EXAMPLE INSTRUCTIONS ONLY

ROUND-SPECIFIC INSTRUCTIONS WILL BE PROVIDED AFTER REGISTERING TO A ROUND

Instructions for performing the EuroFlow LST EQA round

The objective of the EuroFlow LST EQA scheme is to evaluate the technical quality of sample preparation, measurement on the flow cytometer, and analysis of fcs files related to the EuroFlow LST panel. Participation is suitable for laboratories that use the LST panel and related EuroFlow SOPs in their routine diagnostics.

***Note**: In this document, links to EuroFlow SOPs are shown in blue and can be accessed after user login on https://app.euroflow.org/downloads/public.*

Samples:

No samples are provided by the EQA provider. Instead, use three healthy-donor peripheral blood samples drawn locally at your institution. During the complete procedure, the samples should be treated in the same manner as routine samples.

Reagents:

Reagents used for staining are based on the markers and fluorochromes from the EuroFlow LST panel (Table 1).

Table 1 - Composition of the EuroFlow LST panel

PacB	PacO	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7
CD20 and CD4	CD45	CD8 and SmIgλ	CD56 and SmIgκ	CD5	CD19 and TCRγδ	SmCD3	CD38

van Dongen JJ, Lhermitte L, Böttcher S, et al. EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708). EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia. 2012 Sep;26(9):1908-75

For staining of samples, use either one of the following two reagents options:

1. **Reference antibodies of the EuroFlow LST panel**, in the specified amounts (Table 2):

***Note**: Please do not change the reagents nor the amounts used.*

Table 2 – Reference antibodies of the EuroFlow LST panel

Marker	Fluorochrome	Clone	Source	Catalog no.	µL/test
smCD3	APC	SK7	BD Biosciences	345767	2.5
CD4	PacB	RPA-T4	BioLegend	300521	0.5
CD5	PerCPCy5.5	L17F12	BD Biosciences	341109	15
CD8	FITC	UCH-T4	Cytognos	CYT-SLPC-50	20 (Part of LST mixture)
CD19	PECy7	J3-119	Beckman Coulter	IM3628	5

Marker	Fluorochrome	Clone	Source	Catalog no.	µL/test
CD20	PacB	2H7	BioLegend	302320	1
CD38	APCH7	HB7	BD Biosciences	EU: 656646 USA: 653314	3
CD45	PacO	HI30	Invitrogen	MHCD4530	5
CD56	PE	C5.9	Cytognos	CYT-SLPC-50	20 (Part of LST mixture)
SmIgκ	PE	Polyclonal	Cytognos	CYT-SLPC-50	20 (Part of LST mixture)
SmIgλ	FITC	Polyclonal	Cytognos	CYT-SLPC-50	20 (Part of LST mixture)
TCRγδ*	PECy7	11F2	BD Biosciences	EU: 655410 USA: 655434	3

* Please note that TCRγδ is included in the panel but is currently not evaluated in the LST scheme.

As an alternative to the reference antibodies, EuroFlow-approved alternative antibodies may be used but this should be done with caution. Alternative antibodies have been tested as single reagents in comparison with the EuroFlow reference reagents. However, performance of alternative reagents in the EuroFlow 8-color combination as well as modifications in the performance characteristics of these reagents (e.g., in different lots) is out of EuroFlow's control. Alternative antibodies are listed in the following document:

- List of reference and alternative antibodies for hematol malignancies panels Version 1.13 - 13 November 2023

2. **The BD OneFlow™ LST** (Catalog no. 658619) reagents in the format of the premixed LST tube cocktail.

Sample preparation:

For sample preparation, apply either one of the following two options:

1. When the **reference antibodies of the EuroFlow LST panel** (Table 2) are used:
   - Refer to sections 2 and 4 of the EuroFlow SOP for sample preparation:
   - EuroFlow SOP for Sample Preparation Version 1.7 – 1 November 2023

***Note**: Do not add additional washing buffer as is indicated in section 2.10 as you may not succeed in acquiring 50.000 lymphocytes. The sample volume after the washes should be 50 – 100 µL. To stain surface markers, take 50 µL of the sample and add 50 µL of antibody mixture to reach a final volume of 100 µL.*

2. When the **BD OneFlow™ LST** is used:
   - Refer to the instructions enclosed with the product for sample preparation.

Instrument setup:

EuroFlow has designed specific protocols for the setup and compensation of BD Canto, BD Lyric, BD LSR Fortessa, BC Navios, and BC DxFlex cytometer instruments.

For instrument setup and compensation, follow the appropriate EuroFlow SOP:

- EuroFlow SOP for Instrument set-up and compensation for BD LSR II and BD FACS Canto Version 1.2.1 - 29 October 2019
- EuroFlow SOP for instrument set-up and compensation for BD Lyric instrument for the 8-color panels Version 1.8 - 1 July 2019

- EuroFlow SOP for Instrument set-up and compensation for BD LSRFortessa X-20 Instruments Version 2.1 - 15 October 2018
- EuroFlow SOP for Instrument set-up and compensation for Navios Instruments Version 1.5.1 - 29 October 2019
- EuroFlow SOP instrument setup and compensation for DxFlex Version 1.2 - 19 July 2023

Data acquisition:

If applicable for the used cytometer:

- Acquire Rainbow Beads and record Median Fluorescence Intensities (MedFIs) of the 7th peak using the EuroFlow recommended LOT of Rainbow Beads:
- EuroFlow target MFI values for Rainbow beads Version 1.10 - 16 November 2023

***Note**: It is advisable but not mandatory to report the values for Rainbow Beads. In case your performance results are unsuccessful, the reported MedFI values of the 7th peak in each channel will allow us to see whether the instrument has been set up properly.*

- Acquire 3 samples of stained peripheral blood.
- Label each tube with the donor number.
- Acquire 50.000 lymphocytes or the maximum available amount from the tube.

Data analysis (Infinicyt software recommended):

- Load the provided LST EQA Infinicyt Profile (.inp file).
  ***Note**: Do not change the EQA profile in any way (e.g., by adding your own populations, deleting, or renaming the existing ones, etc.)*
  ***Note**: Do not use the EQA profile embedded in the Infinicyt software. Use the provided EQA profile instead.*
- Identify (gate) all required lymphocyte subpopulations according to the LST EQA gating strategy (Figure 1).
  ***Note**: If there are too few lymphocytes, the rare CD56hi NK-cells will be too few to gate with confidence.*
- Obtain MedFI values of:
  - **CD20 PacB** for B cells
    and **CD4 PacB** for CD4$^+$ T cells
  - **CD45 OC-515** for T cells
  - **CD8 FITC** for CD8$^+$ T cells
    and **IgL FITC** for Lambda$^+$ B cells
  - **CD56 PE** for CD56hi NK cells
    and **IgK PE** for Kappa$^+$ B cells
  - **CD5 PerCP-Cy5.5** for T cells
  - **CD19 PE-Cy7** for B cells
  - **CD3 APC** for T cells
  - **CD38 APC-C750** for Monocytes

***Note**: You can export the statistics to a csv file (go to Statistics – Export statistics, check only the values you need and save the configuration for the next files).*

- Save the analyzed file and include the donor number in the file name.

Data reporting:

- Log in to https://eqa.eslho.org/
- Complete the following:
  - Institute details including contact details of the main contact person
  - Cytometer used for data acquisition.
  - Rainbow Beads LOT no.
  - MedFI values of the 7th peak of the Rainbow Beads in each channel, rounded to whole numbers.
  - MedFI values of the populations in each donor sample, rounded to whole numbers.
    ***Note 1**: Report MedFI values even if you used mean fluorescence intensities for instrument setup according to the EuroFlow SOP.*
    ***Note 2**: DxFlex users need to either convert the fcs files to 2^{18} scale in Infinicyt before starting the analysis or use the following formula to recalculate the numbers:*
    *x = (((y * 1.024) / 4.000.000) * 262.144) / 1.024, where y is the original value in the DxFlex scale and x is the result in the 2^{18} scale.*
    ***Note 3**: Navios users should follow the TrueView setup instructions from the EuroFlow SOP for Navios Instruments (section 4):*
    - *EuroFlow SOP for Instrument set-up and compensation for Navios Instruments Version 1.5.1 - 29 October 2019*
  - Reagent catalog numbers.
  - Upload the three analyzed cyt files, ensuring that each file is coupled to the correct donor (cytometry file 1 = donor 1, etc.).
    ***Note**: If you do not use Infinicyt software and therefore have not generated cyt files, we will not be able to include you in the graphical comparison to the other participants of this EQA round.*
- After filling in the form, click on "Submit results". Note that the blue fields are mandatory; submission of results is only possible when all mandatory fields are filled in. You will receive a confirmation that the results are submitted in the browser.

Questions/comments:

Please provide any feedback on clarity, issues with reporting, etc., as a remark in the EQA form or e-mail to EuroFlow.EQA@eslho.org (please state your name and institution/lab in the e-mail communication).

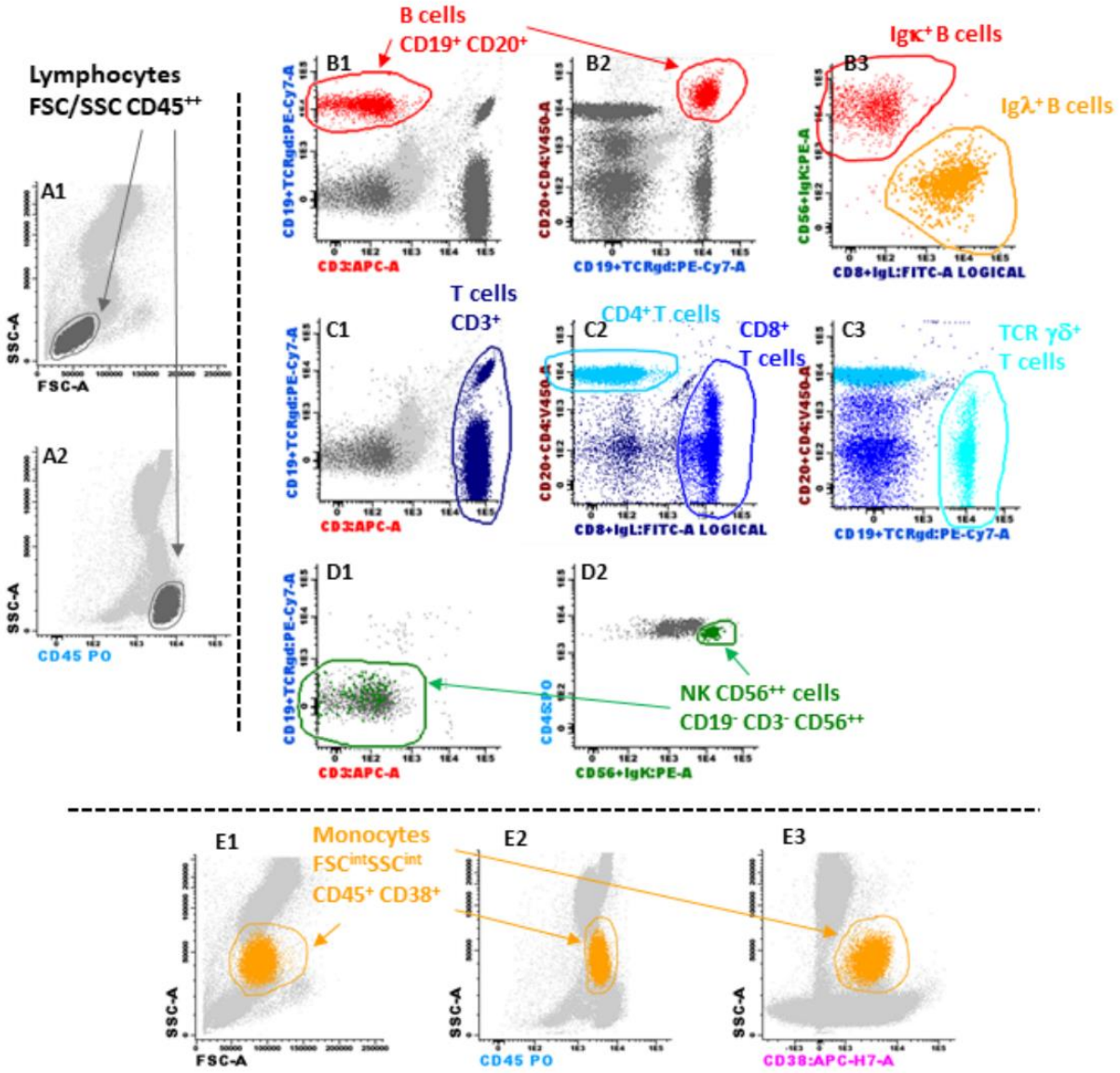

Figure 1 – LST gating strategy

Lymphocytes were gated based on their forward scatter (FSC) / side scatter (SSC) and CD45 profile (grey events; panels A1 and A2). The lymphocytes were classified as B cells ($CD19^+$ $CD3^-$ $CD20^+$ lymphocytes, red events; panels B1 & B2) and T cells ($CD3^+$ lymphocytes, dark blue events; panel C1). B cells were then subdivided into IgK and IgL B cells based on surface expression of IgK or IgL immunoglobulins (red and orange events respectively in IgK versus IgL plot, panel B3). T cells were subdivided into $CD4^+$ and $CD8^+$ and then $TCR\gamma\delta^+$ T cells based on their CD4, CD8, and TCRγδ expression (light blue, blue, and cyanine events respectively; C2 and C3 panels). $CD56^{++}$ NK cells were gated as $CD19^-$ $CD3^-$ and $CD56^{++}$ mature lymphocytes (green events; panels D1 and D2). Finally, monocytes were gated as FSC^{int}, SSC^{int}, $CD45^+$, $CD38^+$ leukocytes (orange events; panels E1, E2, and E3).