Optimized 7-color REAfinity™ Antibody Cocktail for Immunophenotyping

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Introduction

Immunophenotyping by multicolor flow cytometry is a powerful diagnostic tool to assess the status of a patient's immune system, e.g. for immune monitoring and complementary research in clinical trials or the detection of minimal residual disease after treatment for leukemia [1]. Other possible applications include the quality control of cellular therapy products or preclinical research, e.g. mouse xenograft models. Having reliable, consistently performing antibodies and dyes is crucial to all the aforementioned applications. Here we present an improved ready-to-use seven color immunophenotyping cocktail, made up solely of our Miltenyi Biotec REAfinity™ antibodies, for the differentiation of nine leukocyte subsets. REAfinity™ are recombinant antibodies of human IgG1 isotype with a mutated region within the Fc portion, which prevents Fcγ receptor binding. Furthermore, they are characterized by high antigen specificity and consistent quality compared to conventional hybridoma-derived monoclonal antibodies. Here we show their superior performance in a multicolor flow cytometry application.

Results

1 Comparison of hybridoma and REAfinity™ based immunophenotyping cocktails

Specificity	Fluorochrome	AB clone	Purpose
CD3	PE	REA613	T cell lineage
CD4	VioBright 667	REA623	T_H
CD8	APC-Vio 770	REA734	CTL
CD14	VioBlue	REA599	Monocytes
CD16	VioBright 515	REA423	Granulocytes
CD19	PE-Vio 770	REA675	B cells
CD45	VioGreen	REA747	Leukocytes
CD56	VioBright 515	REA196	NK cells
Dead cells	7-AAD	n/a	Dump

Specificity	Fluorochrome	AB clone	Purpose
CD3	APC	BW264/56	T cell lineage
CD4	PerCP	VIT4	T_H
CD8	APC-Vio 770	BW135/80	CTL
CD14	FITC	Tük4	Monocytes
CD16	PE	VEP13	Granulocytes
CD19	PE-Vio 770	LT19	B cells
CD45	VioBlue	5B1	Leukocytes
CD56	PE	AF12-7H3	NK cells

Table 1

The new REAfinity™ based cocktail includes two new Vio© dyes, VioBright™ 515 and VioBright™ 667, and it allows the addition of 7-AAD for dead cell exclusion (table 1). The specificities of antibodies in both cocktails are identical. The antibody cocktails were used to stain whole blood of healthy donors with a lyse/no wash protocol, i.e. the cocktails as well as 7-AAD were added to 100µL of human whole blood, incubated and subsequently the blood was lysed with Red Blood Cell Lysis Solution. Afterwards the cells were acquired with the MACSQuant® Analyzer 10.

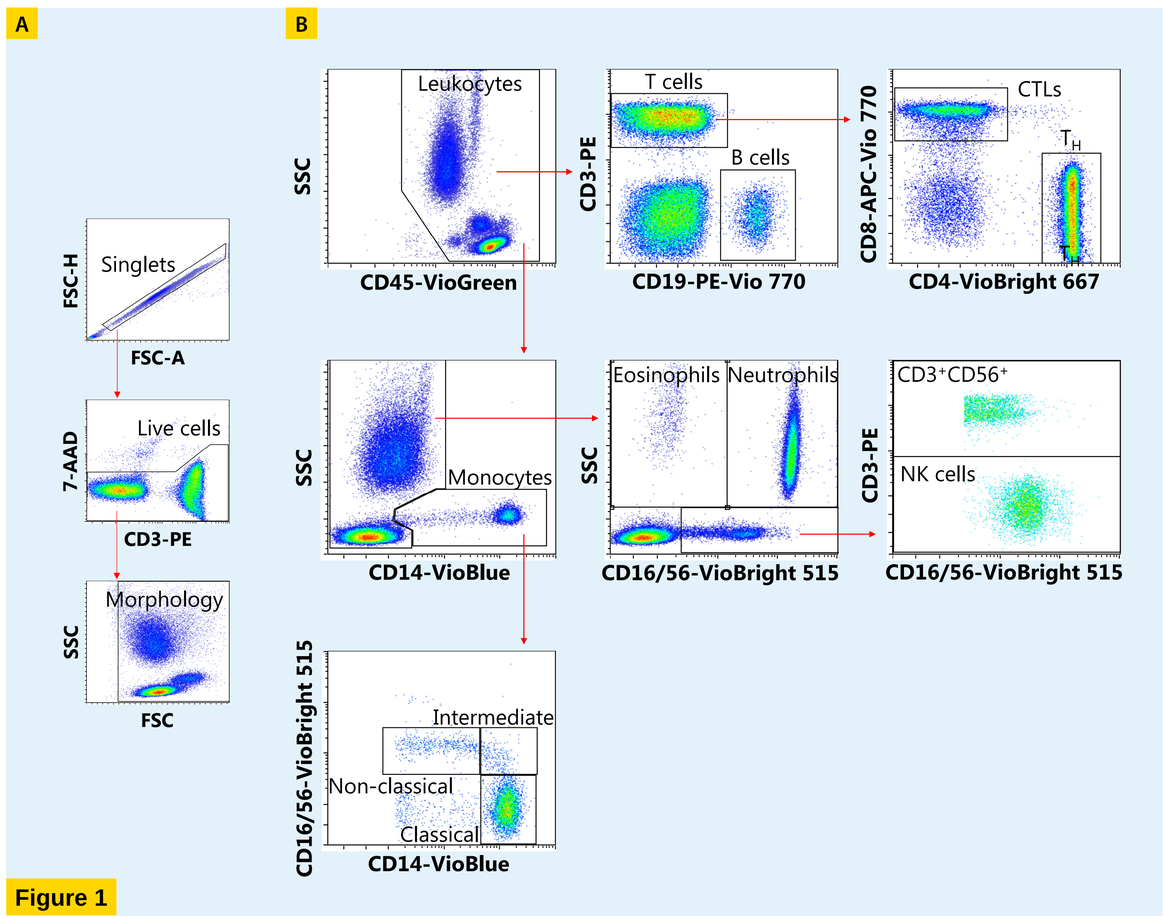

Figure 1

After gating on singlets, viable cells and FSC/SSC (fig. 1A), all leukocytes were gated as $CD45^+$ cells. Among leukocytes, T cells and B cells were identified as $CD3^+$ and $CD19^+$, respectively. T cells were further separated into $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ T helper (T_H) cells. Monocytes were discriminated based on their CD14 expression and further divided into classical, intermediate and non-classical monocytes via CD16. Among the non-monocyte population, CD16 and SSC were used to distinguish neutrophils ($CD16^+SSC^{high}$) and eosinophils ($CD16^-SSC^{high}$). Lastly, NK cells were identified as $CD56^+$ as well as a $CD3^+CD56^+$ population, which included NKT cells among others.

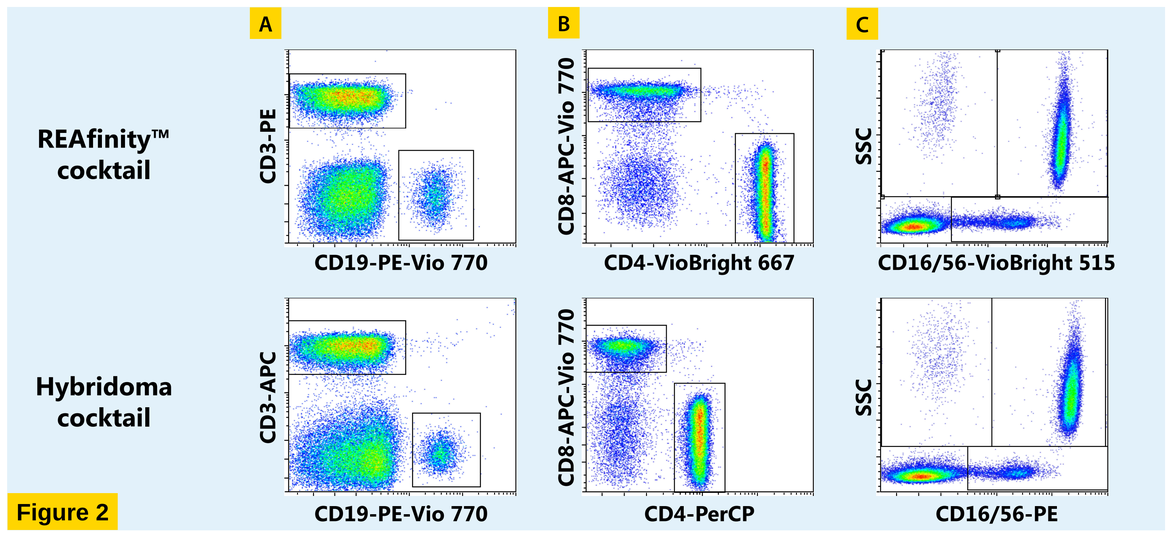

Figure 2

Figure 2 shows three examples of a direct comparison between the staining performance of the two antibody cocktails. The separation of B cells was better with the new REAfinity™ based cocktail with a lower unspecific background (fig. 2A). Due to the change of CD4-PerCP to CD4-VioBright™ 667, the identification of T_H cells was improved (fig. 2B). The combination of low background due to the REAfinity™ antibody and bright signal with the Viobright™ 515 dye, resulted in a better resolution for the discrimination of $SSC^{low}CD56^+$ cells (fig. 2C).

Stain Index [-]*	CD3	CD4	CD8	CD14	CD16	CD19	CD56
REAfinity	50	68	30	113	162	36	17
Hybridoma	57	12	28	63	98	11	9

Table 2 *n=4

The stain indices (SIs) of both antibody cocktails for all specificities except CD45 were determined and the means of the SIs for four whole blood donors are shown in table 2. Overall, comparable or better separation of each target population was achieved with the new cocktail. Only the stain index for CD3 was slightly lower due to the change from CD3-APC to CD3-PE, but still sufficient for the discrimination of T cells. In particular CD4, CD19 and CD56 antibody staining resulted in a definite improvement compared to the previous cocktail, as the separation for these select markers were only mediocre before.

2 REAfinity™ immunophenotyping cocktail as in-process control

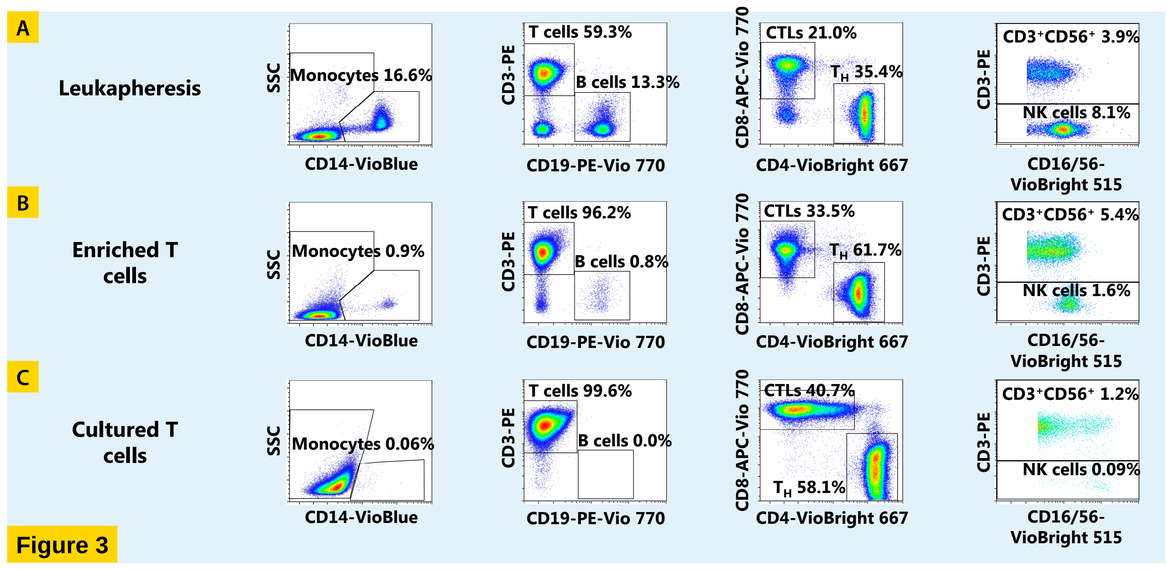

Figure 3

The REAfinity™ immunophenotyping cocktail was used to assess the general cellular composition during the generation of genetically engineered T cells. At different stages of this procedure, samples were stained with the cocktail as part of the in-process control (fig. 3). The gating strategy used was the same as described above and plots are shown for three different time points: the original leukapheresis (fig. 3A), the positive fraction after T cell enrichment with the CliniMACS CD4 and CD8 Reagents (fig. 3B), as well as the genetically engineered T cells after five days of culture (fig. 3C). Frequencies depicted in the plots are among viable leukocytes. For each of these steps the cellular composition could be determined with the new immunophenotyping cocktail.

Conclusion and outlook

- The new REAfinity™ based phenotyping cocktail shows improved performance over the hybridoma-based cocktail with better separation of several populations due to lower background and an optimized panel
- The cocktail can be used in a variety of applications, e.g. as in-process or quality control for cell therapy products
- With the upcoming Express Mode, an easy-to-use and standardized tool for immunophenotyping of human samples will be available

References

1 Van Dongen, JJM et al. (2015) Blood 125: 3996-4009