THESE ARE EXAMPLE INSTRUCTIONS ONLY

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

Instructions for performing the EuroFlow PIDOT EQA round: **Dry part**

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

The objective of the dry part of the EuroFlow PIDOT EQA scheme is to evaluate the ability of participants to analyze and interpret provided fcs files of patients with confirmed primary immunodeficiency (PID), non-PID disease controls in whom PID diagnosis was ruled out (as defined by the treating physician based on standard clinical care), and healthy controls. Participation is suitable for laboratories that are familiar with applying the PIDOT panel and related EuroFlow SOPs in their routine diagnostics.

Fcs files and patient information

All fcs files were generated by processing samples according to the 'EuroFlow SOP for Sample Preparation' and 'EuroFlow SOP for bulk lysis in MRD panels'. Reagents used for staining were 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.

- To obtain the **fcs files**, log in to the ESLHO EQA Portal and go to the "Instructions" tab of the current scheme.
- Download the **PIDOT_2025_II_case#1.fcs** and **PIDOT_2025_II_case#2.fcs** files.

PIDOT_2025_II_case#1: *Female, 4y, 3900 WBC/µL, serum IgG: 13 g/L (on immune replacement therapy), serum IgM: 1.07 g/L, serum IgA: 0.43 g/L History of hospitalisation with septic shock, fulminant varicella virus infection, and interstitial pneumonia). Patient has skeletal dysplasia. Follow-up of known diagnosis.*

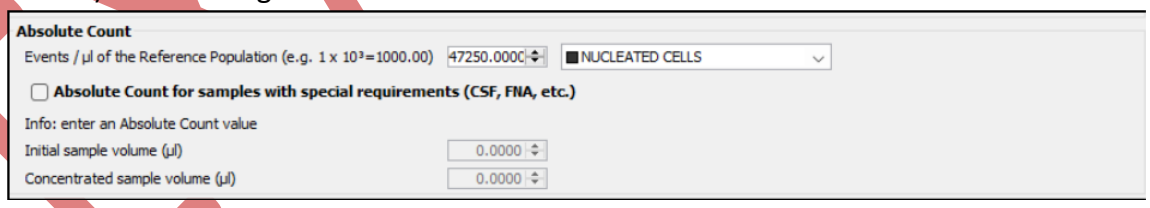
PIDOT_2025_II_case#2: *Male, 44y, 8750 WBC/µL, serum IgG: 10.5 g/L (on immune replacement therapy), serum IgM/A: undetectable. Follow-up sample of known diagnosis (diagnosis is made at age of 1 year with severe and recurrent bacterial infections at presentation).*

Perform data analysis (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 (marked in green 'PIDOT QA Lyric'). The strategy can be used as a guide, click on the magnifying glasses in the hierarchy tree to review or adapt the gates.
   - Identify (gate) all required lymphocyte subpopulations according to the PIDOT EQA gating strategy (Figure 1 and Figure 2).
   - To calculate the Events/µL, fill in the WBC counts for the 'NUCLEATED CELLS' (WBC counts) in the configuration tab:

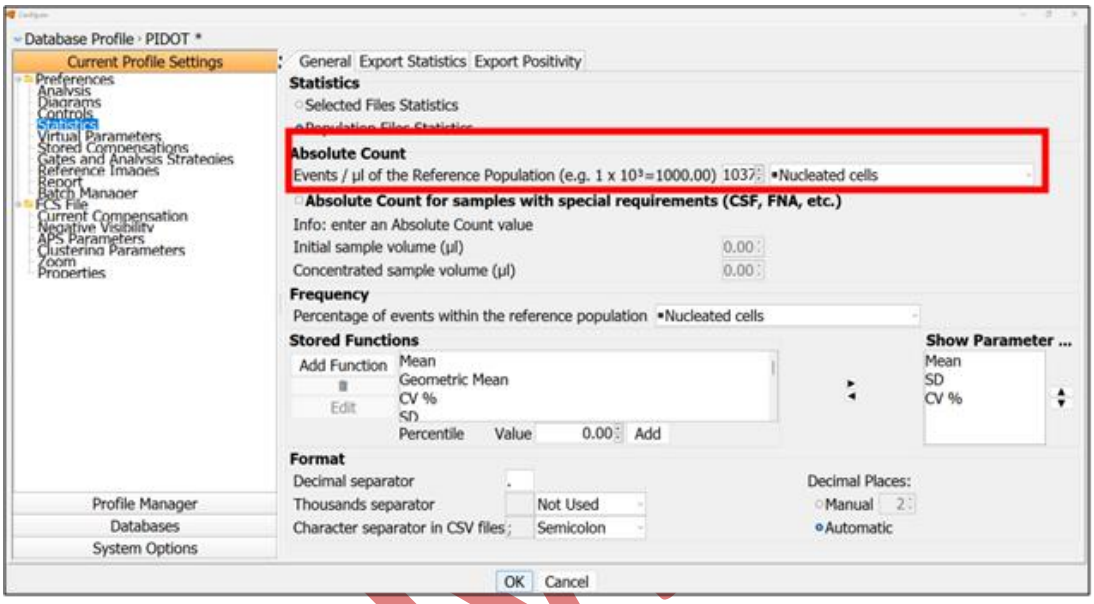


2. **Automated gating**
   - Load the fcs file in the EuroFlow PIDOT database (Automated Gating and Identification; AG&I-tool) of the Infinicyt™ software.

     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.*

- Identify (gate) all required lymphocyte subpopulations through automated gating & identification.
- To calculate the Events/µL, fill in the WBC counts for the 'NUCLEATED CELLS' (WBC counts) in the configuration tab (this tab will pop-up after AG&I is ready):



VIS.	Population	Alerts	Review	Events /	Frequenc	Total %	Events	Partial %	Visibility	Comment
	EVENTS	20					7...		NA	
	Other EVENTS			0	NA	0	0	0	NA	
	Check Populations			10.2	NA	0.085	6...	0...	NA	

Results submission:

- Log in to the ESLHO EQA portal
- Access the PIDOT dry part results form via your Dashboard or in the "Results submission" tab of the current scheme.
- For each case/fcs file, report the following results:
  1. **Section 1:** Fill in absolute cell counts (/µL) and population frequencies (% of parent population) of the populations listed in Table 2.
  2. **Section 2:** Select which cell populations are absent, decreased, normal, or increased.
     Use the official EuroFlow reference ranges:
     Reference values for PB leukocyte populations in absolute numbers of cells (EuroFlow PIDOT). Version 1.1 - July 2024 (available on

https://app.euroflow.org/downloads/public), or the reference ranges available in the AG&I-tool in Infinicyt in case you use the AG&I-tool for data analysis.

3. **Section 3:** Report the combined interpretation of the T and B cell maturation patterns.
4. **Section 4:** Report the combined interpretation of the compatibility of the immunophenotype with common PIDs.
5. **Section 5:** Write your interpretation for the clinician.

Table 2 – PIDOT EQA lymphocyte subpopulations

POPULATION	Corresponding plots in Figure 1 and Figure 2	
	Figure 1	Figure 2
B cells *(% of lymphocytes)*	A1	
Pre-germinal center B cells *(% of B cells)*	A4	
Unswitched memory B cells + plasma cells *(% of B cells)*	A3	
Switched memory B cells + plasma cells *(% of B cells)*	A2	
T cells *(% of lymphocytes)*	B1	
CD4+ T cells *(% of T cells)*	B3	A
CD4+ Naive T cells *(% of CD4+ T cells)*		
CD4+ Central/Transitional Memory T cells *(% of CD4+ T cells)*		
CD4+ Effector Memory T cells *(% of CD4+ T cells)*		
CD4+ Effector TD T cells *(% of CD4+ T cells)*		
CD8+ T cells *(% of T cells)*	B4	B
CD8+ Naïve T cells *(% of CD8+ T cells)*		
CD8+ Central/Transitional Memory T cells *(% of CD8+ T cells)*		
CD8+ Effector Memory T cells *(% of CD8+ T cells)*		
CD27+CD8+ Effector TD T cells *(% of CD8+ T cells)*		
CD8+ Effector TD T cells *(% of CD8+ T cells)*		
CD4-CD8-/dim TCRgd- T cells (double negative T cells) *(% of T cells)*		
CD4-CD8-/dim TCRgd+ T cells *(% of T cells)*	B2	B2
NK cells *(% of lymphocytes)*	C	C

***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).*

- 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).

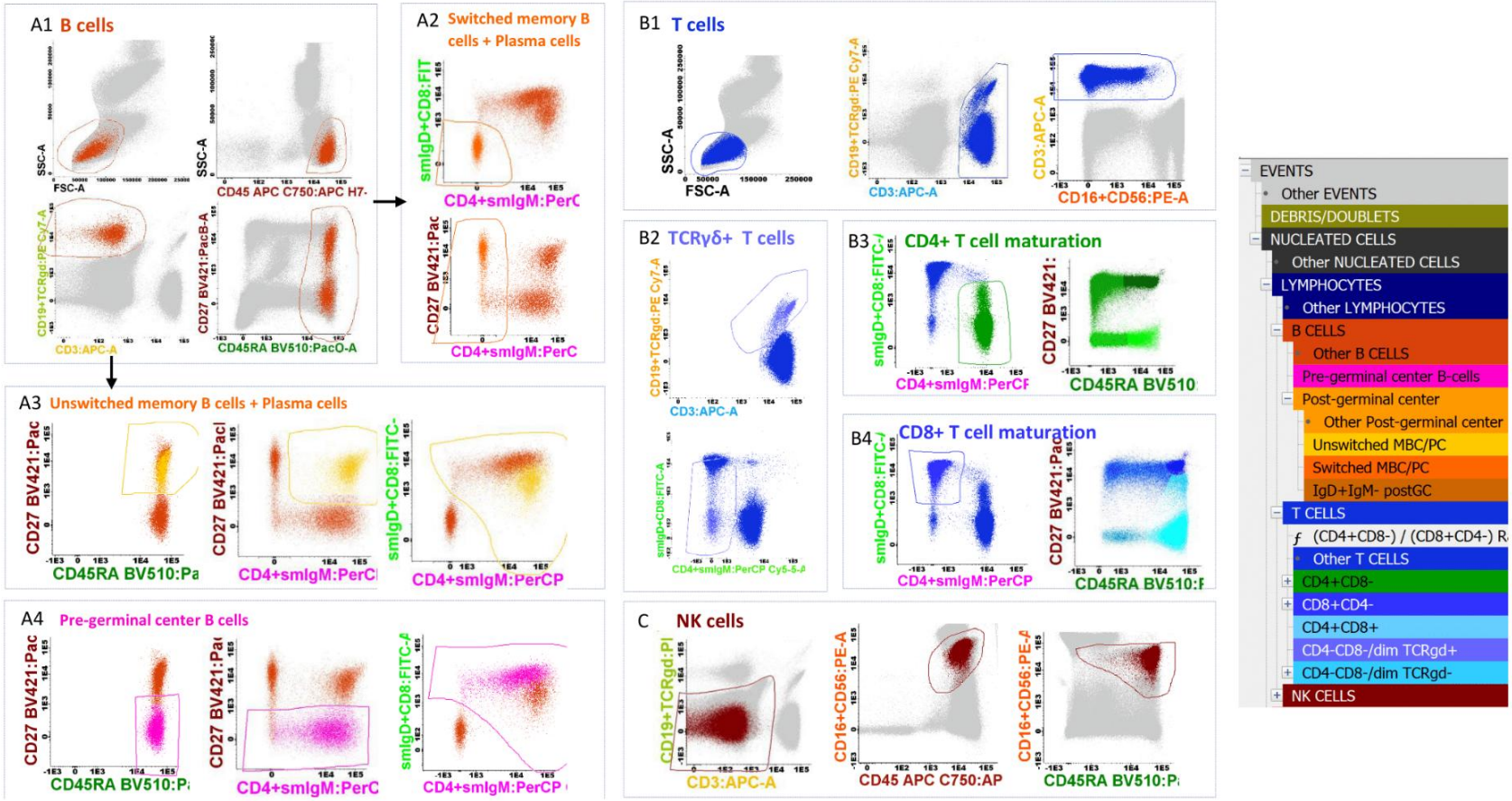


Figure 1 – PIDOT gating strategy

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.

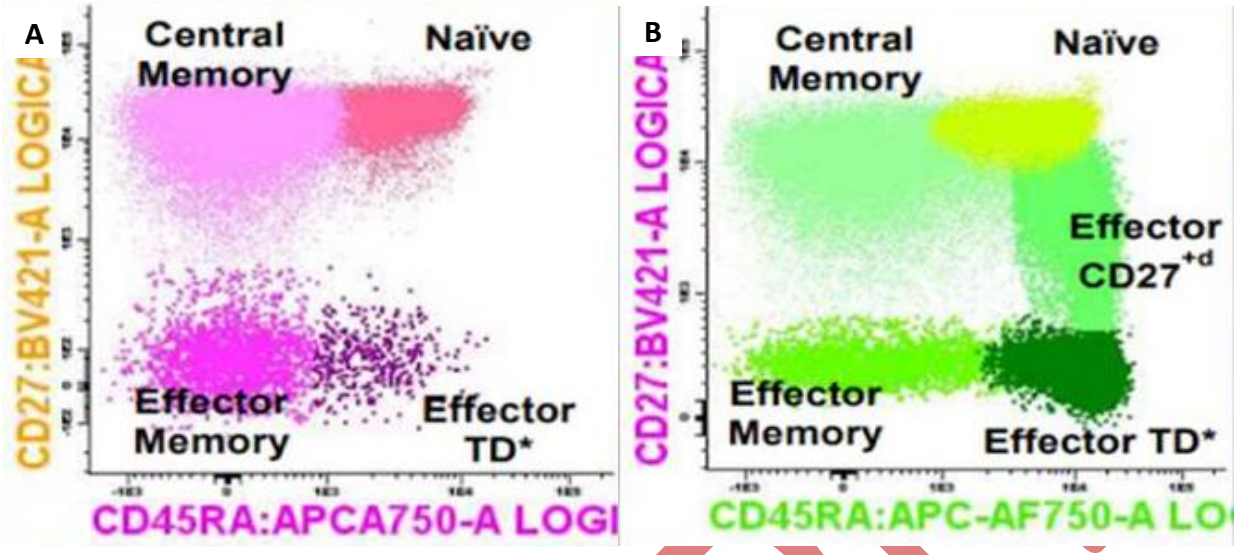


Figure 2 – PIDOT gating strategy: T-cell maturation subsets

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