THESE ARE EXAMPLE INSTRUCTIONS ONLY

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

Instructions for performing the EuroFlow LST EQA round

Scheme: LST
Year: 2025
Round: II
Start reporting period: 1 September 2025 (00:00 CEST)
End reporting period: 29 September 2025 (23:59 CEST)

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.*

You may participate in the LST scheme with multiple reports. Note that you will need to indicate this during registration in the ESLHO EQA Portal.

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

FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Pacific Blue	Pacific Orange
CD8 and SmIgλ	CD56 and SmIgκ	CD5	CD19 and TCRγδ*	SmCD3	CD38	CD20 and CD4	CD45

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
* TCRγδ is included in the panel but is currently not evaluated in the LST EQA scheme.

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
CD8	FITC	UCH-T4	Cytognos	CYT-SLPC-50	20 (Part of LST mixture)
SmIgλ	FITC	Polyclonal	Cytognos	CYT-SLPC-50	20 (Part of LST mixture)
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)
CD5	PerCP-Cy5.5	L17F12	BD Biosciences	341109	15
CD19	PE-Cy7	J3-119	Beckman Coulter	IM3628	5
SmCD3	APC	SK7	BD Biosciences	345767	2.5
CD38	APC-H7	HB7	BD Biosciences	EU: 656646 USA: 653314	3
CD20	Pacific Blue	2H7	BioLegend	302320	1
CD4	Pacific Blue	RPA-T4	BioLegend	300521	0.5
CD45	Pacific Orange	HI30	Invitrogen	MHCD4530	5

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 hematological malignancies panels. Version 1.14 – February 2025

2. **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 **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 BC DxFlex, BD Canto, BD Lyric, BD LSR Fortessa, and BC Navios cytometers.

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

- EuroFlow SOP for instrument set-up and compensation for DxFlex. Version 2.0 - February 2025
- EuroFlow SOP for instrument set-up and compensation for BD LSR II and BD FACS Canto II. Version 2.0 - February 2025
- EuroFlow SOP for instrument set-up and compensation for BD Lyric for 8-color panels. Version 2.0 - June 2025
- EuroFlow SOP for instrument set-up and compensation for BD LSR Fortessa X-20. Version 2.3 - February 2025
- EuroFlow SOP for instrument set-up and compensation for Navios. Version 1.5.1 - October 2019

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 1.20 - April 2025 ***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.
- Acquire 50.000 lymphocytes or the maximum available amount from the tube.

Perform data analysis and obtain MedFI values of the populations in each donor sample (use of Infinicyt software is recommended).

Data analysis with Infinicyt software:

- Load the provided LST EQA Infinicyt Profile (.inp file).
  ***Note**: **Please make sure you download and use the new profile that is provided in this round**. Do not change the EQA profile in any way (e.g., by adding your own populations, deleting, or renaming the existing ones, etc.). The new profile is not compatible with Infinicyt versions older than v2.0.5.*
  ***Note**: Do not use the EQA profile embedded in the Infinicyt software.*
- Apply the population tree, create and organize the diagrams according to Figure 1, and identify (gate) all required lymphocyte subpopulations by following the LST EQA gating strategy (Figure 1).
  ***Note**: You can reuse the saved profile with your analysis strategy in future rounds. We will notify you in the instructions in case a new profile is created. The profile name includes a version date so you can check which version is the current one.*
  ***Note**: If there are too few lymphocytes, the rare CD56hi NK-cells will be too few to gate with confidence.*
- Obtain MedFI values of:
  - **CD8 on** $CD8^+$ T cells
  - **Igλ on** $Lambda^+$ B cells

- **Igκ on** Kappa^{+} B cells
- **CD56 on** CD56++ NK cells
- **CD5** on T cells
- **CD19** on B cells
- **CD3** on T cells
- **CD38** on Monocytes
- **CD4** on CD4^{+} T cells
- **CD20** on B cells
- **CD45** for T cells

***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).*

Anonymize and save your analyzed files: when saving the analyzed files check the Anonymous checkbox and configure what information you want to keep. Make sure that the file does not contain identifiable lab or donor details. The instrument and analysis date should be retained. Save the analyzed .cyt file with the donor numbers in the file name (e.g., Donor 1.cyt, Donor 2.cyt, and Donor 3.cyt). Do not include lab or donor details in the file name.

Data reporting:

- Log in to ESLHO EQA Portal
- Complete the following in the results form:
  - Cytometer used for data acquisition.
  - Rainbow Beads lot no. (optional and if applicable).
  - MedFI values of the 7th peak of the Rainbow Beads in each channel, rounded to whole numbers (optional and if applicable).
  - MedFI values of the populations in each donor sample.

    ***Note 1**: Report MedFI values even if you used mean fluorescence intensities for instrument setup according to the EuroFlow SOP.*

    ***Note 2**: Expected MedFI values and their acceptable ranges in the EQA are derived from instruments that operate with resolution 0 - 262 143 (18-bit) (e.g., BD instruments Canto, Lyric, LSR Fortessa). Users of instruments that operate with another resolution need to make sure that they transform their numbers and report MedFI values in the 18-bit scale.*

    ***Note 2a**: DxFlex users can 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 18-bit scale.*

    ***Note 2b**: Navios users can follow the TrueView setup instructions from the EuroFlow SOP for Navios Instruments (section 4):*

    - *EuroFlow SOP for instrument set-up and compensation for Navios. Version 1.5.1 - October 2019*
  - Reagent type and catalog numbers.
  - Upload the three analyzed .cyt files in the results form, ensuring that each file is coupled to the correct donor (i.e., cyt file 1 = donor 1, etc.).

    ***Note**: If you did not use Infinicyt software and therefore have not generated cyt files, we will not be able to include your results in the graphical comparison of all results of this EQA round.*

- Note that, so long as there is an internet connection, data entries are auto-saved constantly. This is indicated by the "All changes are saved" notification in the top right corner of the results form. This allows you to partially complete the form and return to it later for further completion (or to make changes to the entered data, if needed) so long as the reporting period is open.
- Note that blue fields are mandatory to complete and that submission of results is only possible when all mandatory fields are filled in.
- After filling in the form, click on "Submit results". You will receive a confirmation in the browser that the results are submitted. Additionally, all contacts linked to the round receive a confirmation email, which includes a pdf file with the submitted results.
- Even after submitting the results, you can still make changes to the data entered in the results form and resubmit it so long as the reporting period is open. This allows you to make any corrections and resubmit, as needed.

Questions/comments:

In case you have any questions or comments, please do not hesitate to contact us at EuroFlow.EQA@eslho.org (please state your name and institution/laboratory in the e-mail).

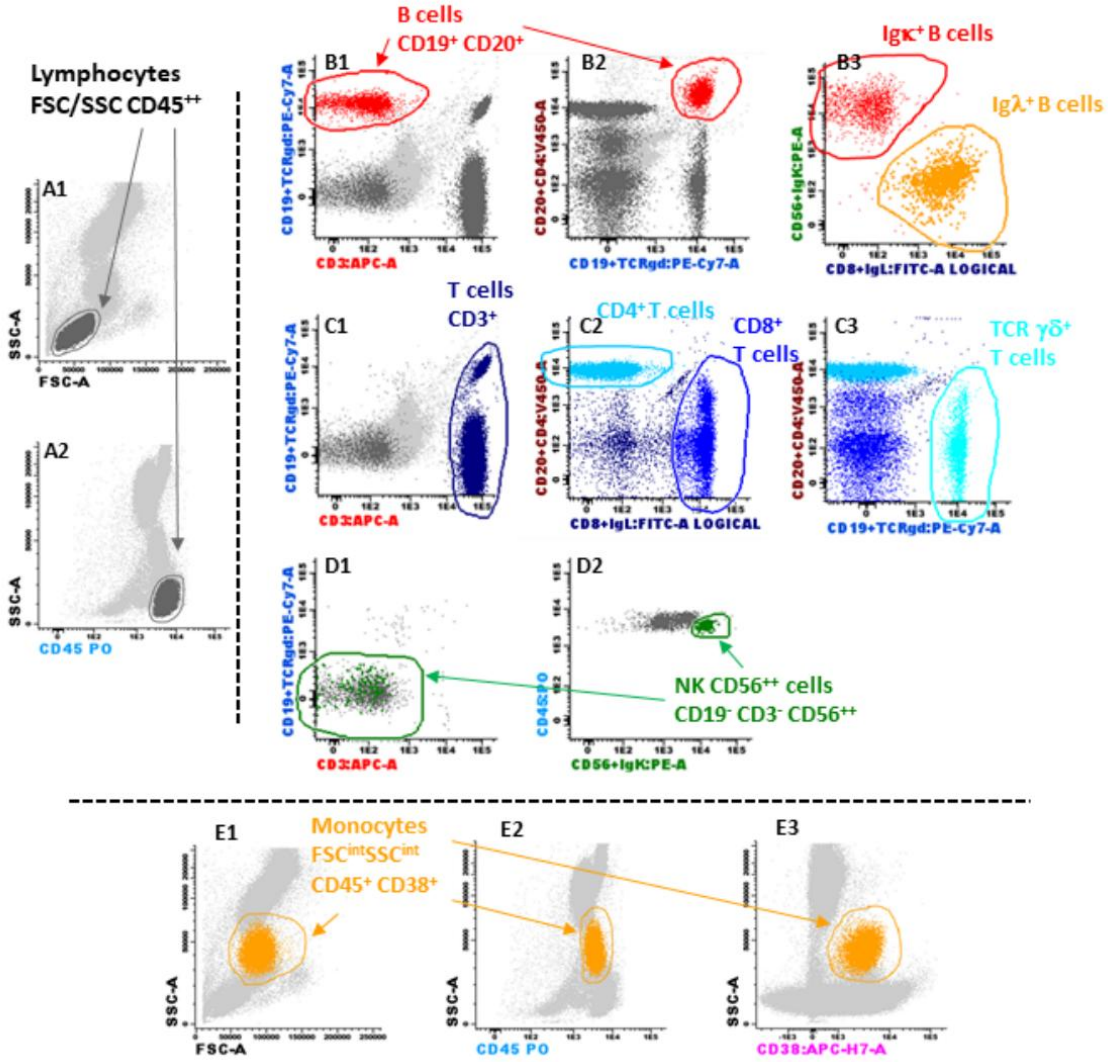

Figure 1 – LST gating strategy

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 Igκ and Igλ B cells based on surface expression of Igκ or Igλ immunoglobulins (red and orange events respectively in Igκ versus Igλ 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).