Miltenyi Biotec

No-lyse whole blood analysis
Simplify flow cytometric analyses with the MACSQuant® Analyzer

Background

For routine flow cytometric monitoring of cell populations within whole blood, there are several critical points to be considered for processing the sample material.
The procedure should be:

1) safe, fast, and reproducible;

2) cause minimal alterations to the blood cell phenotype;

3) applicable for the analysis of various cell populations (e.g., lymphocytes and stem cells).

Many procedures used today for immunophenotyping whole blood cells involve removal of erythrocytes by lysis, as their presence during flow analysis can cause inaccuracies for immunophenotyping as well as cell counting. This lysis step, however, can have a profound effect on light scatter properties of the cells as well as intensities of immunofluorescence staining.[1–3] Additionally, the various methods and reagents for erythrocyte lysis can affect certain cells differently, therefore requiring the use of different procedures depending on the cell analysis required.[2]

CD45-VioBlue®, a pan-leukocyte marker, can be utilized to overcome some of the issues with flow analysis of whole blood. By setting a trigger on V1 (VioBlue), erythrocytes can be efficiently excluded from the analysis, therefore not requiring a lysis step. By performing the analysis with the MACSQuant® Analyzer, absolute cell counts (volumetric) are easily calculated, without the need for expensive counting beads.

This protocol reduces the amount of whole blood, cost, and time necessary for measurement. This application on the MACSQuant Analyzer can be utilized for the analysis of various blood cell populations.

Materials and reagents

Reagents and solutions
- Whole blood anticoagulated with EDTA
- CD45-VioBlue (REA747) (#130-110-637)
- CD3-FITC (REA613) (#130-113-700)
- CD4-PE (REA623) (#130-113-787)
- CD14-APC (REA599) (#130-110-578)
- CD8-APC-Vio®770 (REA734) (#130-110-819)
- Propidium Iodide Solution (#130-093-233)
- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS® BSA Stock Solution (#130-091-376) 1:20 with autoMACS® Rinsing Solution (#130-091-222). Keep buffer cold (2–8 °C).

Materials
- MACSQuant X (#130-105-100)
  MACSQuant Analyzer 10 (#130-096-343) or
  MACSQuant Analyzer 16 (#130-109-803)
- 5mL (12×75 mm) polystyrene round bottom tubes

Procedure

Labeling whole blood for the measurement of T cells and monocytes

1. Aliquot 90 µL of whole blood into a 5 mL tube

2. Add 2 µL each of the following antibodies:
   a. CD45-VioBlue
   b. CD3-FITC
   c. CD4-PE
   d. CD8-APC-Vio 770
   e. CD14-APC

3. Incubate for 10 minutes at 4–8 °C.

4. After incubation, add 1 mL of buffer and centrifuge cells at 455×g for 10 minutes without brake.

5. Remove tube carefully from centrifuge to not disturb the pellet.

Note: Cell pellet is very loose and fragile. Take care not to disturb the pellet when manipulating the tube.

6. Pipet off the supernatant, leaving 1–2 mm of liquid above cell layer.

7. Dilute the original volume of blood (90 µL) 20:1 with buffer to a final volume of 1.8 mL.

8. Place in single tube holder for analysis on the MACSQuant Analyzer.

Data acquisition on the MACSQuant Analyzer

9. Open appropriate instrument settings into the MACSQuantify™ Software (e.g. human blood cells with 8×8 automated compensation matrix).

10. Select the **Height** parameter by navigating to the sidebar channels and the **Advanced** button.

11. Select an appropriate plot template for data display (e.g. plot7). Set one plot to **FSC-H** versus **SSC-H.** Set another plot to **CD45-VioBlue-H** versus **SSC-H.** Set a third plot to **CD4-PE-A** versus **PI-A** (B3 channel). To the additional plots, select axes to represent CD4-PE-A (B2 channel), CD3-FITC-A (B1 channel), CD8-APC-Vio770-A (R2 channel), and CD14-APC-A (R1 channel).

    Note: For best results, CD45-VioBlue-H should be used to set the trigger, although CD45-VioBlue-A can also be used. For gating analysis, CD45-VioBlue-A can be used.

12. Under Experiment tab select the following:
    a. Fluidics mode to **Low.**
    b. Select **Mix sample.**
    c. Select **Standard** processing mode.
    d. Uptake volume = **25 µL.**
    e. Sample volume = **1,800 µL.**
    f. (Optional) Enter a **Sample ID** or **Description.**

13. Under the **Channels** tab, set the Trigger to **V1.** Make sure all fluorescence channels are displayed in **hlog** and the scatter channels are displayed in **linear.**

14. Add 18 µL PI to sample tube and start measurement.

15. As cells are being measured adjust V1 trigger to exclude erythrocytes (cells negative for CD45-VioBlue).

16. Click the **Clear** button to apply new trigger value to all events.

17. When measurement has ended, set new uptake volume to **100 µL.**

18. Start new measurement. (Optional) For comparison, once measurement is complete, set V1 trigger to zero and re-measure sample.

Data analysis with MACSQuantify Software

19. Open acquired whole blood data files into MACSQuantify Software.

20. Open a display layout.

21. Set a gate **P1** on the CD45+ cells (leukocytes) as assessed by VioBlue staining (figure 1, plot A).

22. From P1 gated cells set a gate **P2** (intact cells) on cells by excluding debris in the dotplot FSC vs. SSC (figure 1, plot B).

    Note: It is best to view the FSC and SSC in Height rather than Area for a better display of scatter profiles when analyzing no lysis samples.

23. From P2 gated cells set a P3 gate (live cells) by excluding dead cells in CD4-PE versus PI dotplot (figure 1, plot C).

24. Analyze CD4+ and CD8+ T cells as well CD14+ monocytes by applying live leukocyte gate (P3) to further dotplots (figure 1, plots D–F).

For further details on instrument operation or data analysis, review the MACSQuant Instrument and MACSQuantify Software user manual.
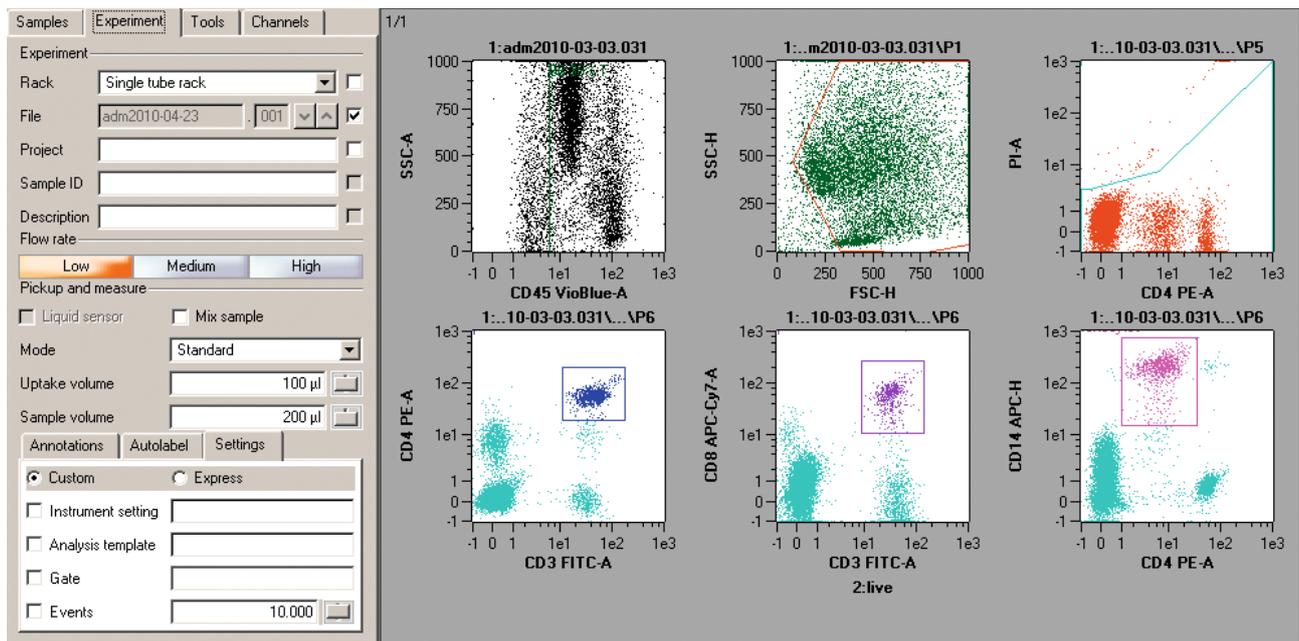

Figure 1: Gating strategy for CD4 and CD8 T cells and monocytes when performing whole blood analysis without red blood cell lysis on the MACSQuant Analyzer.

Cells	Live leukocytes [%]	Total cells [%]	Cells/µL (volume adjusted)	Reference values (µL)
Leukocytes (with trigger)	100	91.28	4600	4500–10000
Leukocytes (without trigger)	100	0.13	1200	4500–10000
CD4+ T cells (with trigger)	10.59	8.21	412	380–1500
CD4+ T cells (without trigger)	11.94	0.01	143	380–1500
CD8+ T cells (with trigger)	5.41	4.20	212	120–1386
CD8+ T cells (without trigger)	3.89	0.00	47	120–1386
Monocytes (with trigger)	10.08	7.28	394	200–900
Monocytes (without trigger)	9.17	0.01	110	200–900

Table 1: Comparison of frequencies measured on the MACSQuant Analyzer with and without appropriate trigger settings for erythrocyte exclusion.

Results

In table 1, the comparisons of frequency and absolute counts of CD4+ T cells, CD8+ T cells and monocytes obtained when applying the CD45-VioBlue trigger and without are presented. As displayed, applying trigger allows the detection and measurement of a greater number of leukocytes (figure 2). Absolute cell counts derived from whole blood after applying the CD45-VioBlue trigger are also more consistent with reported adult reference values.

Conclusion

The use of CD45-VioBlue as a fluorescence trigger in combination with the capabilities of the MACSQuant Analyzer provides an efficient No-Lyse protocol for monitoring various cell populations in whole blood. Further refinement to an automated No-Lyse, No-Wash protocol should provide an effective method for routine monitoring of various cell populations in whole blood.

Figure 2: Comparison of light scatter and CD45 fluorescence when there is no V1 trigger (A) versus the appropriate trigger set for V1 (B) to remove contaminating erytrhocytes.

References

1. Kreissig, C. *et al.* (1994) Characterization and measurement of CD34-expressing hematopoietic cells. J. Hemather. 3: 263–289.
2. Bossuyt, X. *et al.* (1997) Comparative analysis of whole blood lysis methods for flow cytometry. Cytometry Part B. 30: 124–133.
3. Greve, B. *et al.* (2006) The impact of erythrocyte lysing procedures on the recovery of hematopoietic progenitor cells in flow cytometric analysis.
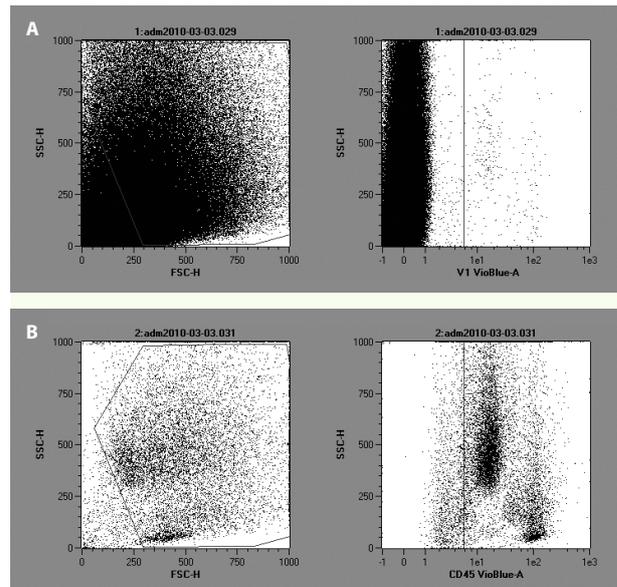
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