Standardization of whole blood immunophenotyping using an automated staining protocol and recombinant antibodies

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Introduction

Monitoring the state of the immune system by flow cytometry is a routine assay in both clinical and research settings. Although human whole blood samples are widely used for these immunophenotyping assays, they represent a critical source of variability and can impede standardization efforts. In general, the analysis includes two steps: staining of cell type–specific markers and lysis of erythrocytes. These steps are influenced by multiple parameters like incubation time, temperature, sample handling by different operators, and choice of reagents. All these parameters can introduce variability in analysis and diminish the overall reproducibility.

In this study, we show that automation of erythrocyte lysis and cell staining not only provides the basis for standardization, but additionally decreases hands-on time. We established an automated lysis and staining protocol on the MACSQuant® Analyzer 10 and verified its proper function with an immunophenotyping panel. Automation not only includes lysis and staining: Pre-selected instrument settings and analysis templates are automatically applied, which simplifies cell analysis considerably.

We also designed an immunophenotyping panel based on recombinantly generated REAfinity™ Antibodies. They are superior to traditional hybridoma antibodies for several reasons: REAfinity Antibodies are recombinantly generated, thus ensuring higher lot-to-lot consistency. Additionally, they are mutated at the Fc region to abolish any background binding to FcγRs and therefore do not require addition of FcR blocking reagent during staining.

Materials and methods

1 Automated versus manual erythrocyte lysis and staining

Whole blood (WB) samples from ten different donors in total were analyzed. For both automated and manual procedures (table 2), one tube was filled manually with a fresh EDTA-treated WB sample (step 1) and a second tube was filled manually with Red Blood Cell Lysis Solution (step 2). The following steps were either performed fully automatically by the instrument or manually. For step 3, FcR Blocking Reagent was applied to the WB sample and incubated for 10 min at room temperature (RT). Then the required antibody panel was added and incubated for 10 min at RT (step 4). Subsequently, a part of the stained WB was transferred into the lysis solution and incubated for 10 min at RT (step 5).

2 Flow cytometry analysis

Samples stained with panel 1 (table 1) were manually taken after steps 4 and 5 of the automated lysis/labeling process to monitor potential lysis effects on various cell types. Samples for immunophenotyping were automatically taken up by the MACSQuant Analyzer 10 for analysis after automated erythrocyte lysis and labeling. Pre-selected instrument settings and analysis templates were automatically applied by the instrument.

We also applied an immunophenotyping panel based on recombinantly generated REAfinity Antibodies (table 1, panel 2).

Panels

	VioBlue®	VioGreen™	FITC	PE	PerCP-Vio® 700	PE-Vio 770	APC	APC-Vio 770
Panel 1 Antigens (clones)	CD45 (REA747)	CD20 (LT20)	CD235a (REA175) CD61 (Y2/51) CD45 (5B1)	CD34 (AC136)	CD14 (Tük4)	CD66b (REA306)	CD61 (Y2/51) CD3 (REA613)	CD123 (AC145)
Panel 2 Antigens (clones)	CD45 (REA747)	CD4 (REA623)	CD3 (REA613)	CD16 (REA423) CD56 (REA196)	7-AAD	CD19 (REA675)	CD14 (REA599)	CD8 (REA734)

Table 1

Results

1 Comparison of automated and manual erythrocyte lysis

To validate the automated procedure, we compared it to the well-established manual standard method. We used antibody panel 1 for assessment of lysis efficiency and potential effects on other cell types by flow cytometry. Analysis of erythrocyte lysis based on cell count gave comparable results for automated and manual procedures. Both processes also did not have an effect on the cell count of thrombocytes and leukocytes. Detailed analysis of the erythrocyte population however revealed that the manual process resulted in the formation of two erythrocyte populations, i.e., damaged erythrocytes with low SSC and erythrocytes with medium SSC representing the majority of the erythrocyte population. In contrast, the automated process generated a single erythrocyte population, indicating a more reliable lysis result.

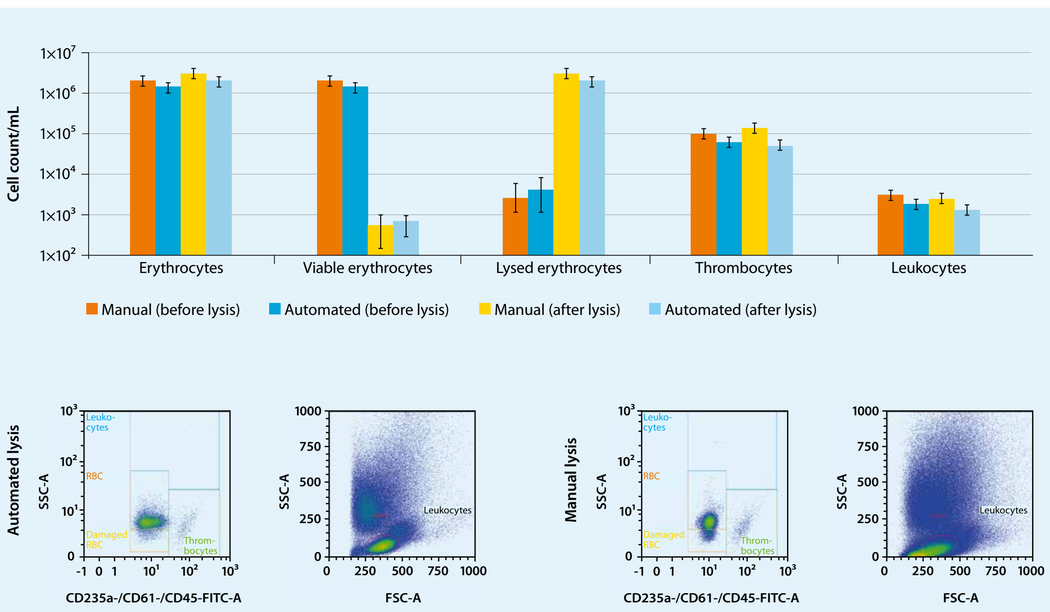


Figure 1

2 Comparison of automated and manual immunophenotyping

Immunophenotyping of cells from samples lysed with the manual or automated process delivered similar results with regard to cell counts, as determined using antibody panel 1. Figure 2A exemplifies the results for an automatically processed sample. The panel enabled the unambiguous identification of major immune cell types and stem cells. Importantly, immunophenotyping based on this panel does not rely on the analysis of scatter properties. Therefore, it was possible to analyze even manually processed cells that showed incomplete lysis (see fig. 1B). The vast majority of automatically processed samples could be analyzed without adapting any gates. In contrast, manually processed samples often required adjustment of the gates and thus made operator's constant attention necessary.

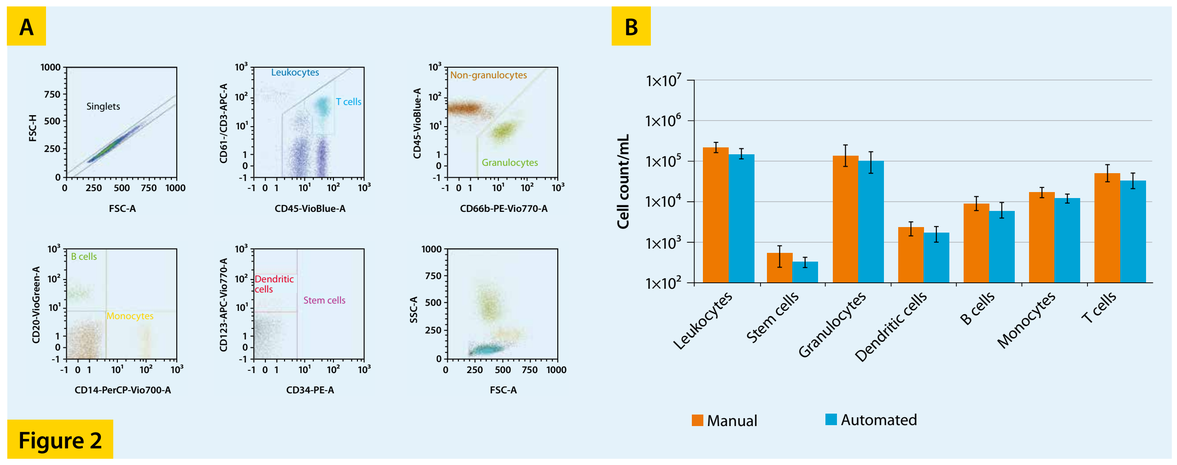
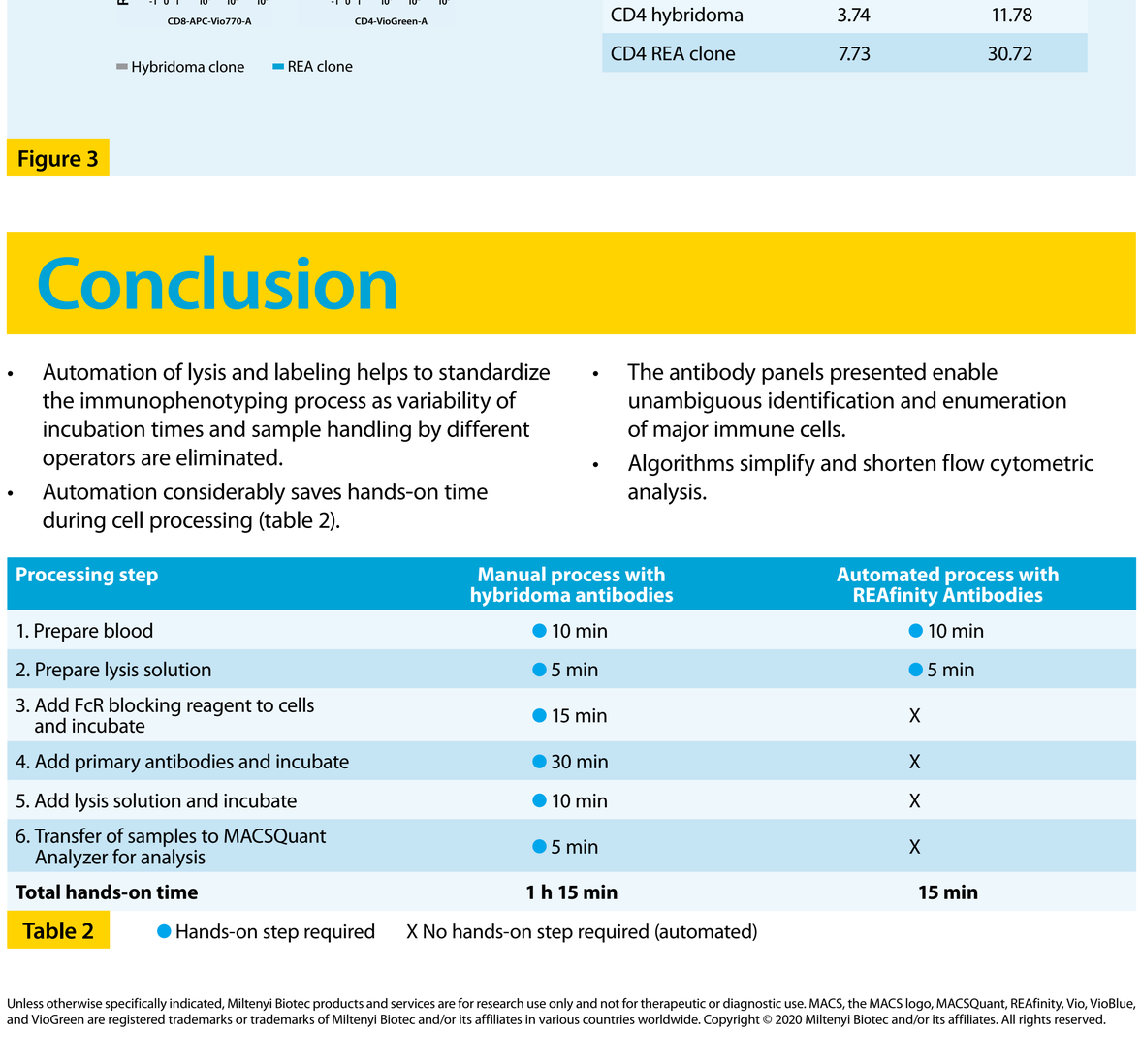


Figure 2

3 REAfinity™ Recombinant Antibody panel enables reliable immunophenotyping

The antibody panel based on REAfinity Antibodies allowed for a clear identification and enumeration of leukocytes, monocytes, eosinophils, neutrophils, natural killer (NK) cells, pan T cells, $CD8^+$ T cells, $CD4^+$ T cells, and B cells, (fig. 3A). Compared to monoclonal hybridoma antibodies, the CD8 and CD4 REA clones, for example, enabled a more specific labeling as indicated by higher stain indices (SI; fig. 3C). Moreover, the CD8 REA clone allowed a more detailed analysis as a population of $CD8^{low}$ cells could be easily identified (fig. 3C).



Antibody	SI	MFI
CD8 hybridoma	18.11	94.51
CD8 REA clone	26.65	87.52
CD4 hybridoma	3.74	11.78
CD4 REA clone	7.73	30.72

Figure 3

Conclusion

- Automation of lysis and labeling helps to standardize the immunophenotyping process as variability of incubation times and sample handling by different operators are eliminated.
- Automation considerably saves hands-on time during cell processing (table 2).
- The antibody panels presented enable unambiguous identification and enumeration of major immune cells.
- Algorithms simplify and shorten flow cytometric analysis.

Processing step	Manual process with hybridoma antibodies	Automated process with REAfinity Antibodies
1. Prepare blood	● 10 min	● 10 min
2. Prepare lysis solution	● 5 min	● 5 min
3. Add FcR blocking reagent to cells and incubate	● 15 min	X
4. Add primary antibodies and incubate	● 30 min	X
5. Add lysis solution and incubate	● 10 min	X
6. Transfer of samples to MACSQuant Analyzer for analysis	● 5 min	X
Total hands-on time	**1 h 15 min**	**15 min**

Table 2 ● Hands-on step required X No hands-on step required (automated)

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