WG2 SOP-04
Data Analysis

Overview

This Standard Operating Procedure (SOP) describes the data analysis of the data generated from both SOP-1 and SOP-03 and to be used for the Working Group 2 (WG2) Inter-Laboratory Study (ILS). The data analysis includes the test samples and QC material acquired during SOP-03 and to which the 3 compensation matrices generated in SOP-02 will be applied. In addition, the data analysis will be performed with data obtained from SOP-1 to calibrate the fluorescence response, in units of Spe and ERF, to estimate the background (B, in units of Spe and ERF) and the detection efficiency (Q, units of Spe/ERF), which can be used to estimate the limit of detection and to predict instrument performance in resolving particles of different brightness, and to obtain the expression levels of CD markers in the unit of ERF.

Each generated .fcs file from SOP-03 will be analyzed with each of the 3 compensation matrices according to the workflow summarized in Figure 1.

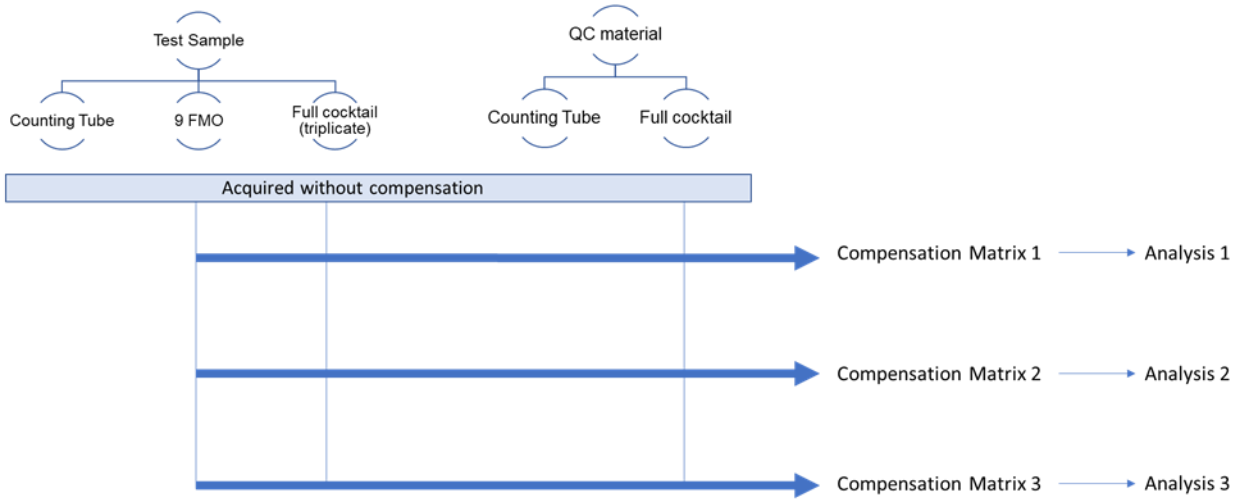


Figure 1. Data Analysis workflow of the WG2 ILS.

Data analysis may be performed on any analysis software but will follow gating plots and guidelines as described in this SOP.

For the analysis of instrument sensitivity, background, ERF calibration, and CD marker expression, etc., an automated WG2 Cytometer Sensitivity and ERF Calibration spreadsheet provided for the study will be used.

Equipment

- Flow Cytometry Analysis Software (use software and version of your institution or acquisition software provided with the cytometer

- WG2 Cytometer Sensitivity and ERF Calibration spreadsheet
- Computer

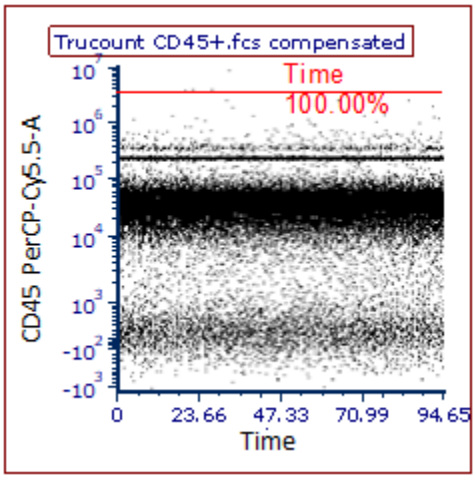
Procedure

1.1. Analysis template for Trucount™ counting tube for Test Sample

Create Single Analysis Template that will be used across all samples and analyses according to the following guidelines.

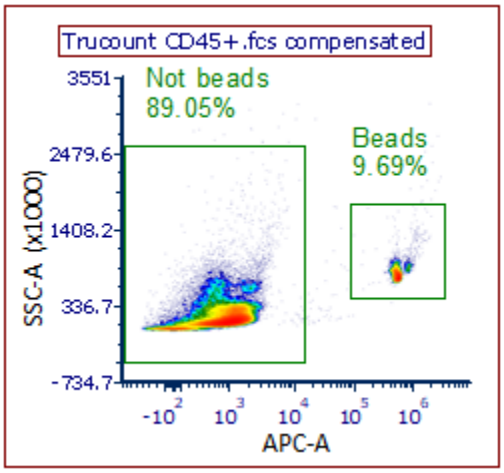
Plot 1

A bivariate dot plot, Time (X-axis) versus CD45 PerCP-Cy5.5-A (Y-axis), of all collected events. This plot is used for acquisition quality monitoring. Note that if a fluctuation is observed, e.g., stark dip or gap in the time profile, participant should consider to re-start sample acquisition or avoid the use of data collected during the fluctuation in the data analysis.



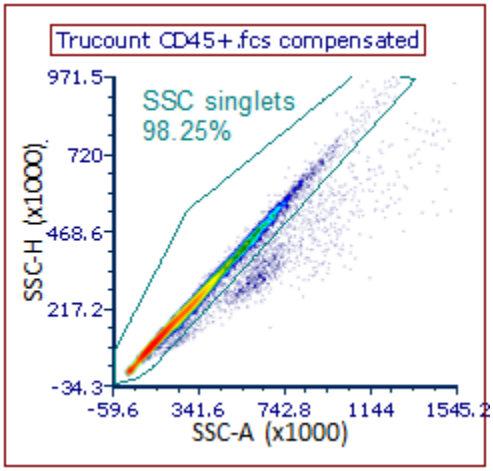
Plot 2

A bivariate dot plot, APC-A (X-axis) versus SSC-A (Y-axis), of events in ‘Time’ gate. This plot is used to draw a region around the “Beads” and the cellular events “Not beads”. Ensure all beads are taken in the gate. Note specific APC fluorescence is not detected because all cellular events are APC negative. Beads autofluorescence is used to detect and gate them.



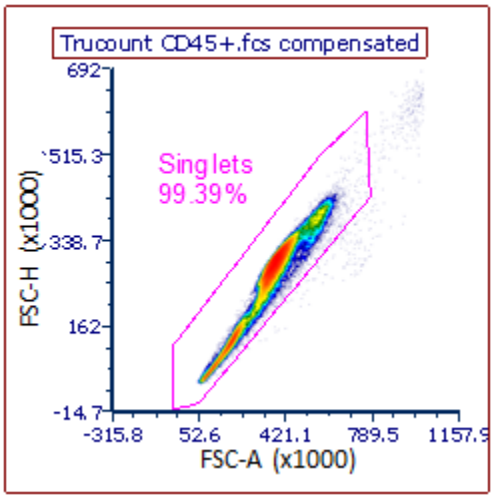
Plot 3

A bivariate dot plot, SSC-A (X-axis) versus SSC-H (Y-axis), of “Not beads”. This plot is used to draw a region around single cells to exclude side scatter doublets, named “SSC Singlets”. Note that singlet events are identified and gated based on Side Scatter (SSC) signal processing of Area (SSC-A) and Height (SSC-H). Doublets will have double the area values of single cells while the height is roughly the same. Therefore, differences between height and area can be used to identify aggregates.



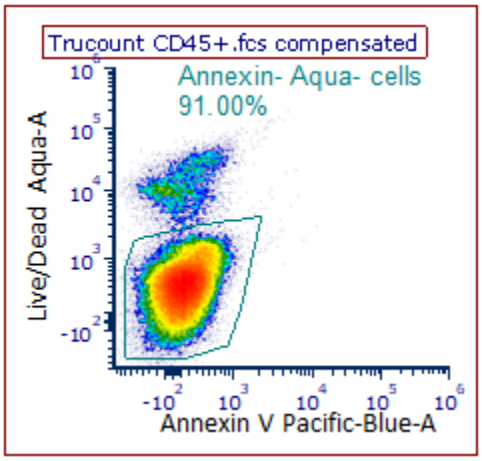
Plot 4

A bivariate dot plot, FSC-A (X-axis) versus FSC-H (Y-axis), of events in ‘SSC singlets’ gate. This plot is used to draw a region around single cells to exclude forward scatter doublets, named “Singlets”. A similar principle of signal processing applies as in Plot 3.



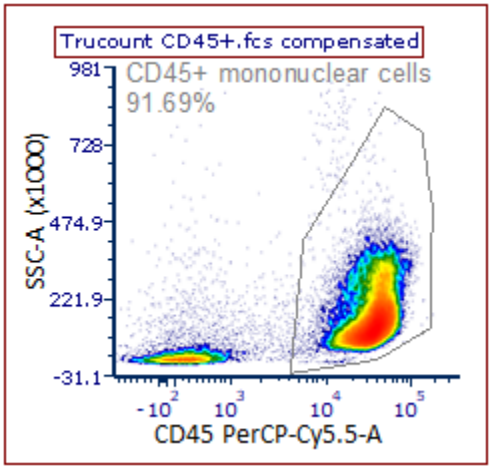
Plot 5

A bivariate dot plot, Annexin V Pacific-Blue-A (X-axis) versus Live/Dead Aqua-A (Y-axis), of events in ‘Singlets’ gate. This plot is used to draw a region around cells to exclude dead and apoptotic cells, named “Annexin-Aqua- cells”.



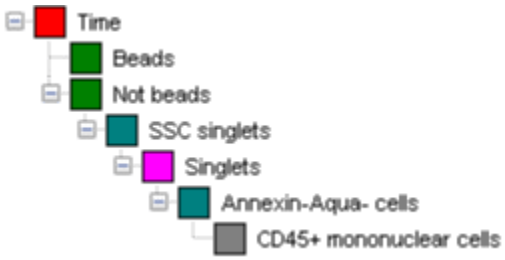
Plot 6

A bivariate dot plot, CD45 PerCP-Cy5.5-A (X-axis) versus SSC-A (Y-axis), of events in ‘Annexin-Aqua- cells’ gate. This plot is used to draw a region around the “CD45+ mononuclear cells”. The events in this gate will be used for the calculation of total cell counts.



Population Hierarchy

Population hierarchy for the counting tube is provided below.

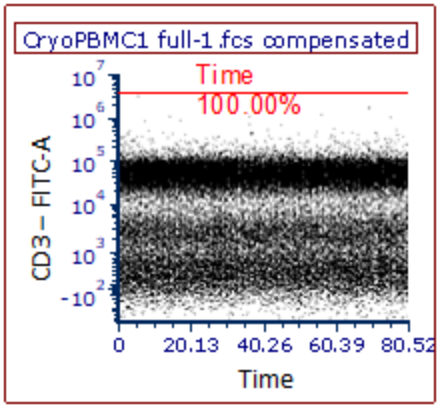


1.2. Analysis template for TBMNK panel for Test Sample

Create Single Analysis Template that will be used across all samples and analyses according to the following guidelines.

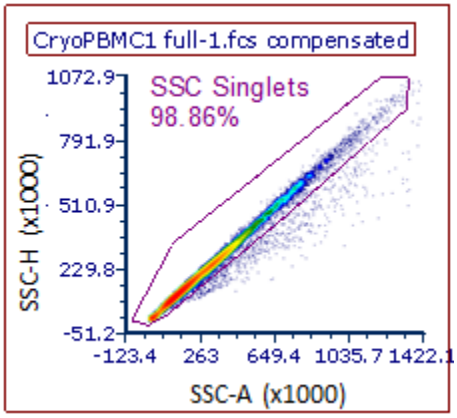
Plot 1

A bivariate dot plot, time (X-axis) versus CD3 FITC-A (Y-axis), of all collected events. This plot is used for acquisition quality monitoring.



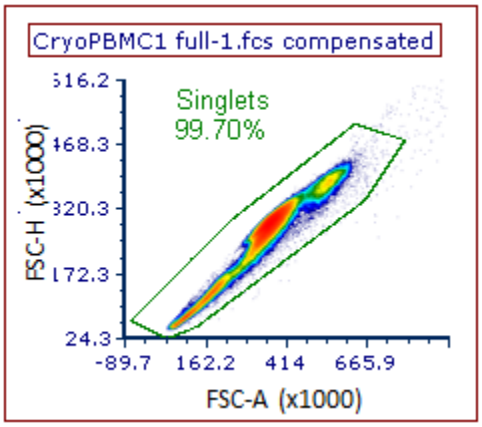
Plot 2

A bivariate dot plot, SSC-A (X-axis) versus SSC-H (Y-axis), of events in 'Time' gate. This plot is used to draw a region around single cells to exclude side scatter doublets, named "SSC Singlets". A similar consideration is applied as above in Plot 3 of Section 1.1.



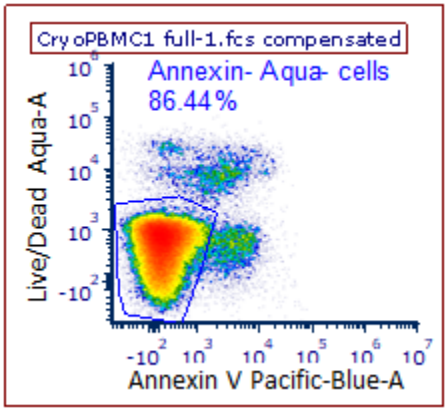
Plot 3

A bivariate dot plot, FSC-A (X-axis) versus FSC-H (Y-axis), of events in 'SSC singlets' gate. This plot is used to draw a region around single cells to exclude forward scatter doublets, named "Singlets". A similar consideration is applied as above in Plot 4 of Section 1.1.



Plot 4

A bivariate dot plot, Annexin V Pacific Blue-A (X-axis) versus Live/Dead Aqua-A (Y-axis), of 'Singlets'. This plot is used to draw a region around single cells to exclude dead and apoptotic cells that are positive for cellular amines and Annexin V, named "Annexin-Aqua- cells".

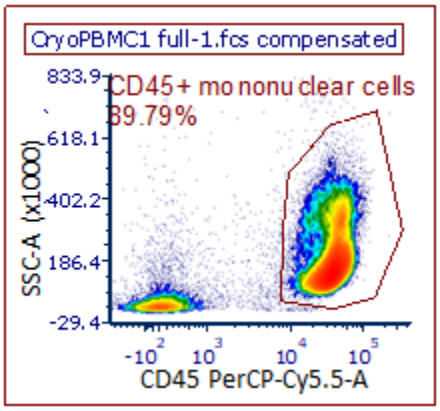


NOTE: QC synthetic cell Trucytes will not be assessed for viability or apoptosis. A separate analysis scheme will be applied, matching as much as feasible the analysis of the test sample.

NOTE: No viability threshold criteria will be applied to test sample. An abnormally low viability should be highlighted to the NIST but data for these must be entered in the spreadsheet.

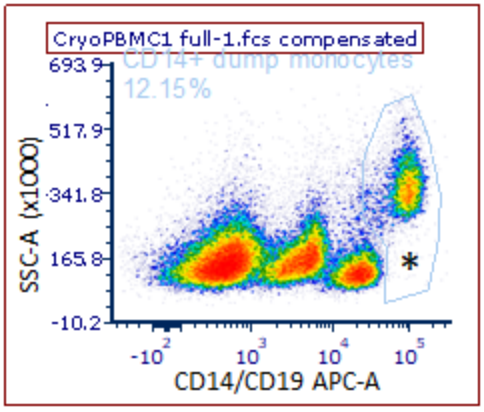
Plot 5

A bivariate dot plot, CD45 PerCP-Cy5.5-A (X-axis) versus SSC-A (Y-axis), of ‘Annexin-Aqua- cells’. This plot is used to draw a region around CD45+ cells that are live and non-apoptotic, named as “CD45+ mononuclear cells”.



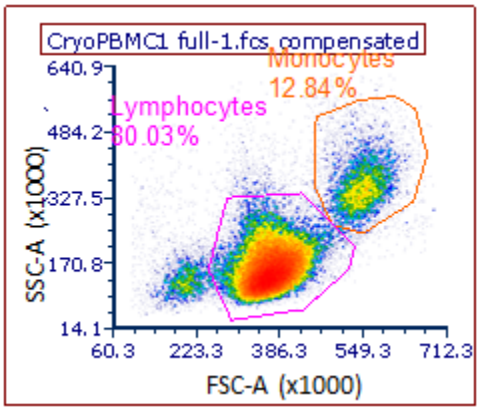
Plot 6

A bivariate dot plot, CD19/CD19 APC-A (X-axis) versus SSC-A (Y-axis), of the “CD45+ mononuclear Cells” gate. This plot is used to draw regions around the CD14bright monocytes, named “CD14+ dump monocytes”. This gate identifies $CD14^{bright}$ monocytes and include both the SSC^{med} (normal monocytes) and SSC^{low} (degranulated monocytes). The scope is to capture any degranulated monocytes in order to be able to subtract them for the ‘Lymphocytes’ gate from Plot 7. Note that the below sample does not contain degranulated monocytes but their expected location is noted with an *.



Plot 7

A bivariate dot plot, FSC-A (X-axis) versus SSC-A (Y-axis), of the 'CD45+ mononuclear cells' gate. This plot is used to draw regions around the following cell subsets: - "Lymphocytes" (CD45+, FSC^{low}, SSC^{low}), - "Monocytes" (CD45+, FSC^{med}, SSC^{med}).



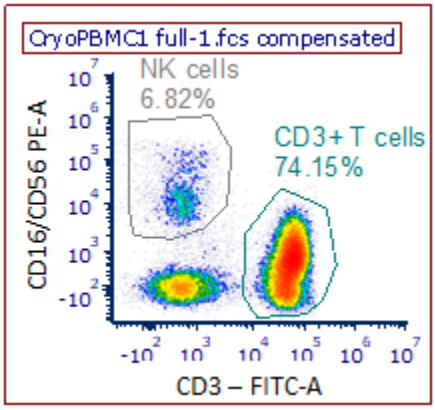
BOOLEAN GATING FOR LYMPHOCYTES GATE

In order to prevent potential contamination from degranulated monocytes, which may contaminate the lymphocyte gate, a boolean gating strategy is applied downstream of the 'Lymphocytes' gate. The events from the 'CD14+ dump monocytes' are substracted from the 'Lymphocytes' gate with the following Boolean equation: Lymphocytes AND NOT 'CD14+ dump monocytes'.

QCing the Boolean formula: Ensure no APC^{bright} cells appear in Plot 10 as correct analysis and Boolean gating would remove these cells from appearing in downstream Lymphocytes plots.

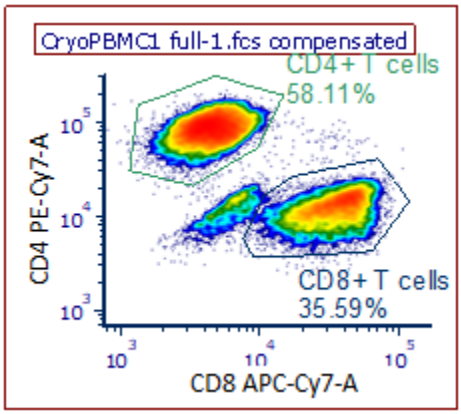
Plot 8

A bivariate dot plot, CD3 FITC-A (X-axis) versus CD16/CD56 PE-A (Y-axis), of the 'Lymphocytes' gate. This plot is used to draw regions around "CD3+ T cells" ($CD45^{+}$, FSC^{low}, SSC^{low}, $CD3^{+}$, $CD16/CD56^{-}$) and "NK cells" ($CD45^{+}$, FSC^{low}, SSC^{low}, $CD3^{-}$, $CD16/CD56^{+}$).



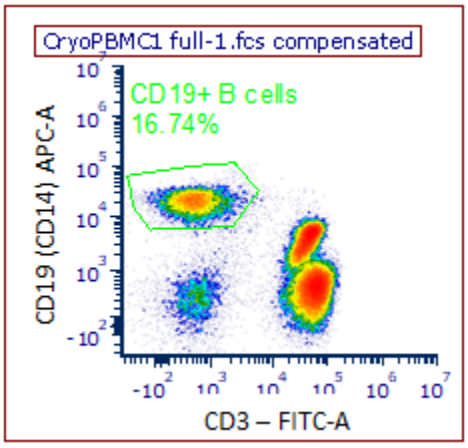
Plot 9

A bivariate dot plot, CD8 APC-Cy7-A (X-axis) versus CD4 PE-Cy7-A (Y-axis), of the 'CD3+ T cells' gate. This plot is used to draw regions around the following cell subsets: - "CD8+ T cells" ($CD45^{+}$, FSC^{low}, SSC^{low}, $CD3^{+}$, $CD8^{+}$) and "CD4+ T cells" ($CD45^{+}$, FSC^{low}, SSC^{low}, $CD3^{+}$, $CD4^{+}$).



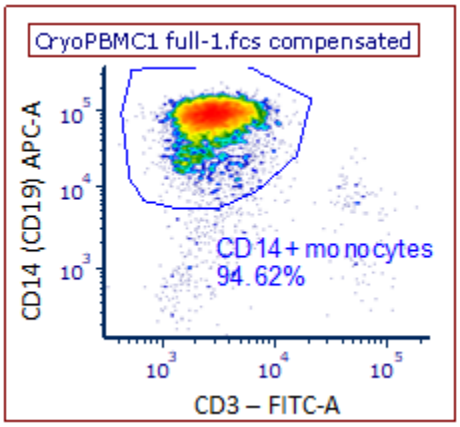
Plot 10

A bivariate dot plot, CD3 FITC-A (X-axis) versus CD19 (CD14) APC-A (Y-axis), of the "Lymphocytes" gate defined in Plot 7. This plot is used to draw regions around the "CD19+ B cells" subset ($CD45^{+}$, FSC^{low}, SSC^{low}, $CD3^{-}$, $CD19^{+}$).



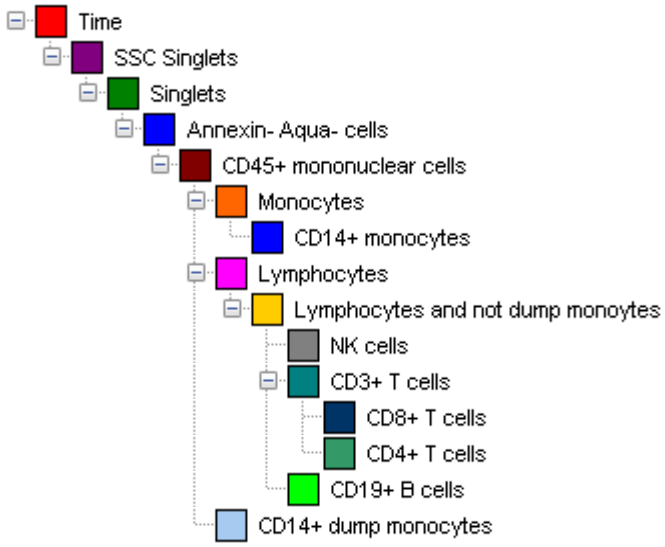
Plot 11

A bivariate dot plot, CD3 FITC-A (X-axis) versus CD14 (CD19) APC-A (Y-axis), of the “Monocytes” gate defined in Plot 7. This plot is used to draw region around “CD14+ monocytes”. Ensure to only include the CD14^{bright} cells similarly to Plot 6.



Population Hierarchy

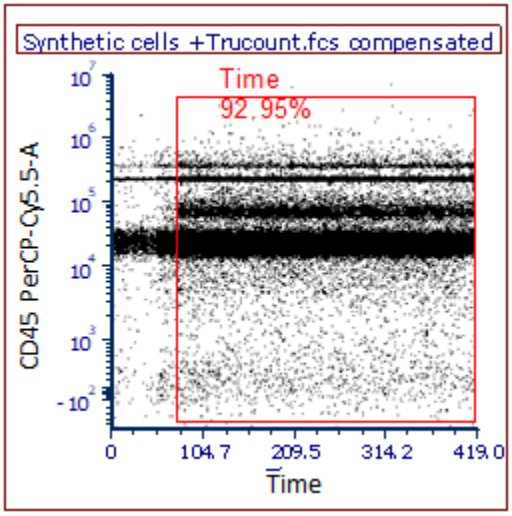
Population hierarchy for the TBNMK tube is provided below.



1.3. Analysis template for Trucount™ counting tube for QC synthetic cell Trucytes

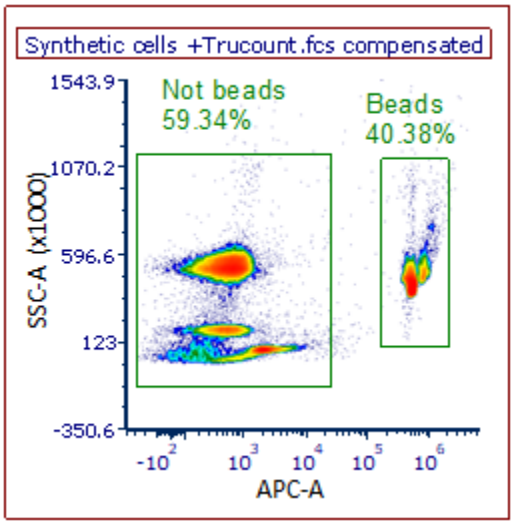
Plot 1

A bivariate dot plot, Time (X-axis) versus CD45 PerCP-Cy5.5-A (Y-axis), of all collected events. This plot is used for acquisition quality monitoring where all populations are equally present.



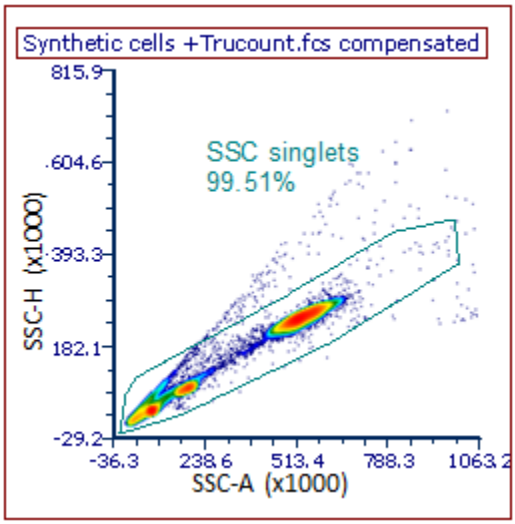
Plot 2

A bivariate dot plot, APC-A (X-axis) versus SSC-A (Y-axis), of events in 'Time' gate. This plot is used to draw a region around the "Beads" and the cellular events "Not beads". Ensure all beads are taken in the gate. Note specific APC fluorescence is not detected because all cellular events are APC negative. Beads autofluorescence is used to detect and gate them.



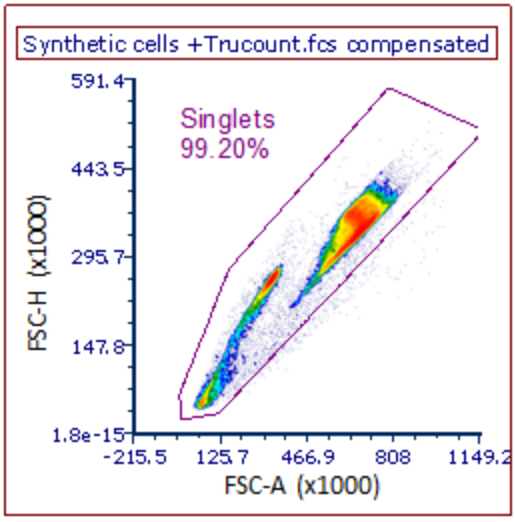
Plot 3

A bivariate dot plot, SSC-A (X-axis) versus SSC-H (Y-axis), of “Not beads”. This plot is used to draw a region around single cells to exclude side scatter doublets, named “SSC Singlets”. Note that scattering profiles of Trucytes are different from cryoPBMC.



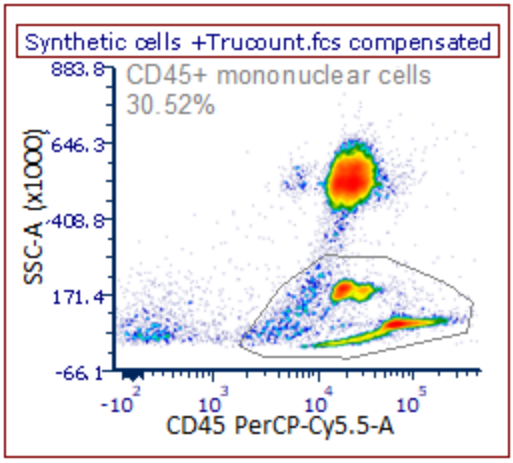
Plot 4

A bivariate dot plot, FSC-A (X-axis) versus FSC-H (Y-axis), of events in ‘SSC singlets’ gate. This plot is used to draw a region around single cells to exclude forward scatter doublets, named “Singlets”. Note that scattering profiles of Trucytes are different from cryoPBMC.



Plot 5

A bivariate dot plot, CD45 PerCP-Cy5.5-A (X-axis) versus SSC-A (Y-axis), of events in ‘Singlets’ gate. This plot is used to draw a region around the “CD45+ mononuclear cells”. The events in this gate will be used for the calculation of absolute counts. Note that Trucytes include granulocytes (CD45+ SSC^{high}) that are outside of the “CD45+ mononuclear cells” gate.



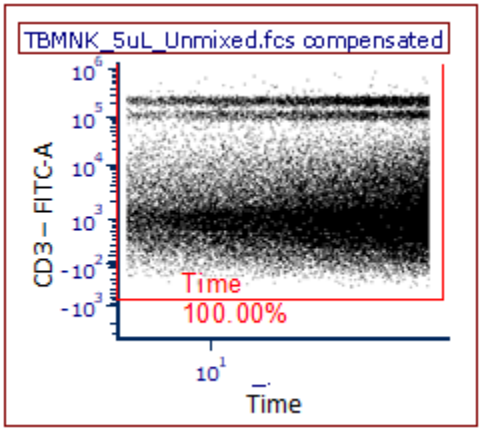
Population Hierarchy

Population hierarchy for the counting tube is provided below.

1.4. Analysis template for TBMNK panel for QC synthetic cell Trucytes

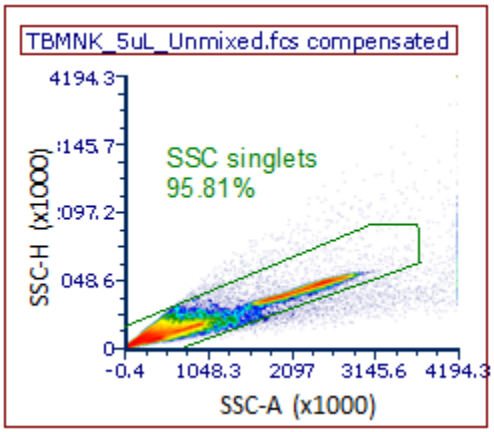
Plot 1

A bivariate dot plot, time (X-axis) versus CD3 FITC-A (Y-axis), of all collected events. This plot is used for acquisition quality monitoring.



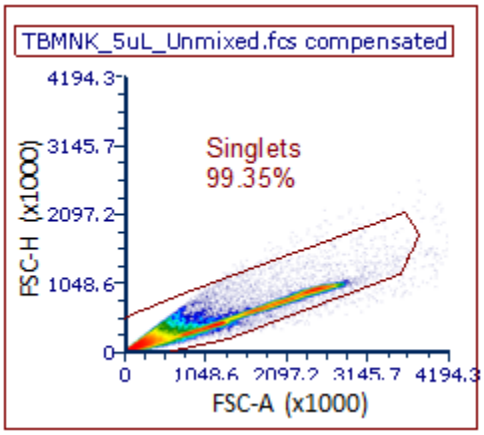
Plot 2

A bivariate dot plot, SSC-A (X-axis) versus SSC-H (Y-axis), of events in 'Time' gate. This plot is used to draw a region around single cells to exclude side scatter doublets, named "SSC Singlets". Do not exclude debris that are SSC^{low} at this stage since they are gated out in downstream Plot 4.



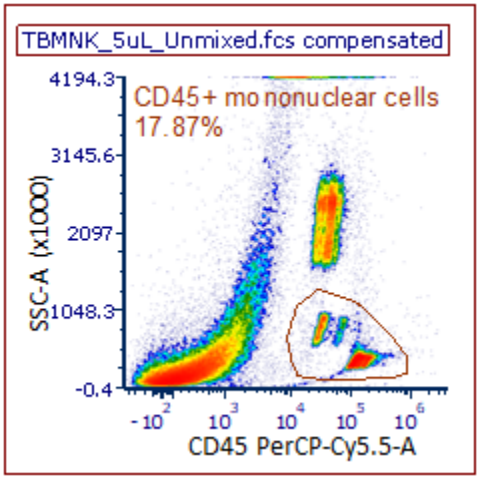
Plot 3

A bivariate dot plot, FSC-A (X-axis) versus FSC-H (Y-axis), of events in ‘SSC singlets’ gate. This plot is used to draw a region around single cells to exclude forward scatter doublets, named “Singlets”. Do not exclude debris that are FSC^{low} at this stage since they are gated out in downstream Plot 4.



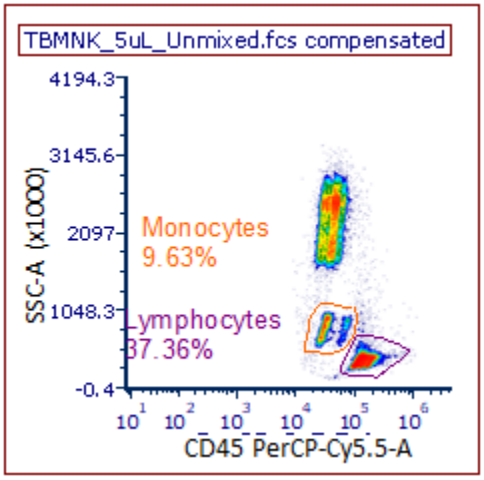
Plot 4

A bivariate dot plot, CD45 PerCP-Cy5.5-A (X-axis) versus SSC-A (Y-axis), of ‘CD45+ cells. This plot is used to draw a region around all of the CD45+ cells to exclude debris and generate a reliable gate for reporting daughter ‘monocytes’ and ‘lymphocytes’ populations. The gate is named “CD45+ mononuclear cells “. Note that Trucytes include granulocytes (CD45+ SSC^{high}) that are above the “CD45+ mononuclear cells” gate.



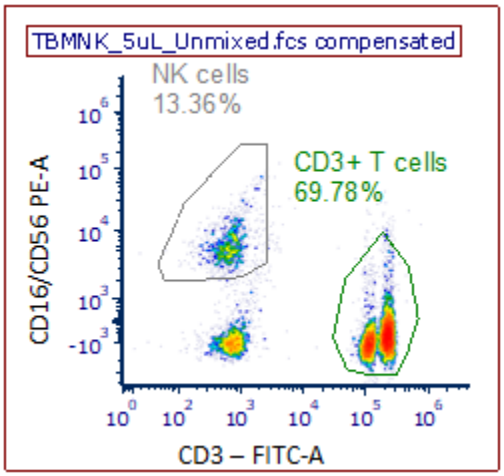
Plot 5

A bivariate dot plot, CD45 PerCP-Cy5.5-A (X-axis) versus SSC-A (Y-axis), of 'C'. This plot is used to draw regions around the following cell subsets: - "Lymphocytes" ($CD45^{bright}$, SSC^{low}), - "Monocytes" ($CD45^{+}$, SSC^{med}). Note that the monocytes of Trucytes consist of two distinct populations, classical and non-classic monocytes (also see Slingshot TDS for Trucytes TBMNK).



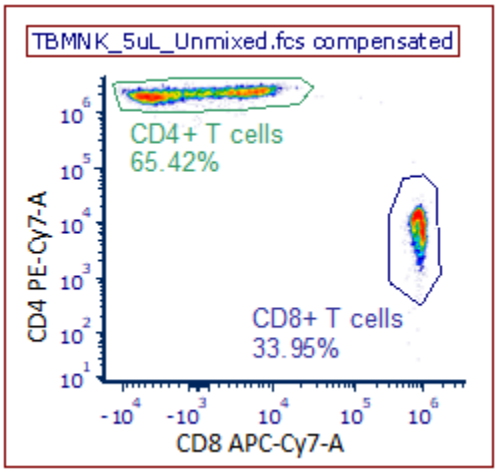
Plot 6

A bivariate dot plot, CD3 FITC-A (X-axis) versus CD16/CD56 PE-A (Y-axis), of the 'Lymphocytes' gate. This plot is used to draw regions around "CD3+ T cells" ($CD45^{bright}$, SSC^{low}, $CD3^{+}$, $CD16/CD56^{-}$) and "NK cells" ($CD45^{bright}$, SSC^{low}, $CD3^{-}$, $CD16/CD56^{+}$). Note that the CD3+ lymphocytes of Trucytes consist of two distinct populations, CD4+ and CD8+ lymphocytes (also see Slingshot TDS for Trucytes TBMNK).



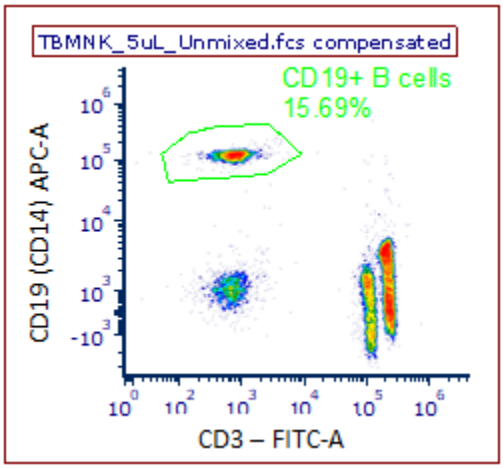
Plot 7

A bivariate dot plot, CD8 APC-Cy7-A (X-axis) versus CD4 PE-Cy7-A (Y-axis), of the 'CD3+ T cells' gate. This plot is used to draw regions around the following cell subsets: - "CD8+ T cells" ($CD45^{bright}$, SSC^{low}, $CD3^{+}$, $CD8^{+}$) and "CD4+ T cells" ($CD45^{bright}$, SSC^{low}, $CD3^{+}$, $CD4^{+}$).



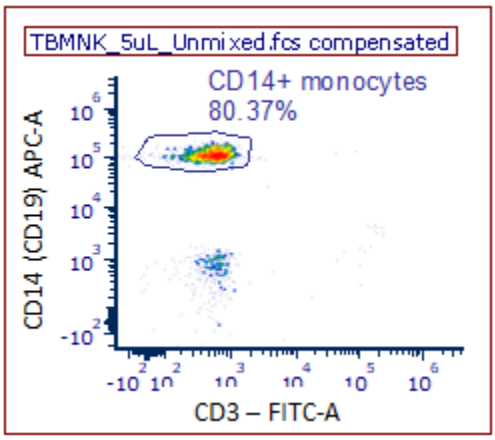
Plot 8

A bivariate dot plot, CD3 FITC-A (X-axis) versus CD19 (CD14) APC-A (Y-axis), of the "Lymphocytes" gate. This plot is used to draw regions around the "CD19+ B cells" subset ($CD45^{bright}$, SSC^{low}, $CD3^{-}$, $CD19^{+}$).



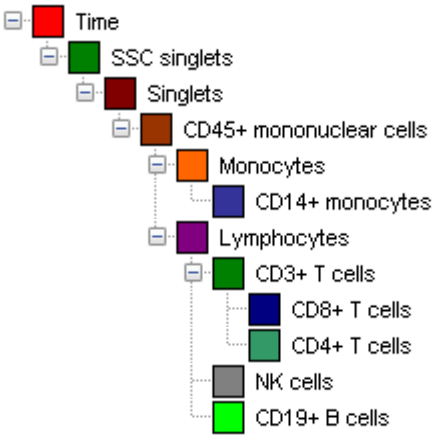
Plot 9

A bivariate dot plot, CD3 FITC-A (X-axis) versus CD14 (CD19) APC-A (Y-axis), of the “Monocytes” gate. This plot is used to draw region around “CD14+ monocytes”.



Population Hierarchy

Population hierarchy for the TBNMK tube for QC synthetic cell Trucytes is provided below.



1.5. WG2 ILS Reporting Spreadsheet

Summary population hierarchy and associated assay reportable and values results needed to generate data to enter in the WG2 ILS Reporting Spreadsheet are listed in Table 1 and Table 2.
The list of reportable results for the test sample and synthetic cells QC material are listed in the below sections.

Table 1. Values to record to obtain reportables for counting tube

Name of population in analysis template	List of parameters to record
Time	NA
Beads	Events*
Not beads	NA
SSC Singlets	NA
Singlets	NA
Annexin-Aqua- cells (for cryo PBMC only)	NA
CD45+ mononuclear cells	Events*

*Will be used for absolute counts calculations

Table 2. Values to record to obtain reportables for immunophenotyping tube

Name of population in analysis template	List of parameters to record			
Time	NA			
SSC Singlets	NA			
Singlets	NA			
Annexin-Aqua- cells (for cryo PBMC only)	% parent	NA	NA	NA
CD45+ mononuclear cells	% parent**	Events*	MdFI PerCP-Cy5.5	rSD PerCP-Cy5.5
Monocytes	% parent	NA	NA	NA
CD14+ monocytes	% parent	Events*	MdFI APC	rSD APC
Lymphocytes	NA			
Lymphocytes and not dump monocytes	% grandparent	NA	NA	NA
NK cells	% parent	Events*	MdFI PE	rSD PE
CD3+ T cells	% parent	Events*	MdFI FITC	rSD FITC
CD8+ T cells	% parent	Events*	MdFI APC-Cy7	rSD APC-Cy7
CD4+ T cells	% parent	Events*	MdFI PE-Cy7	rSD PE-PE-Cy7
CD19+ B cells	% parent	Events*	MdFI APC	rSD APC
CD14+ dump monocytes	NA			

*Will be used for absolute counts calculations

**For cryoPBMC only

1.6. Summary population hierarchy and associated assay reportable results to enter in the WG2 ILS Reporting Spreadsheet

Population Hierarchy is provided in Section 1.2 and the list of reportable results for each sample and QC material are listed in Tables 3 and 4.

Table 3: Test Sample (cryoPBMC) Reportable Results

population	Immunophenotype	Reporting Unit
Annexin-Aqua- cells	Aqua-, Annexin-	% of singlets (% parent)
CD45+ mononuclear cells	Aqua-, Annexin-, CD45+	% Annexin-Aqua- cells (% parent)
		cells/uL*
		MdFI and rSD CD45 PerCP-Cy5.5
Monocytes	Aqua-, Annexin-, CD45+, FSCmed, SSCmed	% CD45+ cells (% parent)
CD14+ monocytes	Aqua-, Annexin-, CD45+, FSCmed, SSCmed, CD14+/hi	% monocytes (% parent)
		cells/uL*
		MdFI and rSD CD14 APC
Lymphocytes And Not Dump Monocytes	Aqua-, Annexin-, CD45+, FSClow, SSClow	% CD45+ cells (% grandparent)
CD3+ T cells	Aqua-, Annexin-, CD45+, FSClow, SSClow, CD3+	% of Lymphocytes And Not Dump Monocytes (% parent)
		cells/uL*
		MdFI and rSD CD3 FITC
CD4+ T cells	Aqua-, Annexin-, CD45+, FSClow, SSClow, CD3+, CD4+	% of CD3+ T cells (% parent)
		cells/uL*
		MdFI and rSD CD4 PE-Cy7
CD8+ T cells	Aqua-, Annexin-, CD45+, FSClow, SSClow, CD3+, CD8+	% of CD3+ T cells (% parent)
		cells/uL*
		MdFI and rSD CD8 APC-Cy7
CD19+ B cells	Aqua-, Annexin-, CD45+, FSClow, SSClow, CD19+	% of Lymphocytes And Not Dump Monocytes (% parent)
		cells/uL*
		MdFI and rSD CD19 APC
NK cells	Aqua-, Annexin-, CD45+, FSClow, SSClow, CD56+/CD16+:	% of Lymphocytes And Not Dump Monocytes (% parent)
		cells/uL*
		MdFI and rSD CD56 CD16 PE

*absolute counts may be derived using volumetric counting or Trucount™ tube method

Table 4. QC Synthetic cell Trucytes Reportable Results

population	Immunophenotype	Reporting Unit
CD45+ mononuclear cells	CD45+	NA (no % will be reported)
		cells/uL*
		MdFI and rSD CD45 PerCP-Cy5.5
Monocytes	CD45+, SSCmed	% CD45+ cells % parent)
CD14+ monocytes	CD45+, SSCmed, CD14+	% monocytes (% parent)
		cells/uL*
		MdFI and rSD CD14 APC

Lymphocytes	CD45bright, SSClow	% CD45+ cells (% parent)
CD3+ T cells	CD45bright, SSClow, CD3+	% of Lymphocytes (% parent)
		cells/uL*
		MdFI and rSD CD3 FITC
CD4+ T cells	CD45bright, SSClow, CD3+, CD4+	% of CD3+ T cells (% parent)
		cells/uL*
		MdFI and rSD CD4 PE-Cy7
CD8+ T cells	CD45bright, SSClow, CD3+, CD8+	% of CD3+ T cells (% parent)
		cells/uL*
		MdFI and rSD CD8 APC-Cy7
CD19+ B cells	CD45bright, SSClow, CD19+	% of Lymphocytes (% parent)
		cells/uL*
		MdFI and rSD CD19 APC
NK cells	CD45bright, SSClow, CD56+/CD16+:	% of Lymphocytes (% parent)
		cells/uL*
		MdFI and rSD CD56 CD16 PE

*absolute counts may be derived using volumetric counting or Trucount™ tube method

1.7. Analysis Procedure for Counting Tube

- Import compensated .fcs files for the Test Samples and the QC material into counting tube analysis template (analysis template guidelines described in Section 1.1 and Appendix A and in Section 1.3 and Appendix C).
- **NOTE**: Compensated Matrix 1 generated with cryoPBMC and lyoLeuk is suggested to be applied to the counting tubes for the test samples and QC material as specified in the WG2 ILS Reporting Spreadsheet.
- Analyze data files as described in Section 1.1 and 1.3 in order to derive CD45+ cells absolute counts. Generate pdf of the batch files and enter reportable results in WG2 ILS Reporting Spreadsheet format.
- Generate pdf of the batch files and use events numbers in order to calculate absolute.

1.7.1. Analysis Procedure for TBMNK Panel Compensation Matrix 1

- Import uncompensated .fcs files for the Test Samples and the QC material into analysis template (analysis template guideline described in Section 1.2 and Appendix B and in Section 1.4 and Appendix D)
- Apply compensation matrix 1 to all of the files (FMOs and fully stained tubes)
- Set gates as described in Section 1.2 for the full stain tube and cross verify that the gates are appropriate by verifying the FMO pattern.
- Once gets are set, run analysis on full stain triplicates as a batch without changing gates between replicates.
- Generate pdf of the batch files and enter reportable results for % of parent in WG2 ILS Reporting Spreadsheet format.
- Follow sections 1.7.4, 1.7.5, and 1.8 for the absolute counts and ERF calculations.

1.7.2. Compensation Matrix 2

- Import uncompensated .fcs files for the Test Samples and the QC material into appropriate analysis template (analysis guideline described in Section 4.2 and Appendix B and in Section 1.4 and Appendix D).
- Apply compensation matrix 2 to all of the files (FMOs and fully stained tubes).
- Set gates as described in Section 1.2 for the full stain tube and cross verify that the gates are appropriate by verifying the FMO pattern.
- Once gets are set, run analysis on full stain triplicates as a batch without changing gates between replicates.
- Generate pdf of the batch files and enter reportable results for % of parent in WG2 ILS Reporting Spreadsheet format.
- Follow sections 1.7.4, 1.7.5, and 1.8 for the absolute counts and ERF calculations.

1.7.3. Compensation Matrix 3

- Import uncompensated .fcs files for the Test Samples and the QC material into appropriate analysis template (analysis template guideline described in Section 4.2 and Appendix B and in Section 1.4 and Appendix D).
- Apply compensation matrix 3 to all of the files (FMOs and fully stained tubes).
- Set gates as described in Section 1.2 for the full stain tube and cross verify that the gates are appropriate by verifying the FMO pattern.
- Once gets are set, run analysis on full stain triplicates as a batch without changing gates between replicates.
- Generate pdf of the batch files and enter reportable results for % of parent in WG2 ILS Reporting Spreadsheet format.
- Follow sections 1.7.4, 1.7.5, and 1.8 for the absolute counts and ERF calculations.

1.7.4. Cell Counting using volumetric method

- For volumetric flow cytometers, cell count can be directly obtained from acquired data files showing a known analytical volume and event and percentage of the cell population.

1.7.5. Cell Counting using Trucount™ method

- Collect data from cell counting Trucount™ tube analysis pdf and perform the following calculation to determine the CD45+ cells absolute count in each sample and each compensation matrix

- $\text{CD45+ mononuclear cells cells/µL} = \frac{(\text{CD45+ mononuclear cells events}) \times (\text{bead counts}) *}{(\text{beads events}) \times (100\ \mu\text{L sample volume used})}$

*lot specific and provided in manufacturer's package insert

- Once the CD45+ cell count has been determined, calculate absolute counts for each CD45+ cell subsets from the full stain tube.

T cells:

- $$\text{CD3+ T cells cells/µL} = \frac{(\text{CD45}^{+}\text{ mononuclear cells absolute count from counting tube}) \times (\text{CD3+ T cells events})}{(\text{CD45+ mononuclear cells events})}$$

CD4 T cells:

- $$\text{CD4+ T cells cells/µL} = \frac{(\text{CD45}^{+}\text{ mononuclear cells absolute count from counting tube}) \times (\text{CD4+ T cells events})}{(\text{CD45+ mononuclear cells events})}$$

CD8 T cells:

- $$\text{CD8+ T cells cells/µL} = \frac{(\text{CD45}^{+}\text{ mononuclear cells absolute count from counting tube}) \times (\text{CD8+ T cells events})}{(\text{CD45+ mononuclear cells events})}$$

B cells:

- $$\text{CD19+B cells cells/µL} = \frac{(\text{CD45}^{+}\text{ mononuclear cells absolute count from counting tube}) \times (\text{B cells events})}{(\text{CD45+ mononuclear cells events})}$$

CD14+ monocytes cells:

- $$\text{CD14+ monocytes cells/µL} = \frac{(\text{CD45}^{+}\text{ mononuclear cells absolute count from counting tube}) \times (\text{CD14+ monocytes events})}{(\text{CD45+ mononuclear cells events})}$$

NK cells:

- $$\text{NK cells cells/µL} = \frac{(\text{CD45}^{+}\text{ mononuclear cells absolute count from counting tube}) \times (\text{NK cells events})}{(\text{CD45+ mononuclear cells events})}$$

- Enter absolute cell counts into WG2 ILS Reporting Spreadsheet.

1.8. Determination of instrument performance characteristics and ERF values of CD markers

- Insert (copy and paste values) raw data, MdFI, rCV and rSD of the Rainbow 8-peak beads obtained for the 8 fluorescence channels in the 'Column G', 'Column H', and 'Column I', respectively, in the 'Instrument' sheet of the WG2 ILS Reporting Spreadsheet to the respective columns in Row 30-37 for each fluorescence channel (labeled as FITC,-A, APC,-A, etc. in Row 29) in the 'Raw Data' sheet (2nd sheet) of the WG2 Cytometer Sensitivity and ERF Calibration spreadsheet. Raw data for the two fluorescence channels, FITC and APC is shown as examples in Figure 2. [**Note**: i) Users should make a copy of the original WG2 Cytometer Sensitivity and ERF Calibration spreadsheet and insert your data in the copy of the spreadsheet; ii) Users are not allowed to make any changes in cells highlighted in blue; iii) Data from minimal 5 peaks with distinguishable fluorescence signals should be entered starting from Row 30. Peak_01 does not have to be the corresponding peak 1 in the Rainbow 8-peak beads.

28	Sample	FITC-A			APC,-A			APC-Cy7,-A
29	Statistic	FITC-A	CV,FITC-A	SD,FITC-A	APC,-A	CV,APC,-A	SD,APC,-A	APC-Cy7,-A
30	Peak_01	17.22	1.4825784	25.53	58.32	0.720679	42.03	4.86
31	Peak_02	291.92	0.12078652	35.26	613.17	0.2076096	127.3	44.55
32	Peak_03	826.56	0.06618999	54.71	1674.67	0.1337398	223.97	118.26
33	Peak_04	2415.72	0.04076217	98.47	4774.95	0.0767087	366.28	349.92
34	Peak_05	5971.65	0.02799226	167.16	11856.38	0.0497825	590.24	865.89
35	Peak_06	17266.74	0.02084122	359.86	34175.52	0.0371424	1269.36	2536.11
36	Peak_07	51448.44	0.01775797	913.62	97947.63	0.0306639	3003.46	7610.36
37	Peak_08	108846.39	0.01710576	1861.9	165218.95	0.0308551	5097.85	14037.3
38	Peak_09							
39	Peak_10							
40	FS Bead ERF	35072.16			42203.06			32284.44
41	FS Bead Mean	21311.00			35279.00			13758.00
42	FS Bead rSD	1590.00			2006.00			
43	CDx Mean				20000.00			
44	CDx rSD							
45	CDy Mean				25000.00			
46	Cdy rSD							

Figure 2. A portion of the data sets in the 'Raw Data' sheet of the WG2 Cytometer Sensitivity and ERF Calibration spreadsheet.

- Enter MdFI and rSD of respective FC bead obtained from SOP-1 and recorded in the 'Instrument' sheet of the WG2 ILS Reporting Spreadsheet in Row 41 and 42 in the same 'Raw Data' sheet as shown in Figure 2.
- Insert an ERF value assigned by NIST to each FC bead in Row 40 in the 'Raw Data' sheet. The NIST assigned ERF values for the 8 FC beads will be provided to participants separately.
- Enter the MdFI and rSD values of the different cell markers in the respective Column and Rows in the 'Raw Data' sheet of the WG2 Cytometer Sensitivity Spreadsheet (Rows 43-46).

- Press **only once** the 'Analyze Data' button on the top of the 'Raw Data' sheet. The automatic calculations will be proceeded using the 'Analysis Template' sheet (1st sheet of the spreadsheet) and raw data entered in the "Raw Data' sheet. The outputs of the automatic calculations are the individual fluorescence channel/detector worksheets added to the spreadsheet before the 'Analysis Template' sheet. Note: for more information on the use of the automated WG2 Cytometry Sensitivity and ERF Calibration spreadsheet, please see 'Using the Excel Spreadsheet to Analyze Multi-Intensity Ranging Data'.
- The cytometer performance characteristics generated from the 'Analysis Data' program in Step above are reported in the 'Summary' sheet (last sheet) of the spreadsheet. An example summary report is provided in Figure 3.

Summary

Date: 19-May-2022

0

0

Sample Name	Spe (Q_{Spe})	B_{Spe}	B_{IU}	$CV_{Intrinsic}$	Q_{ERF}	B_{ERF}	CDx_{ERF}	CDy_{ERF}	Goodness of Fit	Model Used
Analysis Template	1.82E-01	9.65E+02	5.30E+03	1.37E-02	1.82E-01	5.30E+03			9.94E-01	QUAD FIT
FITC-A	4.36E-01	4.78E+02	1.10E+03	1.65E-02	2.65E-01	6.66E+02			9.58E-01	QUAD FIT
APC,-A	4.13E-01	1.74E+04	4.22E+04	3.05E-02	3.45E-01	3.53E+04	2.39E+04	2.99E+04	8.28E-01	QUAD FIT
APC-Cy7,-A	1.32E-01	-1.70E+01	-1.29E+02	1.62E-02	5.63E-02	-5.49E+01			1.84E-40	QUAD FIT
PE,-A	4.07E-01	2.08E+03	5.12E+03	2.31E-02	6.17E-02	7.77E+02			8.48E-01	QUAD FIT
PE-Cy7,-A	1.54E+01	3.68E+05	2.39E+04	1.96E-02	6.97E-01	1.08E+03			9.33E-01	QUAD FIT
Pacific Blue,-A	-3.66E-01	4.01E+03	-1.10E+04	2.10E-02	-3.66E-01	-1.10E+04			6.12E-01	QUAD FIT
Aqua,-A	-1.92E-02	7.60E+00	-3.96E+02	1.10E-01	-1.92E-02	-3.96E+02			4.33E-04	QUAD FIT
PerCP-Cy5.5,-A	1.82E-01	9.65E+02	5.30E+03	1.37E-02	1.82E-01	5.30E+03			9.94E-01	QUAD FIT

Figure 3. Example summary report on the instrument performance characteristics and ERF values of CD markers.

- Report Q_{ERF} values for 8 fluorescence channels/detectors in Column 'J' and the analysis date/operator in Column 'K', respectively in the 'Instrument' sheet of the WG2 ILS Reporting Spreadsheet.
- Report ERF values for 7 CD markers in Column 'J' and the analysis date/operator in Column 'K', respectively in the 'Sample Data Analysis' sheet of the WG2 ILS Reporting Spreadsheet. Optional: Participant can record sample filename (either Local Filename from the 'Test and QC Samples' sheet or FCSC ILS Filename from the 'FCSC ILS Filenames' sheet) used for the analysis in Column 'L' using copy/paste function keys. This Column 'L' serves as a sample file place holder for participant to track the fcs file used for value determination.
- Copy and paste the summary report table alike Figure 3 in the 'Summary' sheet as an image at the bottom of the 'Sample Data Analysis' sheet of the WG2 ILS Reporting Spreadsheet (below Row 314).

1.9. File Export for WG3

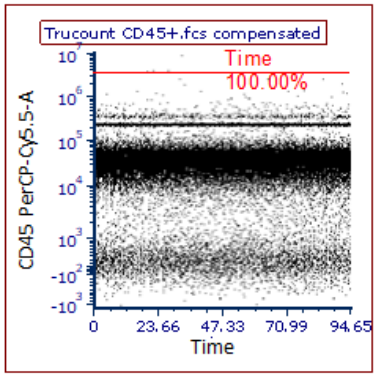
- Complete the WG2 ILS Reporting Spreadsheet and return to NIST.

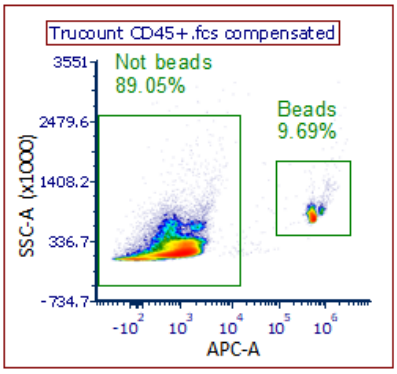
Attachments

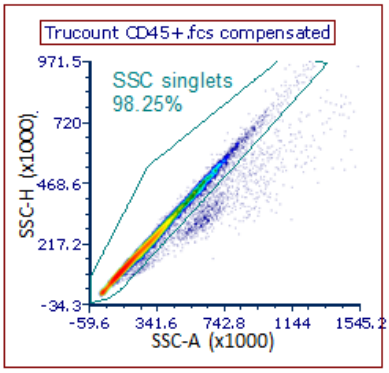
Appendix A: Screenshot of counting tube for Cryopreserved PBMCs
Appendix B: Screenshot of analysis template for full stain of Cryopreserved PBMCs
Appendix C: Screenshot of Trucount™ counting tube for QC Synthetic Cell Trucytes
Appendix D: Screenshot of analysis template for full stain of QC Synthetic Cell Trucytes
Slingshot TDS for Trucytes TBMNK

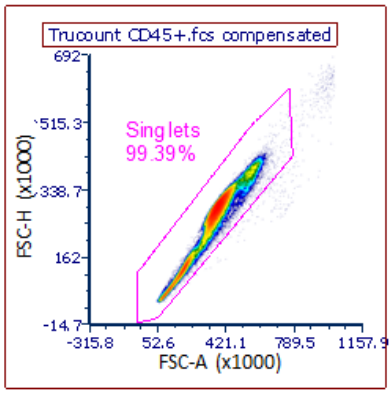
Appendix A: Analysis Template for Counting Tube:

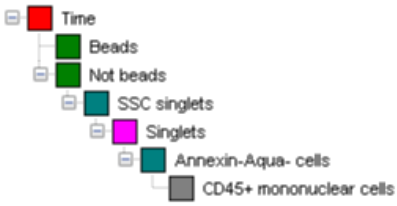
Cryopreserved PBMCs

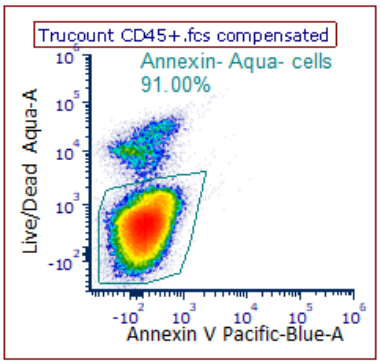


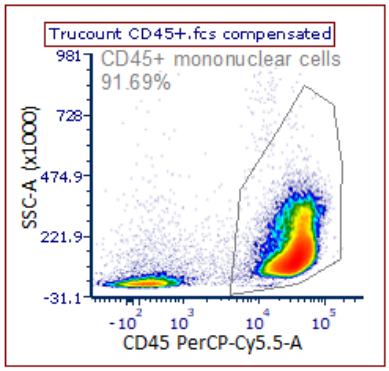






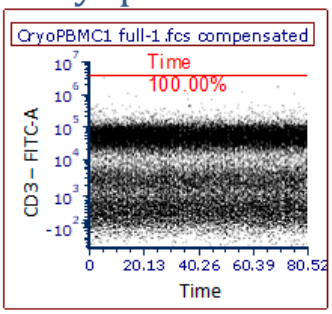


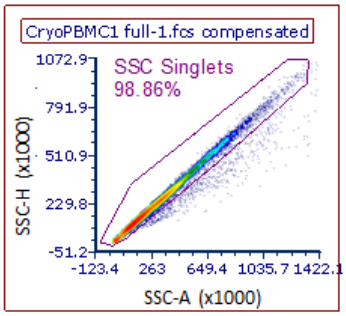


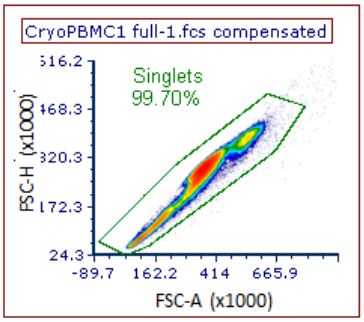


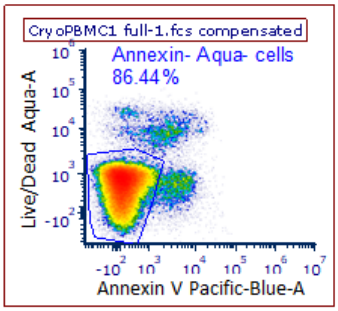
Appendix B: Analysis Template for TBMNK Assay:

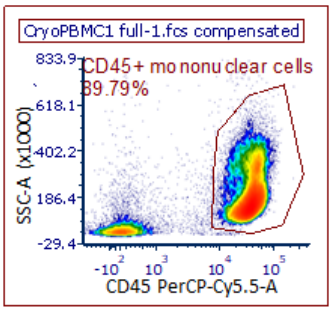
Cryopreserved PBMCs

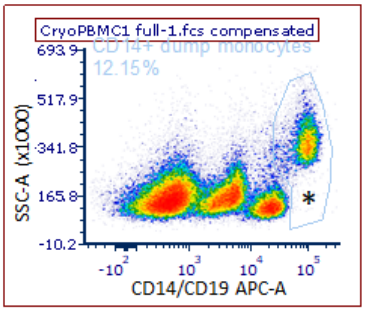


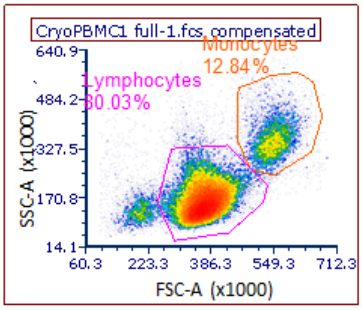


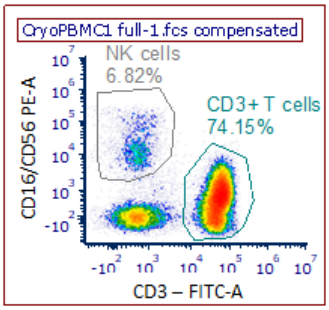


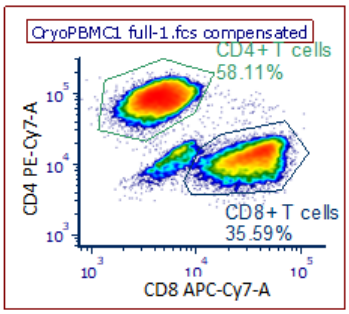


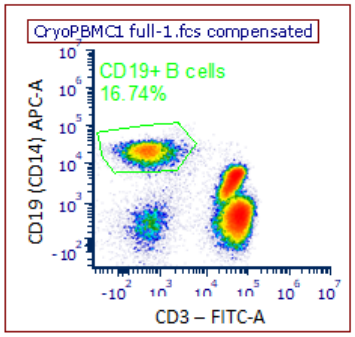


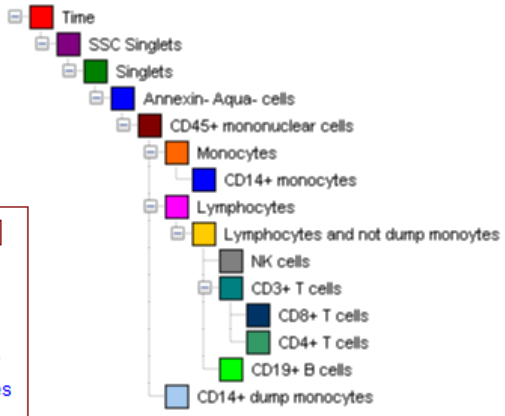


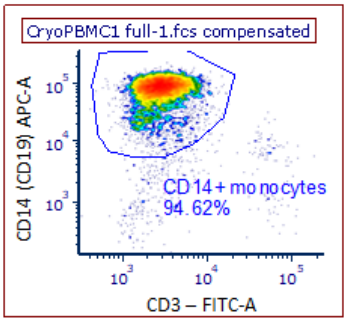




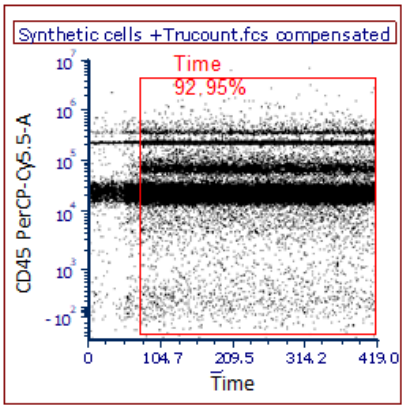


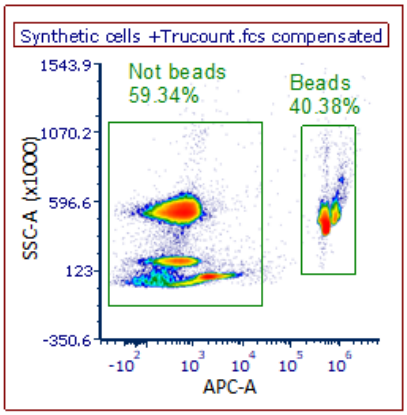


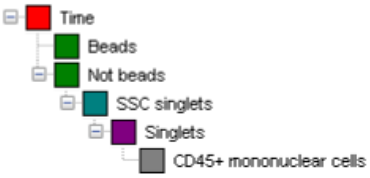


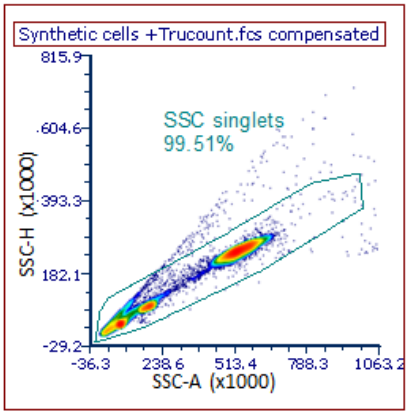


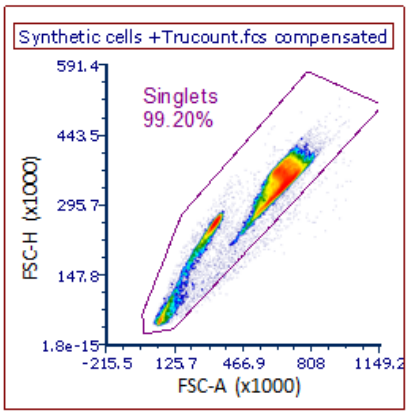
Appendix C: Analysis Template for Trucount™ Counting Tube: QC Synthetic Cell Trucytes

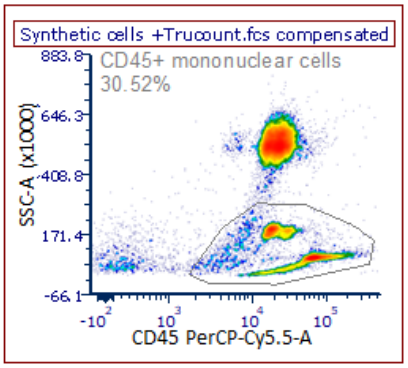












Appendix D Analysis Template for TBMNK Tube:

QC Synthetic Cell Trucytes