THESE ARE EXAMPLE INSTRUCTIONS ONLY

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

Instructions for performing the EuroFlow BCP-ALL MRD EQA round

Scheme: BCP-ALL MRD
Year: 2025
Round: II
Start reporting period: 30 September 2025 (00:00 CEST)
End reporting period: 31 October 2025 (23:59 CET)

The objective of the wet lab part of the EuroFlow BCP-ALL MRD 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 BCP-ALL MRD panel. Participation is suitable for laboratories that use the BCP-ALL MRD panel and related EuroFlow SOPs in their routine diagnostics.
The dry part is designed to evaluate a laboratory's ability to analyze and interpret flow cytometry standard (FCS) files obtained from BCP-ALL patient samples using the EuroFlow methodology.

***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.

Wet lab part

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 BCP-ALL MRD Tube 1 panel (Table 1).

Table 1 - Composition of the EuroFlow BCP-ALL MRD (Tube 1) panel

Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-AF750
CD20	CD45	CD81	CD66c and CD123	CD34	CD19	CD10	CD38

Theunissen P, Mejstrikova E, Sedek L, et al., on behalf of the EuroFlow Consortium. Standardized next-generation flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood 2017; 129: 347-357.

For sample staining, use the **reference antibodies of the EuroFlow BCP-ALL MRD Tube 1 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 BCP-ALL MRD (Tube 1) panel

Marker	Fluorochrome	Clone	Source	Catalog no.	µL/test
CD10	APC	HI10a	BD Biosciences	332777	5
CD19	PE-Cy7	J3-119	Beckman Coulter	IM3628	5
CD20	Pacific Blue	2H7	BioLegend	302320	1
CD34	PerCP-Cy5.5	8G12	BD Biosciences	347222	7
CD38	APC-AF750	LS198-4-3	Beckman Coulter	B49200	1
CD45	Pacific Orange	HI30	Invitrogen	MHCD4530	5
CD66c	PE	KOR-SA3544	Beckman Coulter	IM2357U	10
CD81	FITC	JS-81	BD Biosciences	551108	5
CD123	PE	AC145	Miltenyi Biotec	130-113-326	2

Alternatives for sample staining:

1. 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 against 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. As an alternative to the reference antibodies, the Cytognos BCP-ALL MRD (Catalog no. CYT-BCP-ALL-MRD) reagents in the format of the premixed BCP-ALL MRD Tube 1 dried cocktail can be used but this should be done with caution as EuroFlow at this stage cannot confirm whether this has been validated as an equally viable alternative.

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

1. **When the reference antibodies of the EuroFlow BCP-ALL MRD Tube 1 panel** (Table 2) are used:
   - Section 3 'Sample processing procedure' of
     EuroFlow SOP for bulk lysis in MRD panels. Version 2.0 – June 2025
   - Measure the WBC and calculate the volume of the sample needed for the bulk lysis. Generally, the aim is to measure ≥ $5x10^6$ of cells (**note that the number of cells measured is one of the evaluation parameters**). Therefore, a sample containing at least $10x10^6$ cells should undergo bulk lysis. **Note that you will report the initial WBC and the volume used for the bulk lysis in the results form**.
   - After bulk lysis, resuspend the cells so that the final concentration is $10x10^6$ cells/100 µL. **You will report this number (concentration of the cells after bulk lysis but before staining) in the results form**. In the next step, use 100 µL of the sample solution for staining.

2. In case the premixed Cytognos BCP-ALL MRD dried cocktail 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 FACSCanto II, BD FACSLyric, BD LSRFortessa, 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

In case you are using the BD FACSLyric and FACS Suite Software, use the "EF_BCP-ALL MRD_Lyric_v1 UD.ud" assay file that is available at https://app.euroflow.org/downloads/public ("Downloads for the BD Lyric" section > "Assay files (zip)" folder) and acquire CS&T Beads.

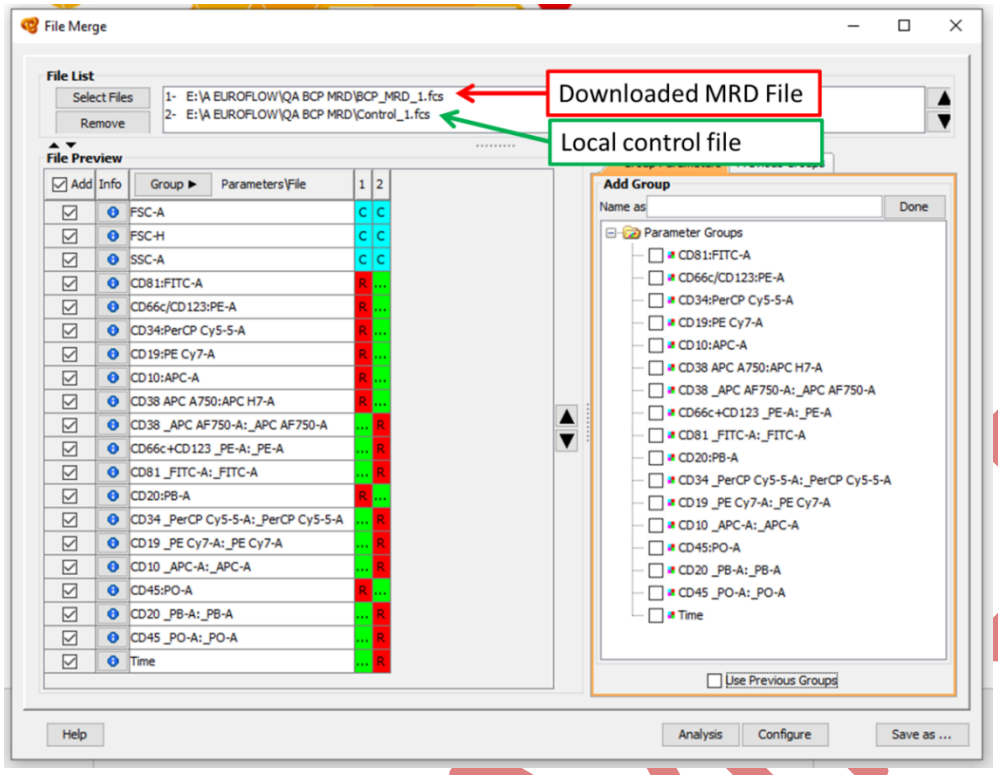
- Name the files obtained from the three donors as "*Control_1*", "*Control_2*", and "*Control_3*"

Dry part

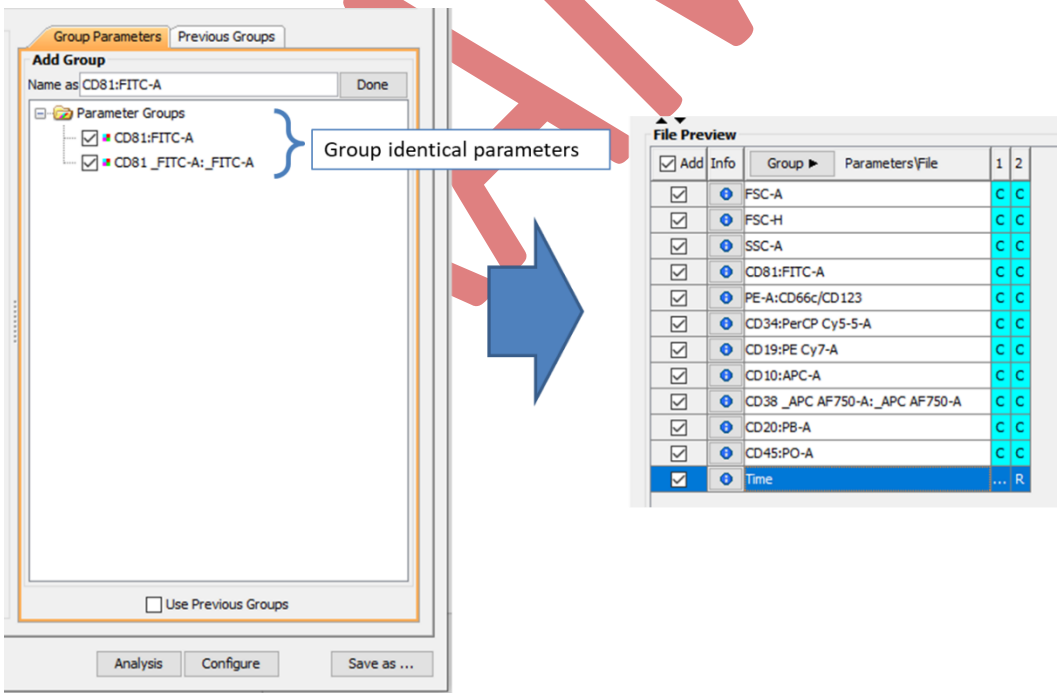
Data analysis with Infinicyt software (recommended):

- Download the following files from the "Instructions" tab of the scheme in your Dashboard in the ESLHO EQA Portal:
  - MRD fcs files named "*BCP_MRD_1*", *BCP_MRD_2*", and "*BCP_MRD_3*"
  - BCP MRD QA profile 2022-2.inp
- Open Infinicyt and load the Infinicyt BCP-ALL MRD EQA Profile (in the software: go to tab 'Profile' and select 'Load Profile from Folder', navigate to your download folder and select the correct profile).
- Prepare the merged files:
  - merge "*BCP_MRD_1*" file with "*Control_1*" file (all events)
  - merge "*BCP_MRD_2*" file with "*Control_2*" file (all events)

- merge "*BCP_MRD_3*" file with "*Control_3*" file (all events)



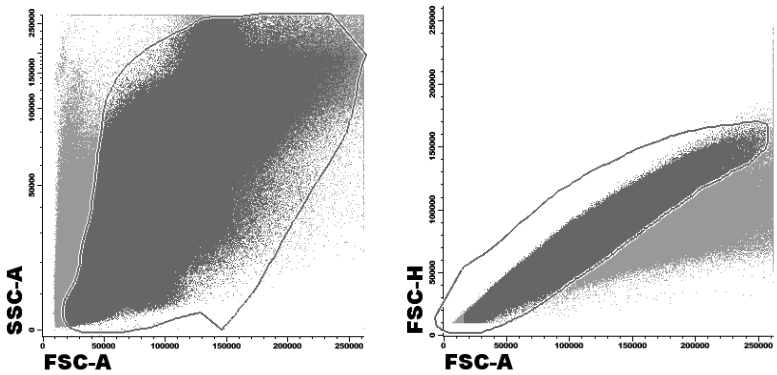
If necessary, group the parameters so that all the parameters are marked with "C" as "common".



- Save and analyze the merged files:

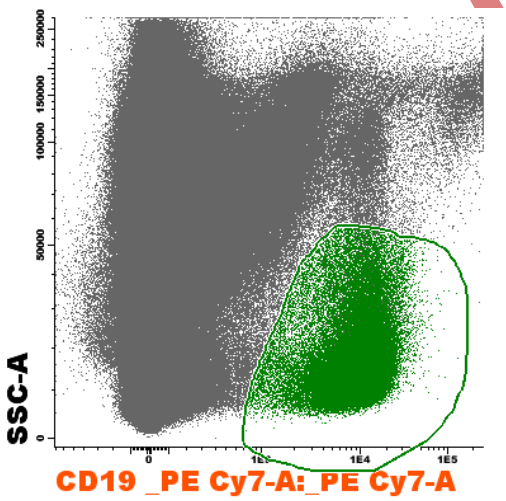
a. Assign nucleated cells (exclude debris and doublets). This number will be reported and used as denominator for MRD analysis.

***Note**: reducing the number of visible events in the Configuration might help to distinguish between the cells and the debris/doublets.*



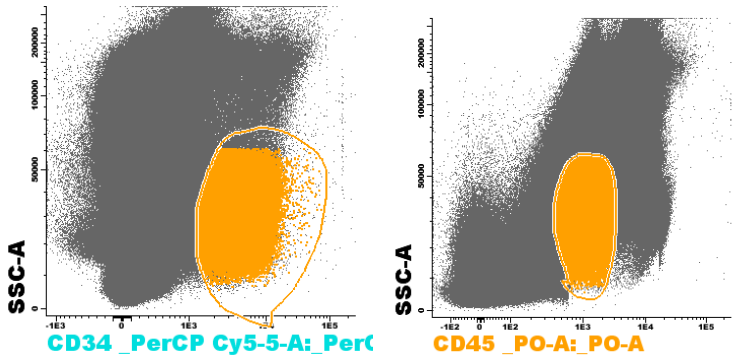
Within the nucleated cells:

b. Assign B cells

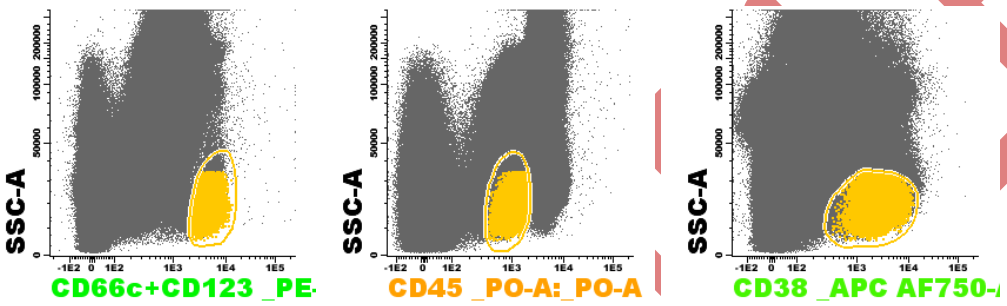


c. Assign CD34+ myeloid precursors

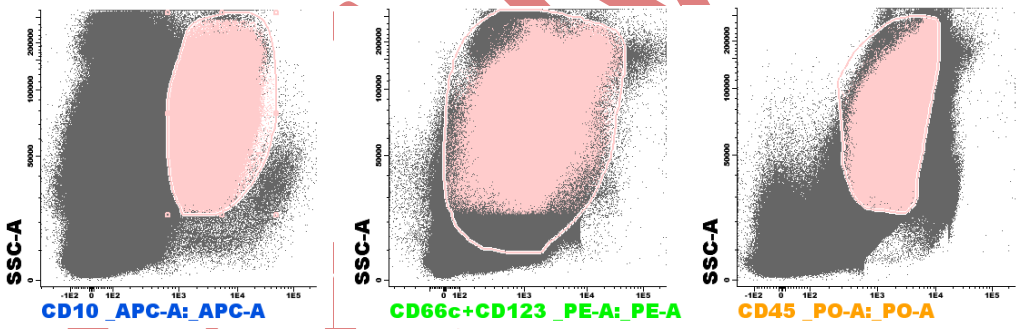
***Note**: increasing the number of visible events in the Configuration might help to identify rare populations in the sample.*



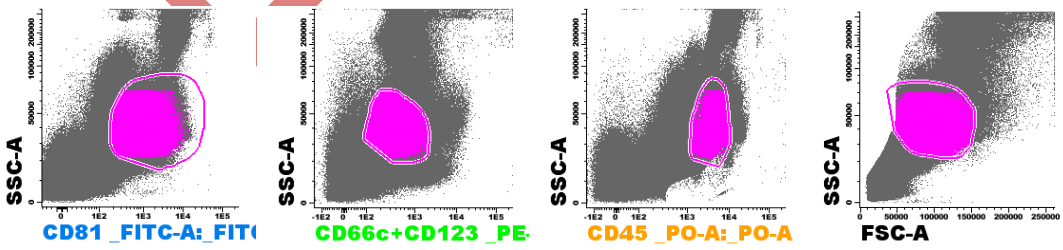
d. Assign dendritic cells (note: distinguish CD66c/CD123bright dendritic cells from CD66c/CD123dim basophils).



e. Assign CD10+ neutrophils

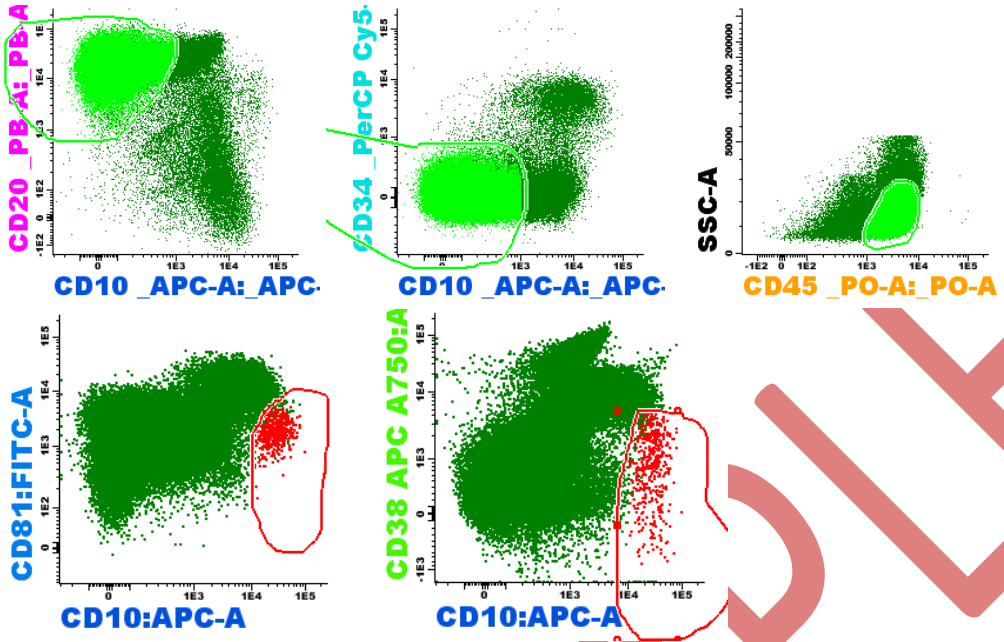


f. Assign CD81+ monocytes



g. Within the CD19+ B cells, first assign mature B cells and then identify abnormal blasts, if present. Gating below is an example, all the markers may be used for identification of

blast cells. Different subpopulation of B cells including plasma cells are defined in the population hierarchy to help step by step dissection of B cell compartment, if needed.



Data reporting:

- Log in to ESLHO EQA Portal
- Access the BCP-ALL MRD results form via your Dashboard or in the "Results submission" tab of the current scheme.

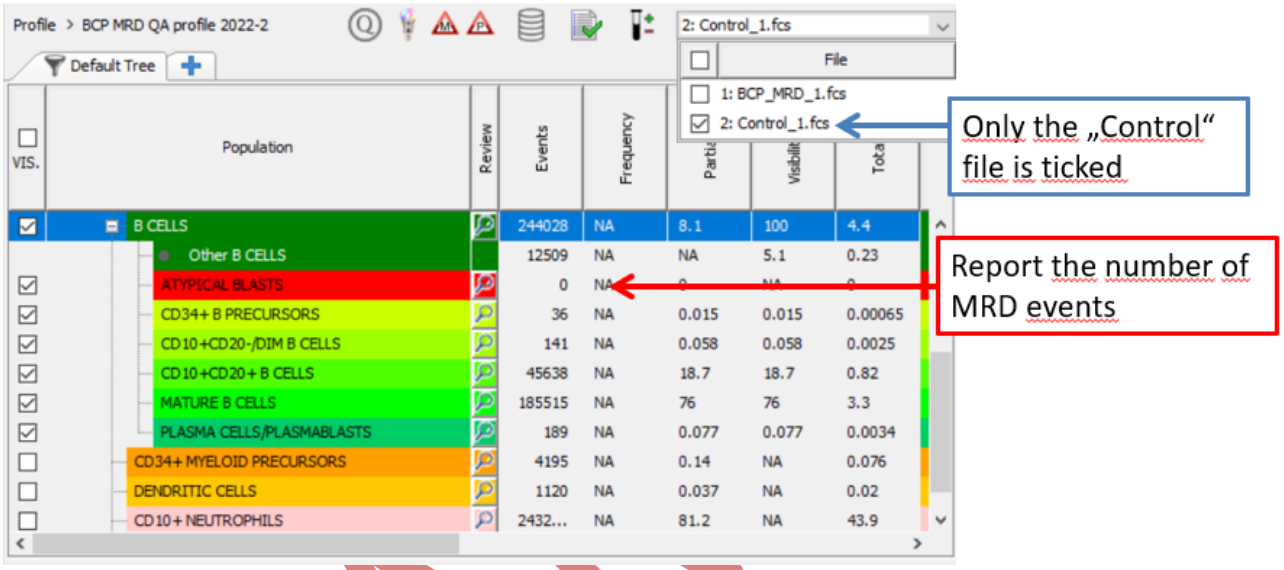
Wet part:

- Report the cytometer used for data acquisition.
- Report the initial WBC of the sample, volume of sample used for bulk lysis, cell concentration after bulk lysis but before staining, and the number of nucleated cells measured (excluding debris and doublets).
- Choose the "Control" file only and export the statistics. Report the following:
  - MedFI values of given markers for the following populations, rounded to whole numbers:
    - Mature B cells: CD81 FITC, CD19 PE-Cy7, CD20 Pacific Blue, CD45 Pacific Orange
    - CD34+ myeloid precursors: CD34 PerCP-Cy5.5
    - Neutrophils: CD10 APC
    - Dendritic cells: CD66c+CD123 PE, CD38 APC-AF750
    - Monocytes: CD38 APC-AF750
  - MedFI values of FSC and SSC for mature B cells
  - The number of MRD events (the background)

***Note 1**: If a population is missing in your sample and you are unable to provide values, it is strongly recommended to obtain a new sample with all populations present.*

***Note 2**: Report MedFI values even if you used mean fluorescence intensities for instrument setup according to the EuroFlow SOP.*
***Note 3**: 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 FACSCanto II, FACSLyric, LSRFortessa). 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 3a**: Navios users can follow the TrueView setup instructions from the EuroFlow SOP for Navios Instruments (section 4):*

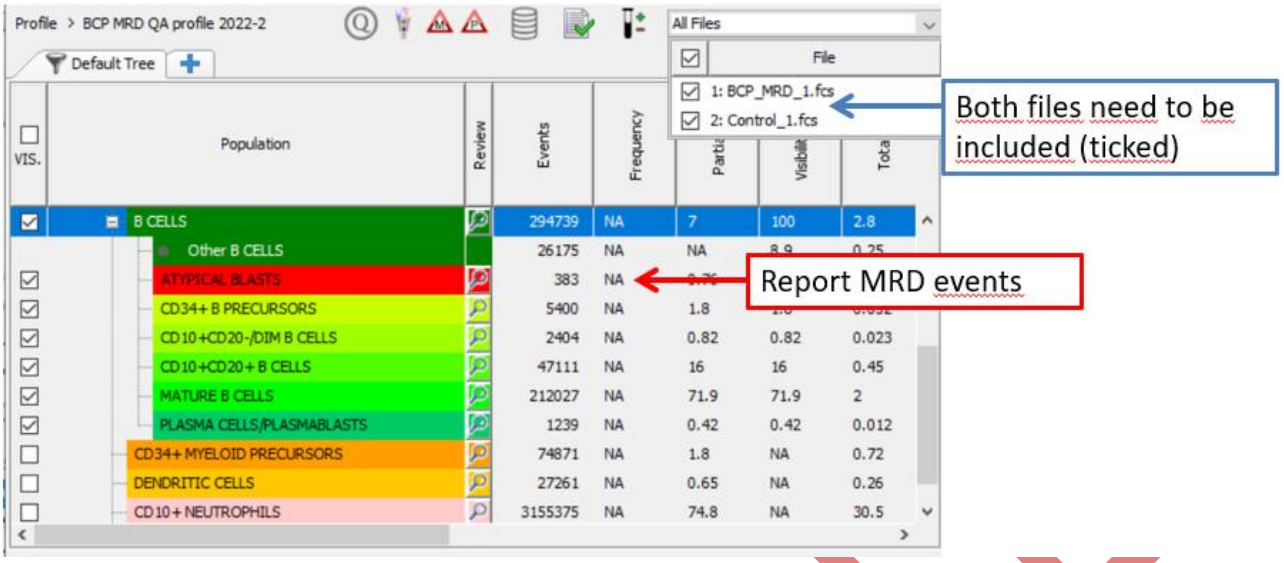
- EuroFlow SOP for instrument set-up and compensation for Navios. Version 1.5.1 - October 2019



- Report the details of the reagents used.

<u>Dry part:</u>

- Report how the file was analyzed (manually or AG&I tool)
- From the Merged file:
  - Report the number of nucleated cells measured (excluding debris and doublets)
  - Quantify the MRD population within the merged file and report:
    - the number of MRD events
    - the % of MRD within all nucleated cells
    - LoD (the limit of detection)
    - LoQ (the limit of quantitation)



- If present, phenotypically characterize the MRD population for each marker and classify the expression as negative, heterogeneous, or positive, or not evaluated.

Perform a similar analysis with the other two merged files.

- Note that, as 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 to complete it (or to make changes to the entered data, if needed) as 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 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).