EXAMPLE INSTRUCTIONS ONLY

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

Instructions for performing the EuroFlow PIDOT dry part EQA round

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.

***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.

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 (**Error! Reference source not found.**).

Table 1 - Composition of the EuroFlow PIDOT panel

BV421	BV510	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7/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 (https://eqa.eslho.org/) and go to the "Instructions" tab of the current scheme.
- Download the **PIDOT_YYYY_I/II_case #1** and **PIDOT_YYYY_I/II_case #2** fcs files.

PIDOT_YYYY_I/II_case #1*: [include patient's information in format: sex, age (y or m), WBC/µL, immunoglobulin levels (indicate if immunoglobulin replacement therapy), relevant clinical information.]*

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PIDOT_YYYY_I/II_case #2: *[include patient's information in format: sex, age (y or m), WBC/µL, immunoglobulin levels (indicate if immunoglobulin replacement therapy), relevant clinical information.]*

Data analysis (Infinicyt™ software recommended):

- Apply one of following data analysis strategies:
  1. **Manual gating**
  - Download 'PIDOT EQA Infinicyt Profile.inp' from the "Instructions" tab of the current scheme in the ESLHO EQA portal
  - Load the provided PIDOT EQA Infinicyt Profile (.inp file), and the included PIDOT EQA Analysis Strategy for population identification.
  - Identify (gate) all required lymphocyte subpopulations according to the PIDOT EQA gating strategy (Figure 1).
  2. **Automated gating**
  - Load the fcs file in the EuroFlow PIDOT database of the Infinicyt™ software
  - Identify (gate) all required lymphocyte subpopulations through automated gating & identification.

Data submission:

- Log in to the ESLHO EQA portal and access the PIDOT dry part datasheet via your dashboard or in the "Data submission" tab of the current scheme.
- For each case/fcs file, report the following results (a template of the different sections of results is provided in Attachment 1):
  1. **Section 1:** Fill in absolute cell counts (/µL) and population frequencies (% of parent population) of the populations listed in **Error! Reference source not found.**.
  2. **Section 2:** Select which cell populations are absent, normal, increased, or decreased.

Use the official EuroFlow reference ranges 'Reference values for PB leukocyte populations (EuroFlow PIDOT tube), version 1.0, 24 May 2023' (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 stages.
  4. **Section 4:** Report the combined interpretation of the most compatible PID subtype with the immunophenotype
  5. **Section 5:** Write your interpretation for the clinician.
- The form is saved automatically, so you can leave the form without losing any of the data that you filled in.
- After completely 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. All

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contact persons of the scheme will receive an email confirmation that the results are submitted successfully, with in the attachment an overview of the submitted data.

- During the data submission period, the form can still be viewed and edited.
- It is not possible to submit or edit your data after the deadline for data submission has passed (see “End of the reporting period” at the top of these instructions).

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Table 2 – PIDOT EQA lymphocyte subpopulations

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

Questions/comments:

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

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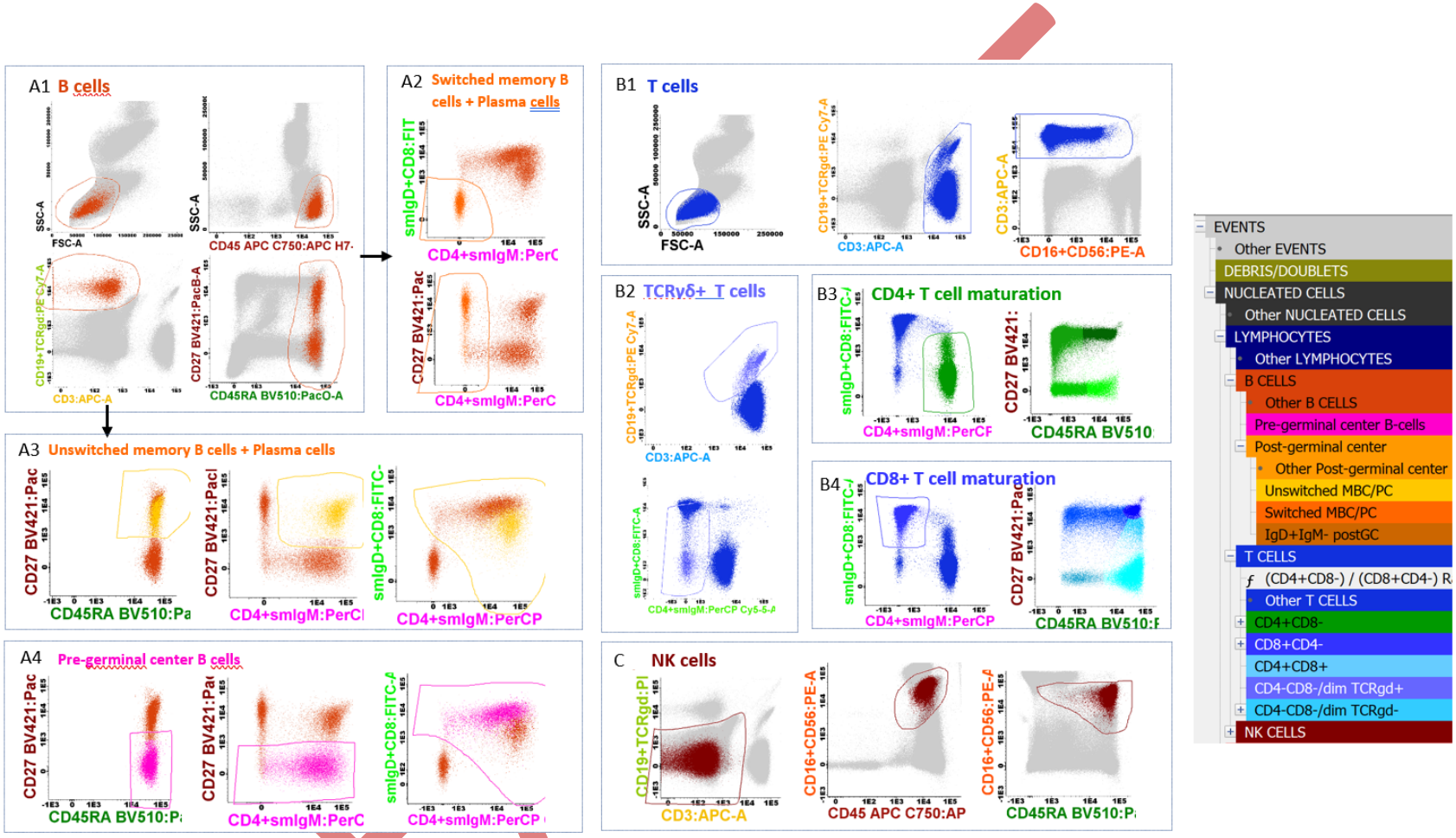

Figure 1 – PIDOT gating strategy

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.

Attachment 1 – Template for reporting the PIDOT EQA scheme dry part

SECTION 1: cell count reporting				SECTION 2: cell count interpretation*				
POPULATION	**Figure 1. dot-plot**	**Absolute cell counts (/µL)**	**% of parent**	**Absent**	**Normal**	**Increased**	**Decreased**	**NA**
B cells *(% on lymphocytes)*	A1			☐	☐	☐	☐	☐
Pre-germinal center B cells *(% on B cells)*	A4			☐	☐	☐	☐	☐
Unswitched memory B cells + plasma cells *(% on B cells)*	A3			☐	☐	☐	☐	☐
Switched memory B cells + plasma cells *(% on B cells)*	A2			☐	☐	☐	☐	☐
T cells *(% on lymphocytes)*	B1			☐	☐	☐	☐	☐
CD4+ T cells *(% on T cells)*				☐	☐	☐	☐	☐
CD4+ Naive T cells *(% on CD4+ T cells)*				☐	☐	☐	☐	☐
CD4+ Central/Transitional Memory T cells *(% on CD4+ T cells)*	B3			☐	☐	☐	☐	☐
CD4+ Effector Memory T cells *(% on CD4+ T cells)*				☐	☐	☐	☐	☐
CD4+ Effector TD T cells *(% on CD4+ T cells)*				☐	☐	☐	☐	☐
CD8+ T cells *(% on T cells)*				☐	☐	☐	☐	☐
CD8+ Naïve T cells *(% on CD8+ T cells)*				☐	☐	☐	☐	☐
CD8+ Central/Transitional Memory T cells *(% on CD8+ T cells)*				☐	☐	☐	☐	☐
CD8+ Effector Memory T cells *(% on CD8+ T cells)*	B4			☐	☐	☐	☐	☐
CD8+CD27+ Effector TD T cells *(% on CD8+ T cells)*				☐	☐	☐	☐	☐
CD8+CD27+ Effector TD T cells *(% on CD8+ T cells)*				☐	☐	☐	☐	☐
CD4-CD8-/dim TCRgd- T cells (double negative T cells) *(% on T cells)*				☐	☐	☐	☐	☐
CD4-CD8-/dim TCRgd+ T cells *(% on T cells)*	B2			☐	☐	☐	☐	☐
NK cells *(% on lymphocytes)*	C			☐	☐	☐	☐	☐

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* Use the official EuroFlow reference ranges 'Reference values for PB leukocyte populations (EuroFlow PIDOT tube), version 1.0, 24 May 2023' (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&-tool for data analysis

Section 3: Combined interpretation of the T and B cell maturation stages	
B cell compartment	
Normal peripheral B cell maturation, with no relevant deviations	☐
B cells are absent, B cell maturation is not interpretable	☐
B cell lymphopenia, with normal B cell maturation	☐
B cell lymphopenia, with disturbed B cell maturation	☐
Disturbed B cell maturation, with weak/absent memory compartment (switched + unswitched memory B cells)	☐
Disturbed B cell maturation, with weak/absent switched memory compartment	☐
T cell compartment	
Normal peripheral T cell maturation, with no relevant deviations	☐
T cells are absent, T cell maturation is not interpretable	☐
T cell lymphopenia, with normal T cell maturation	☐
T cell lymphopenia with disturbed T cell maturation	☐
Disturbed T cell maturation (CD4+ and/or CD8+), with enriched naive compartment	☐
Disturbed T cell maturation (CD4+ and/or CD8+), with enriched memory compartment	☐
Decreased CD4+ (naive) T cell compartment	☐
Decreased CD8+ (naive) T cell compartment	☐
Increased double-negative T cells	☐

Section 4: Immunophenotyping most compatible with				
PID subtype	**YES**	**POSSIBLE**	**NO**	**NA**
SCID (T-B+/T-B-)	☐	☐	☐	☐
SCID with maternal engraftment	☐	☐	☐	☐
CID	☐	☐	☐	☐
XLA	☐	☐	☐	☐
CVID(-like)	☐	☐	☐	☐
HIGM	☐	☐	☐	☐
ALPS	☐	☐	☐	☐
Other	*Free text:*			

Section 5: Interpretation for a clinician
Write your interpretation for the clinician
Free text:

Disclaimer: no diagnosis made based on flow cytometry only

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