THESE ARE EXAMPLE INSTRUCTIONS ONLY

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

Instructions for performing the EuroFlow PIDOT EQA round: **Wet lab part**

Scheme: PIDOT
Year: 2025
Round: II
Part: Wet lab
Start reporting period: 30 September 2025 (00:00 CEST)
End reporting period: 31 October 2025 (23:59 CET)

The objective of the wet part of the EuroFlow PIDOT 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 PIDOT panel. Participation is suitable for laboratories that use the PIDOT panel and related EuroFlow SOPs in their routine diagnostics.
***Note**: In this document, EuroFlow SOPs and other EuroFlow files 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 PIDOT panel (Table 1).

Table 1 - Composition of the EuroFlow PIDOT panel

BV421	BV510	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7/APC-C750
CD27	CD45RA	CD8 and SmIgD	CD16 and CD56	CD4 and SmIgM	CD19 and TCRγδ	CD3	CD45

van der Burg M, Kalina T, Perez-Andres M, et al. The EuroFlow PID Orientation Tube for Flow Cytometric Diagnostic Screening of Primary Immunodeficiencies of the Lymphoid System. Front Immunol. 2019 Mar 4;10:246.

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

1. **Reference antibodies of the EuroFlow PIDOT 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 PIDOT panel

Marker	Fluorochrome	Clone	Source	Catalog no.	µL/test
CD3	APC	SK7	BD Biosciences	345767	2.5
CD4	PerCP-Cy5.5	SK3	BD Biosciences	332772	7
CD8	FITC	SKI	BD Biosciences	345772	5
CD16	PE	3G8	BD Biosciences	555407	5
CD19	PE-Cy7	J3-119	Beckman Coulter	IM3628U	5
CD27	BV421	M-T271	BD Biosciences	562513	1
CD45	APC-H7	2D1	BD Biosciences	641417	2
CD45RA	BV510	HI100	BD Biosciences	563031	2.5
CD56	PE	CS.9	Cytognos	CYT-56PE	5
SmIgD	FITC	IA6-2	BioLegend	348205	1.25
SmIgM	PerCP-Cy5.5	MHM-88	BioLegend	314511	2
TCRγδ	PE-Cy7	11F2	BD Biosciences	655410	1

EuroFlow-PID antibody panels version 1.2 14 September 2022

2. Alternative reagents in the format of the PIDOT premixed dried cocktail from Cytognos (Catalog no. CYT-PIDOT8).

Sample preparation:

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

1. When the **reference antibodies of the EuroFlow PIDOT panel (**Table 2**)** are used:
   - Section 3 'Sample processing procedure' of
     EuroFlow SOP for bulk lysis in MRD panels. Version 2.0 – June 2025
   - Section 4 'Staining of surface markers only' of
     EuroFlow SOP for sample preparation. Version 1.7 – November 2023
     ***Note:** for more details, see **Attachment 1** below*

2. In case the PIDOT premixed dried cocktail from Cytognos 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 cytometer instruments.

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 LSRFortessa 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.

In case you are using the BD Lyric and FACS Suite Software, use the 'EF_PIDOT_Lyric_v1 UD.ud' assay file that is available on https://app.euroflow.org/downloads/public ("Downloads for the BD Lyric" section > "Assay files (zip)" folder) and acquire CS&T Beads.

Acquire at least 1 million events per sample.

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:

Apply one of following data analysis strategies:

1. Manual gating:

- Download the '**PIDOT EQA Infinicyt Profile.inp**' file from the "Instructions" tab of the current scheme in the ESLHO EQA Portal.
- Load the PIDOT EQA Infinicyt Profile.inp file (in the software: go to tab 'Profile' and select 'Load Profile from Folder', navigate to your download folder and select the correct profile).
- Load the included PIDOT EQA Analysis Strategy for population identification (in the software: go to tab 'Profile' and select 'Load Analysis Strategy', select the strategy linked to the profile. The strategy can be used as a guide, click on the magnifying glasses in the hierarchy tree to review or adapt the gates.

  ***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 PIDOT EQA gating strategy (**Figure 1** and **Figure 2**).

2. Automated gating:

- Load the fcs file in the EuroFlow PIDOT database (Automated Gating and Identification; AG&I-tool) of the Infinicyt™ software.

***Note:** For more detailed information on the use of the AG&I-tool, please check the Infinicyt Manual (https://www.cytognos.com/infinicyt/resources/) or the following webinar: 03-0001 - NanoZoom - Intro2 - NL (youtube.com)*
***Note:** The 'Check Populations' included in the cell population tree contain events that have not been unequivocally identified as similar to the populations in the database and need further revision to assign the events to the correct population.*

- Obtain MedFI values of the cell populations provided in Table 3.

Table 3 – Cell subset marker combinations

Reagent	Positive population	Figure 1. dot-plot
CD27-BV421	Unswitched memory B-cells + plasma cells	A3
CD45RA-BV510	B-cells	A1
CD8-FITC	CD8+ T-cells	B4
SmIgD-FITC	Pre-germinal center B-cells	A4
CD16+CD56-PE	NK cells	C
CD4-PerCP-Cy5.5	CD4+ T-cells	B3
SmIgM-PerCP-Cy5.5	Unswitched memory B-cells + plasma cells	A3
CD19-PE-Cy7	B-cells	A1
TCRγδ-PE-Cy7	TCRγδ+ T-cells	B2
CD3-APC	T-cells	B1
CD45-APC-C750	T-cells	B1

1) Diks AM, Bonroy C, Teodosio C, et al. Impact of blood storage and sample handling on quality of high dimensional flow cytometric data in multicenter clinical research. J Immunol Methods. 2019 Dec;475:112616.

2) van der Velden VHJ, Flores-Montero J, Perez-Andres M, et al. Optimization and testing of dried antibody tube: The EuroFlow LST and PIDOT tubes as examples. J Immunol Methods. 2019 Dec;475:112287.

***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).*

Results submission:

- Log in to ESLHO EQA Portal

- Access the PIDOT wet lab part results form via your Dashboard or in the "Results submission" tab of the current scheme.
- Complete the following in the results form:
  - 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**: 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**: 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 Instruments Version 1.5.1 - October 2019*
  - Reagent type including manufacturer(s), catalog number(s), and/or lot number(s). and catalog numbers.
- 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.
- It is not possible to submit or edit your data after the deadline for results submission has passed (see "End of the reporting period" at the top of these instructions).

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).

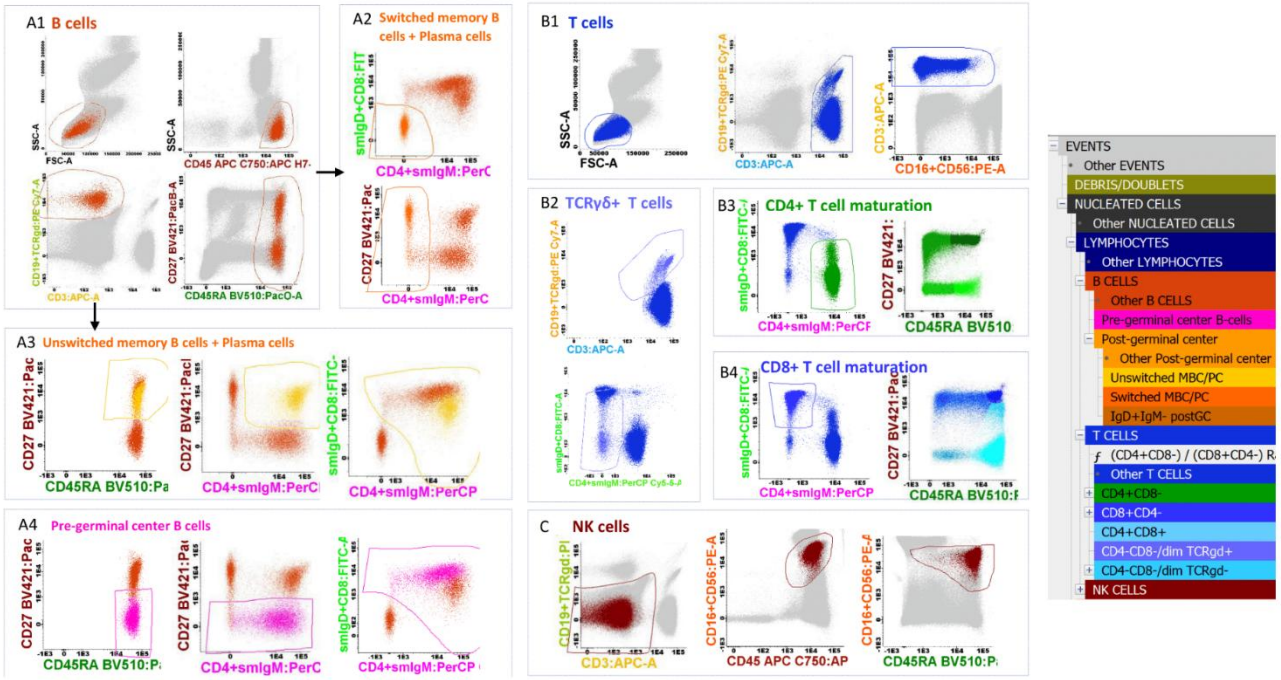

Figure 1 – PIDOT gating strategy

EuroFlow PIDOT gating strategy and population tree. The markers CD3, CD19 in combination with TCRγδ and CD56^{+}CD16^{+} were used to define B-cells (plot A1), T-cells (B1), TCRγδ^{+} T-cells (B2), and NK-cells (C). B-cell subsets could be further subdivided into pre-germinal center B-cells (Pre-GC; CD27^{-}smIgM^{+}smIgD^{+}, plot A4), unswitched memory B-cells (CD27^{+}smIgM^{+}smIgD^{+}, plot A3) and switched memory B-cells (CD27^{+}smIgM^{-}smIgD^{-}, plot A2). T-cell subsets could be further subdivided into CD4^{+} T-cells and CD8^{+} T-cells (B3 & B4). Plot C illustrates the NK cells gating strategy.

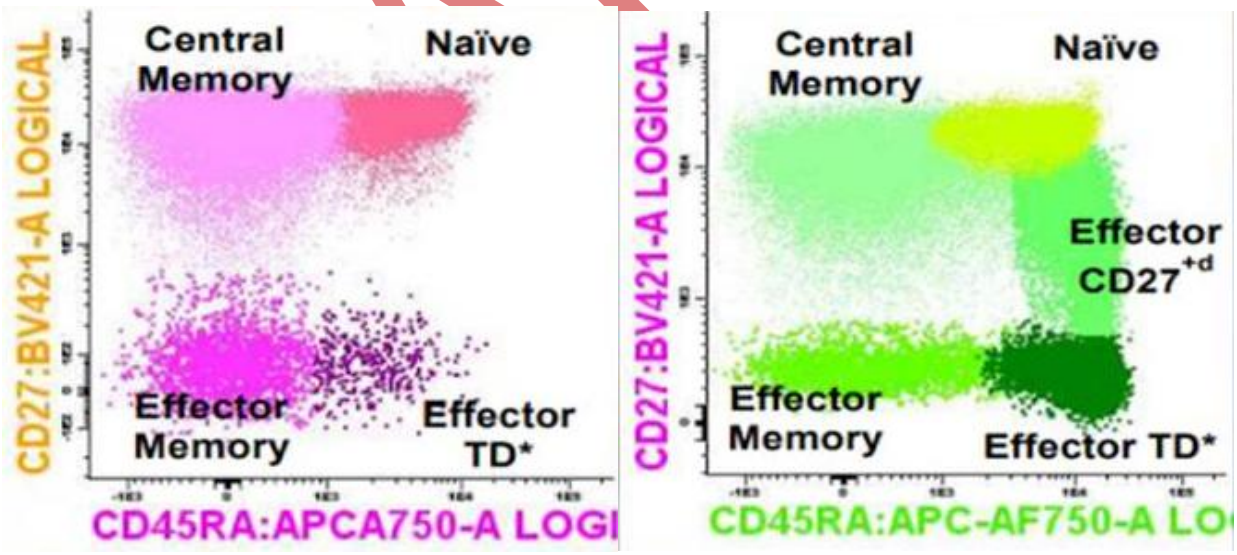

Figure 2 – PIDOT gating strategy: T-cell maturation subsets

T-cell subsets could be further subdivided into CD4^{+} T-cells (with naïve, central memory (CM), effector memory (EM), effector terminal differentiated (ETM) subpopulations) and CD8^{+} T-cells (with naïve, CM, EM, ETM and ETM CD27+ subpopulations).

Attachment 1 – Sample preparation

1. **In case the reference antibodies of the EuroFlow PIDOT panel (Table 2) are used:**

Bulk lysis: Refer to section 3 'Sample processing procedure' of the EuroFlow SOP for bulk lysis:

- Transfer no more than 2 mL of the sample containing at least 10 x 10^6 nucleated cells to a 50 mL Falcon tube.
- Fill the tube up to reach 50 mL volume using the diluted BulkLysis™ (10X BulkLysis™ solution, Cytognos ref: CYT-BL, diluted 1/10 in dH_2O).
- Mix gently and incubate for 15 min. on a roller at RT.
- Centrifuge (10 min at 800g) and remove the supernatant using a Pasteur pipette or a vacuum system without disturbing the cell pellet.
- Vortex and add 2 mL of PBS+BSA and resuspend the cell pellet vigorously, preferably by vortexing.
- Complete the volume of the tube containing the cell suspension up to 50 mL final volume with PBS+BSA.
- Mix well by inverting the tube 3-5 times.
- Centrifuge (10 min at 800g) and remove the supernatant using a Pasteur pipette or a vacuum system without disturbing the cell pellet.
- Vortex and add 2 mL of PBS+BSA and resuspend the cell pellet by gently vortexing and transfer this volume to a 5 mL polystyrene round-bottom Falcon tube ("FACS tube").
- Wash the 50 mL Falcon tube with 2 mL of washing buffer to recover cells that might have been left in the tube. Add this volume to the 5 mL Falcon tube containing the rest of the transferred sample.
- Centrifuge at 540 g for 5 min and remove the supernatant by decanting or using a Pasteur pipette. The residual sample volume after the washes should be 50 - 100 µL.

Staining of surface markers: Refer to section 4 'Staining of surface markers only' of the EuroFlow SOP for sample preparation:

- Add the appropriate volumes of liquid antibodies (Table 2). If necessary, use washing buffer to reach a final volume of 100 µL.
- Vortex and incubate for 30 min. at RT and protected from light.
- Add 2 mL of 1X FACS Lysing Solution (10X FACS Lysing Solution, BD Biosciences ref: 349202, diluted 1/10 in dH2O).
- Vortex and incubate (10 min. at RT and protected from light) and centrifuge (5 min. at 540g).
- Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.
- Vortex and add 2 mL PBS+BSA and resuspend by gently vortexing, and centrifuge (5 min. at 540g).
- Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.
- Vortex and resuspend the cell pellet in 200µL PBS+BSA by gently vortexing.
- Acquire at least 1 x 10^6 cells after staining or after storage of maximum 1h at 4°C.