Recombinant antibodies for improved standardization in flow cytometry

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Introduction

REAfinity™ Antibodies are recombinant antibodies developed by Miltenyi Biotec that allow reproducible flow cytometry analysis. They are expressed in mammalian cells under standardized *in vitro* culture conditions and highly controlled manufacturing practices that ensure long-term consistency across all lots[1]. To ensure homogeneity within and among lots, each of these recombinant antibodies is generated from a carefully defined sequence encoding a single heavy chain and a single light chain. Furthermore, all REAfinity Antibodies include the same engineered human IgG1 constant region allowing the use of one universal isotype control. Miltenyi Biotec offers 5,000 flow cytometry-validated REAfinity Antibodies.

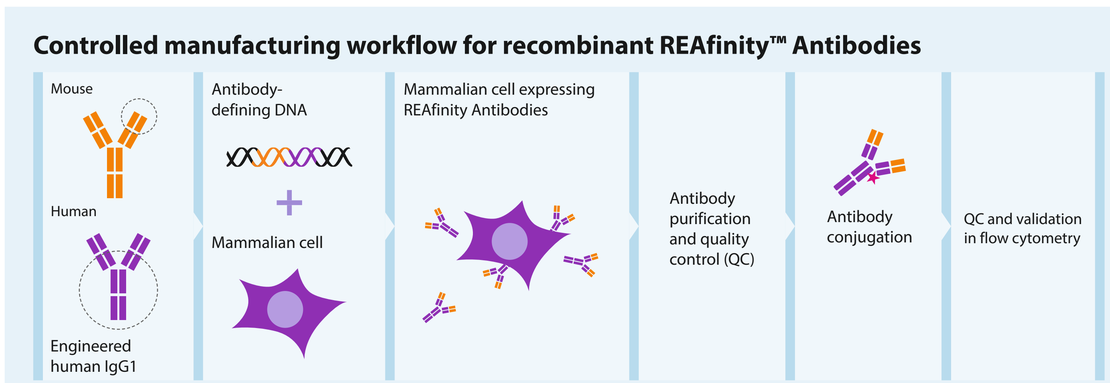


Figure 1

REAfinity™ Antibodies are recombinantly expressed in a mammalian expression system. After purification and validation of the purity of the antibodies, they are conjugated to fluorochromes. These conjugated antibodies are then purified again to remove unbound fluorochromes. A standardized and controlled manufacturing workflow with strict specifications ensures a consistently high product quality. The usage of a recombinant system makes sure that one DNA sequence translates into exactly one product, and the control over the sequence allows for engineering and improvement of antibody functions.

Results

1 REAfinity™ Antibodies are highly pure and standardized in composition

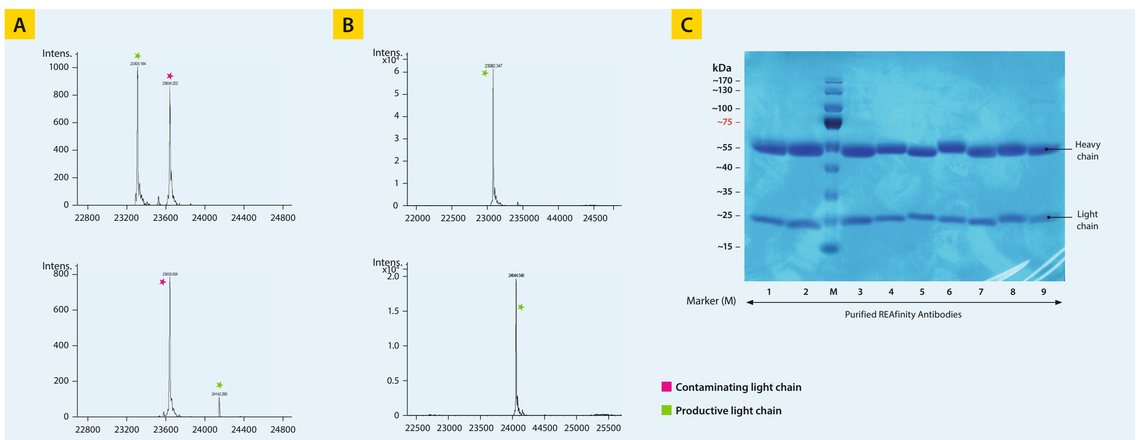


Figure 2

Purified antibodies were reduced and analyzed by ESI LC-MS. REAfinity™ Antibodies are defined products, while hybridoma-generated antibodies can be a mixture. In figure 2A two examples of hybridoma-generated antibodies each containing a second light chain with a molecular weight of approx. 23,635 Da are presented. We have found that this is the mouse myeloma MOPC-21 light chain[2], descending from a rearranged kappa variable IGKV-J gene. In one example, the amount of the contaminating light chain was by far exceeding the productive light chain (24,142 Da). We have identified also other contaminating light chains in mouse and rat hybridomas with masses of ~22,147 Da and ~24,227 Da (not shown). Figure 2B shows two examples of recombinant REAfinity Antibodies. Both had the expected light chains without the contaminating light chain. Figure 2C shows an SDS-PAGE of nine purified REAfinity Antibodies: 10 µg each of the antibodies were analyzed under reducing conditions and stained by Coomassie® Brilliant Blue. Heavy chains migrate at ~50 kDa, light chains at ~25 kDa (fig. 2C).

2 Engineered REAfinity™ Antibodies do not bind to Fc gamma receptors

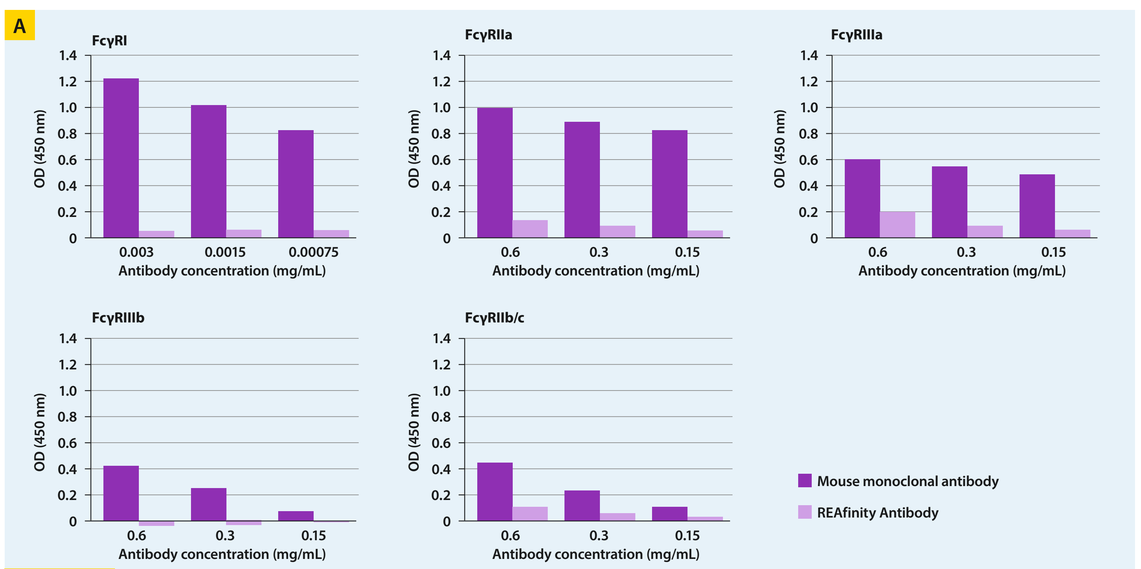


Figure 3

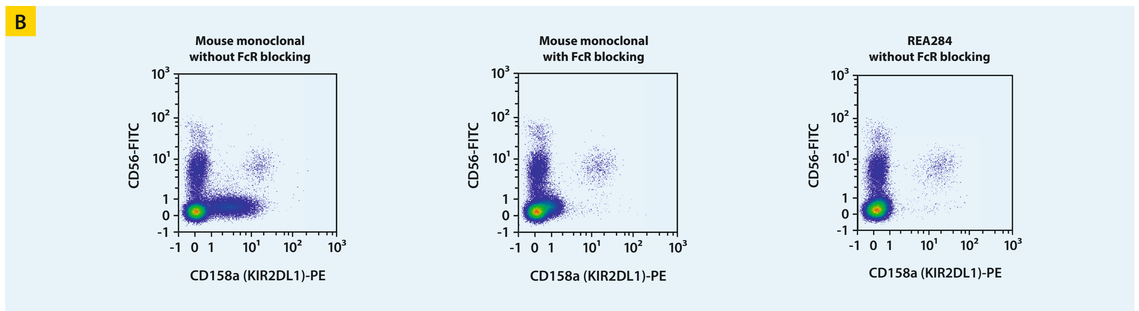


Figure 3 (cont.)

Hybridoma-generated mouse monoclonal antibodies bind to the high affinity FcγRI (CD64) as well as the low affinity FcγRIII (CD16) and FcγRII (CD32). In contrast, REAfinity™ Antibodies are engineered and have a human Fc portion with a mutated Fc gamma receptor binding site, resulting in virtually no interaction with Fc gamma receptors, as demonstrated by using recombinant soluble Fc-gamma receptors (extracellular domains, fig. 3A). Human PBMCs were stained with CD158a (KIR2DL1)-PE conjugates, as well as with CD56-FITC and analyzed by flow cytometry using the MACSQuant® Analyzer. Left column: Hybridoma-generated antibody-PE +/– FcγR blocking reagent. Right column: REAfinity Antibody-PE. While for a hybridoma antibody FcγR blocking was required to reduce non-specific Fc gamma receptor binding, the same or even a better result could be achieved with a REAfinity Antibody without blocking (fig. 3B).

3 High lot-to-lot reproducibility by multiple quality control steps

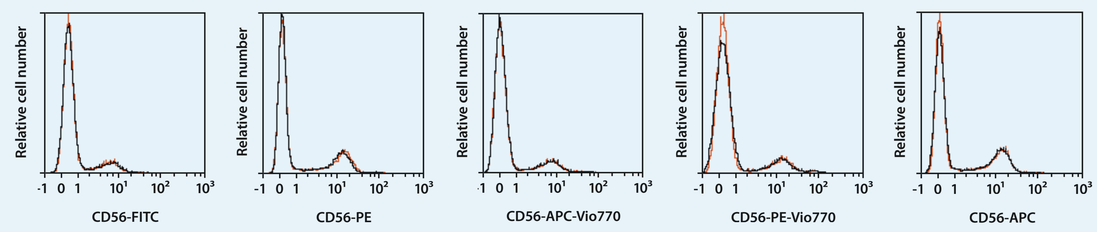


Figure 4

Every manufactured lot is tested at the stage of purified antibody as well as at the stage of the antibody-fluorochrome product. Test procedures comprise analytical (such as MS), biochemical, and cell-based methods. The final product is always tested for staining performance with cells.

Two manufacturing lots of five different CD56 REAfinity Antibody conjugates were compared by flow cytometry using the MACSQuant Analyzer (fig. 4). The two histogram curves (black and red) represent the two lots. Lot-to-lot staining performance was nearly identical. Human PBMCs from a single donor were used.

4 Validated for specificity and flow cytometry applications

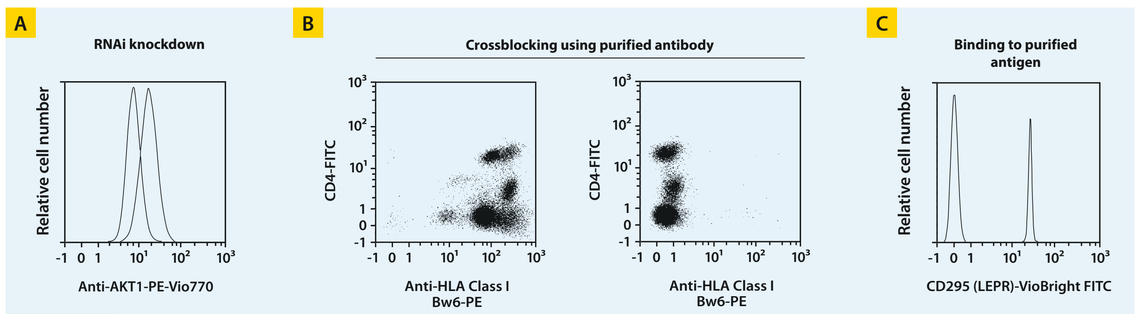


Figure 5

To validate the specific binding of REAfinity Antibodies to their target antigens, we have introduced several methods for validation of antibodies, for example, knockdown of target antigens, cross-blocking using validated antibodies as reference, and antibody binding to purified target antigen. Figure 5A shows a knockdown of the target antigen by RNAi. The left peak indicates HeLa cells transfected with AKT1 RNAi, the right peak is the non-transfected control. Cells were fixed, permeabilized, stained with Anti-AKT1 antibody-fluorochrome conjugates, and analyzed by flow cytometry using the MACSQuant Analyzer. Cell debris was excluded based on scatter signals. Fig. 5B shows crossblocking experiments, where the specificity of the REAfinity Antibody was confirmed by blocking the binding to the ligand on PBMCs, using pure non-conjugated reference antibodies. Cells were then stained with REAfinity Antibody anti-HLA Class I Bw6-PE conjugates, as well as with CD4-FITC and analyzed by flow cytometry using the MACSQuant Analyzer (left: staining signals of the REAfinity Antibody; right: blocked signals). Cell debris and dead cells were excluded based on scatter signals and PI fluorescence. Figure 5C shows antibody binding to purified antigen. Latex beads were coated with recombinant human CD295 protein. Left peak: staining of beads with appropriate REA Control (S) antibody. Right peak: staining with REAfinity Antibody CD295 (LEPR)-VioBright FITC. Flow cytometry was performed with the MACSQuant Analyzer.

Conclusion

REAfinity Antibodies are engineered "next-generation" antibodies that comply with novel research requirements that cannot be fulfilled anymore by hybridoma-generated antibodies:

- One DNA – one product
- Standardized and controlled manufacturing workflow
- No hybridoma antibody restrictions (light chain contaminations, FcγR binding)
- State-of-the-art analytical, biochemical, and cell-based quality control
- High lot-to-lot reproducibility, strict release specifications
- Validation of antibody specificity for flow cytometry applications

References
1. Bradbury, A. and Plückthun, A. (2015) 518: 27–29.
2. Svasti, J. and Milstein, C. (1972) Biochem J. 128: 427–44

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