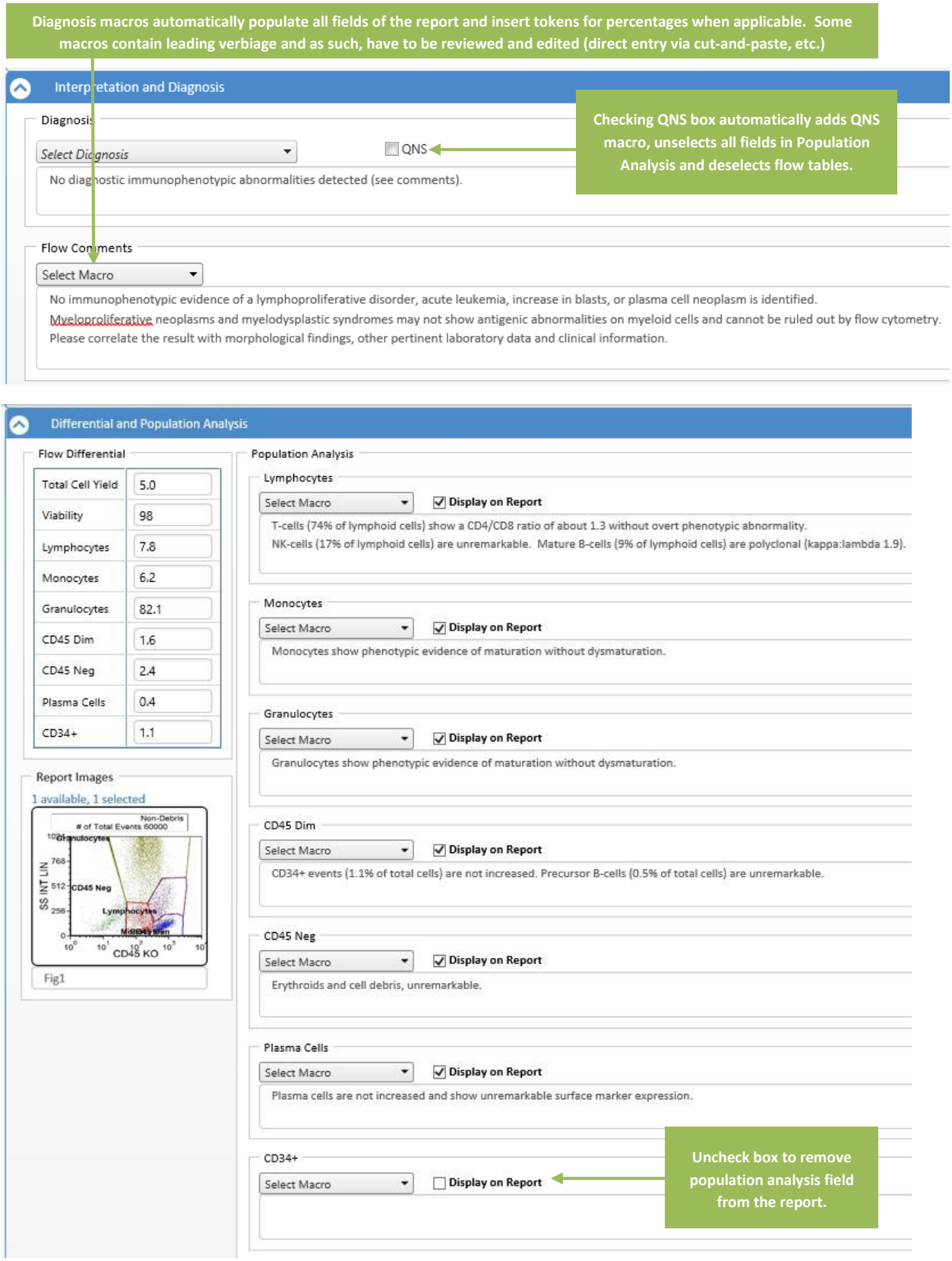
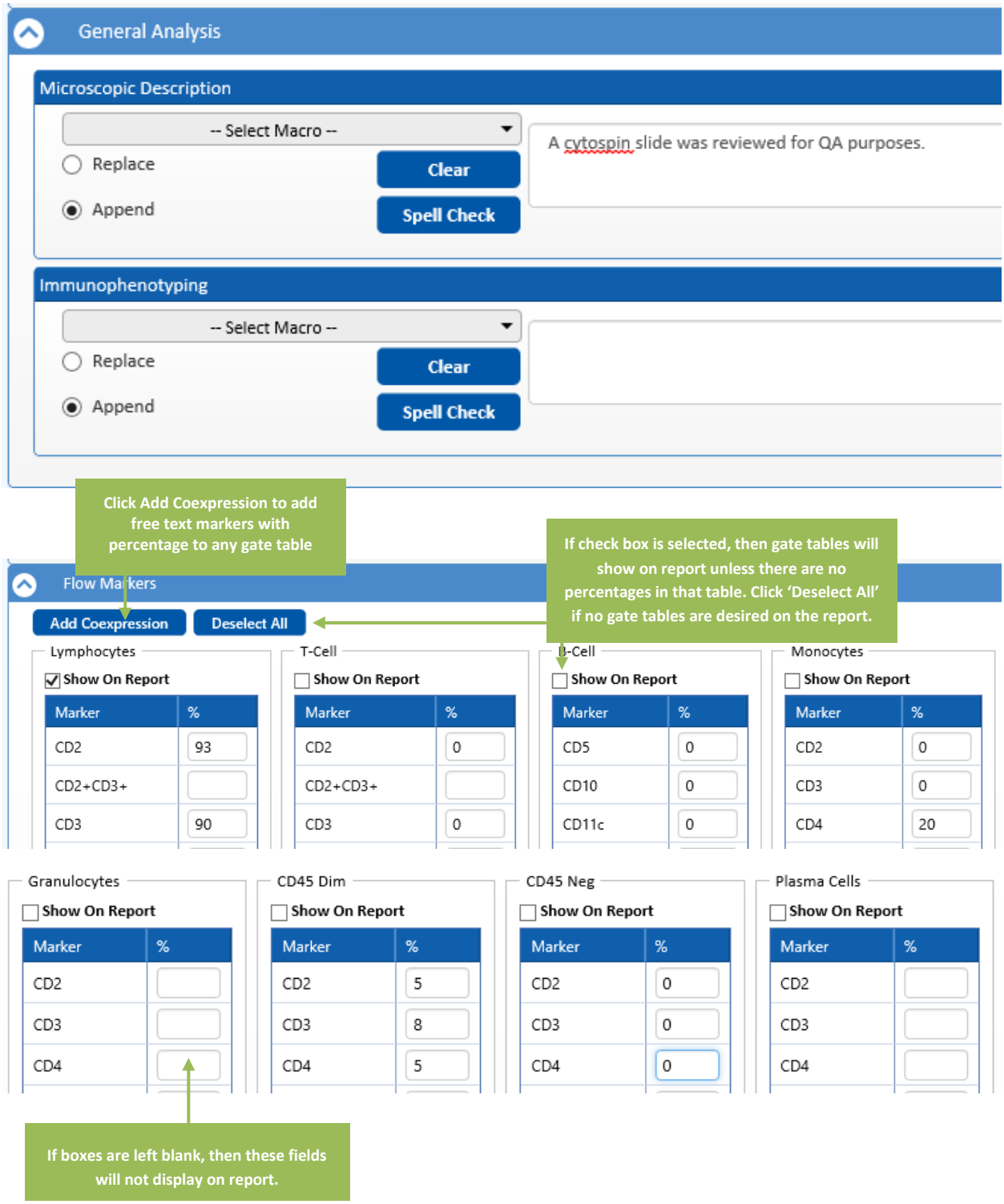
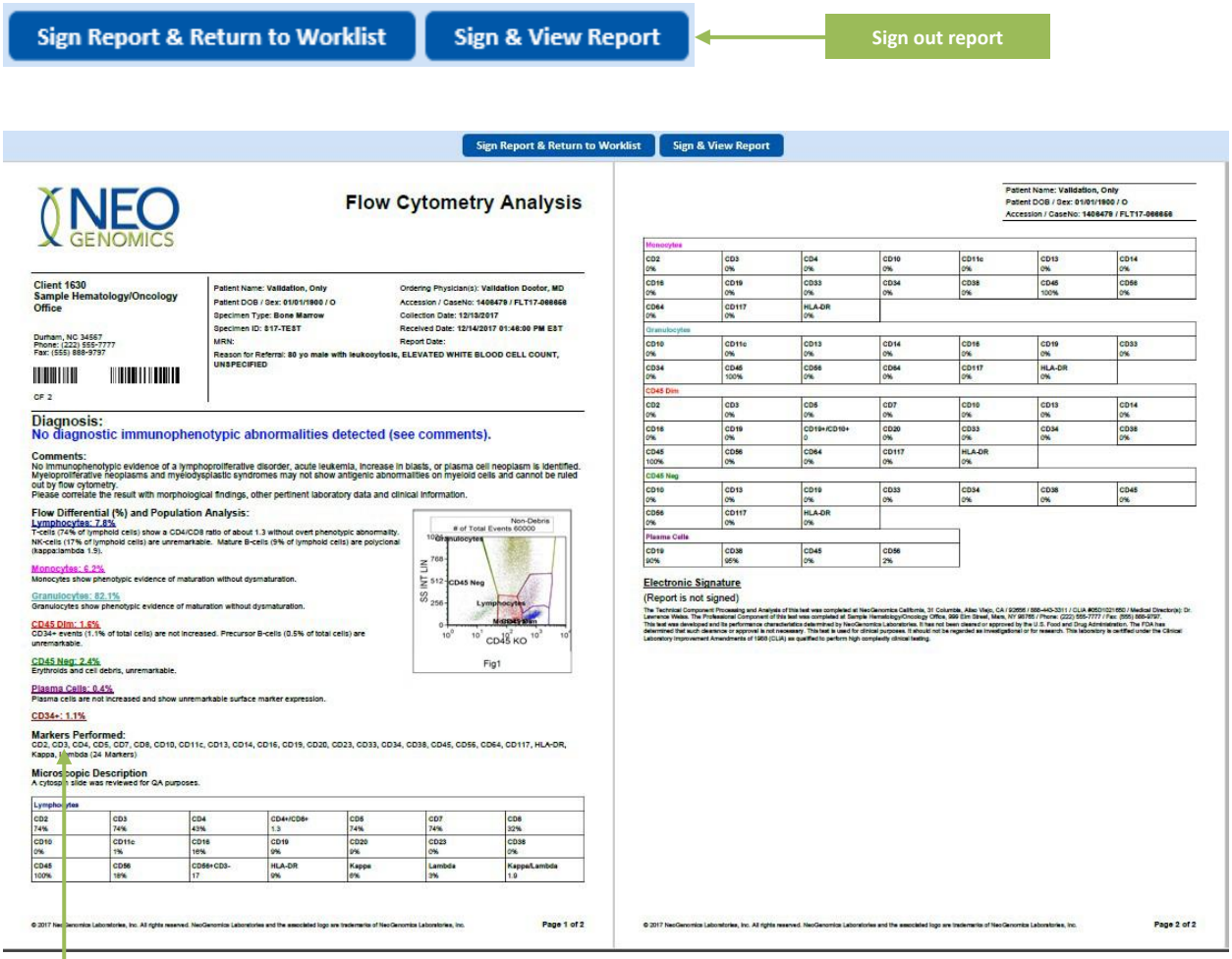


Figure 14: Report input screen continued



- After adding interpretation to the Flow input screen, click on the "Preview Report" button.
- By clicking this button, changes will be saved and the report preview will display.
- At this point, options are to go back to make more changes and create new preview or sign the report to complete the case **(Figure 15)**.

Figure 15: Preview and sign report



- After a case is signed out, the Flow workbook containing the data plots and the report is available from the work list in the column labeled "Report" **(Figure 16)**.
- If more than one Flow workbook was created, such as when add on markers are requested, the flow workbook (FWB) icon will contain a '+' sign FWB. Click the '+' sign to display a list of available workbooks in a small pop-up window. Click on the title of a workbook to open it.
- Alternatively, click on the button in the Status / Test Add column and select from the choices in the Reports box to view all of the workbooks after a case is complete.
    - In this screen, both the flow workbooks and reports will appear as a .pdf icon. Hover over the FWB icon to see the name of the workbook file. Signed reports may also have a Word file associated with them depending on client preferences **(Figure 17)**.

Figure 16: View flow workbook and report after case is complete via worklist

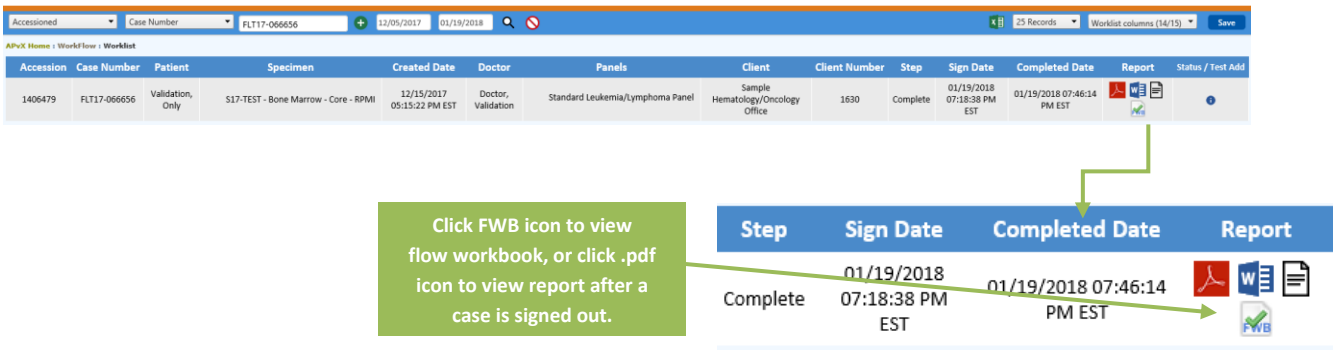
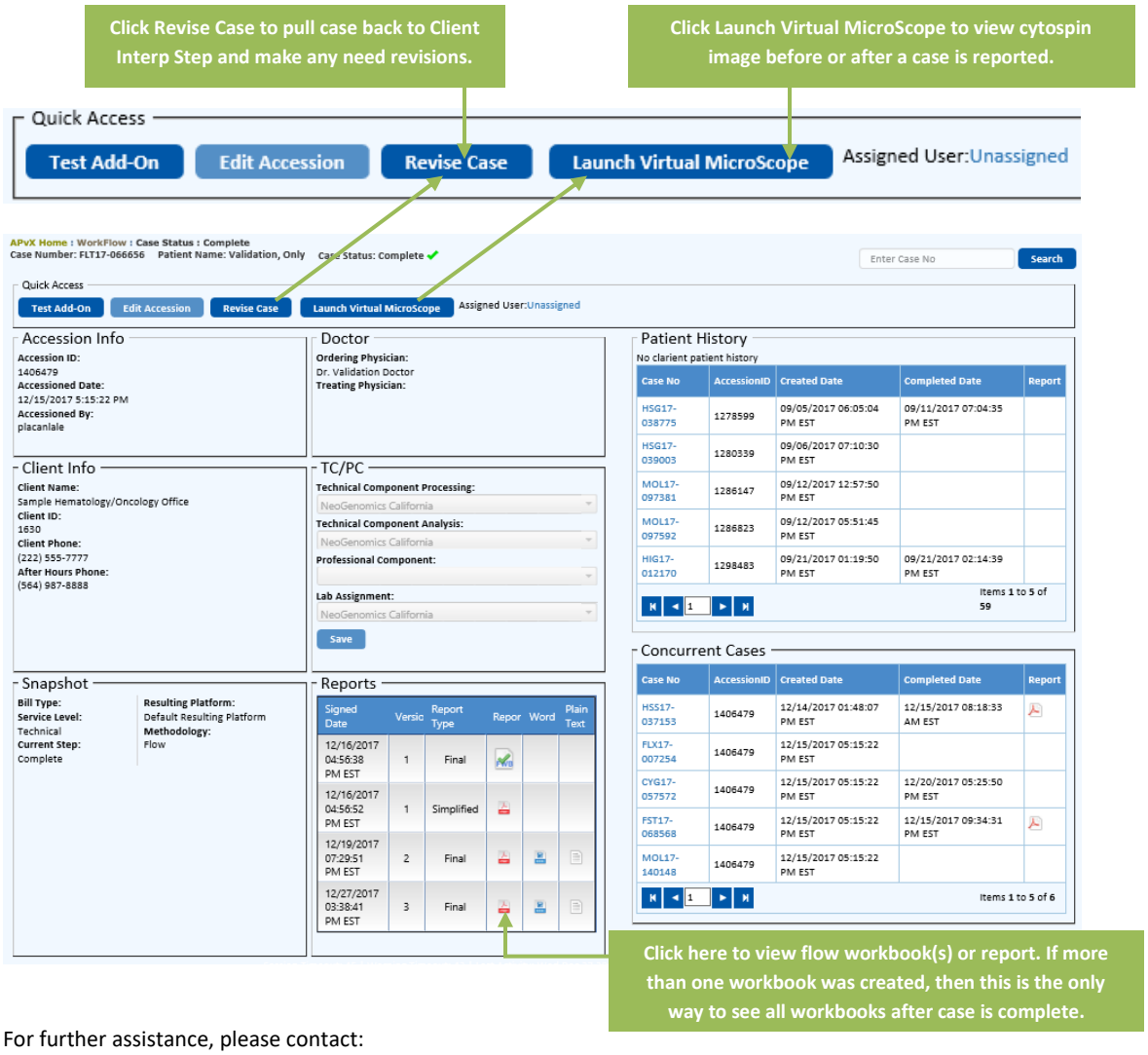


Figure 17: Alternative method for viewing flow workbook and report after case is complete via case status screen



For further assistance, please contact:
NeoGenomics Client Services at 866.776.5907 and select option #3