MACS
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Flow cytometry: Antibodies and controls

Introduction

Fluorochrome-conjugated antibodies are powerful tools for the detection of particular cell types by multiparameter flow cytometry.
As antibodies can be generated against almost any kind of molecular structure, they provide a high specificity for labeling individual cell subsets. However, due to their complex structure, antibodies also can undergo multiple interactions with any kind of molecule on or inside the cells. Therefore, it is vitally important to use the reagents correctly and have reliable and reasonable controls to obtain high-quality data. This guideline outlines important tips and tricks, including necessary controls, for using antibodies in flow cytometry.

Desired and undesired antibody-cell interactions

Antibodies and cells can interact in various ways with each other. Besides binding to specific epitopes on the cell surface or inside cells, fluorochrome-conjugated antibodies can also bind non-specifically to cellular structures.

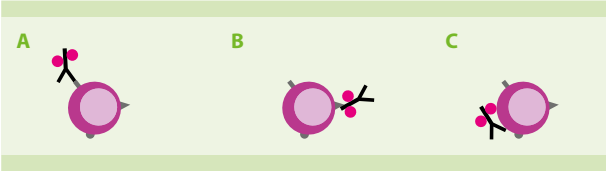

Figure 1: Fluorochrome-conjugated antibodies can bind to a cell in various ways. (A) Antigen-specific binding (B) Non-specific binding to FcR (C) Unclassified non-specific binding

As shown in figure 1 the desired interaction is the antigen-specific binding of the antibody to the molecule of interest, such as a cell type–specific marker (A). This interaction enables flow cytometry analysis of a particular cell type.
In contrast, antibody binding to Fc receptors (FcR) is undesired (B). These receptors are expressed on certain leukocyte subpopulations and capture the antibody at the constant Fc part, inducing a positive signal during flow cytometry, although the cell might not express the molecule of interest. This is a false positive signal and would result in misinterpretation of the data.
A third way of antibody-cell interaction is the non-specific binding ("sticking") of the conjugated molecules, such as fluorochromes, to cells (C). In most cases the precise molecular interaction in this process is not known and can therefore not be prevented by blocking.
Although unavoidable during the experiment, this non-specific binding can be accounted for by running appropriate controls.

Preventing non-specific binding of fluorochrome-conjugated antibodies to cells

There are various options to avoid undesired interaction of flow antibodies with cells, for example, using blocking reagents or genetically engineered antibodies.

Eliminating binding of antibodies to FcRs

FcR blocking reagents saturate FcRs, so that antibodies cannot bind to the cells with their constant parts.
This blocking avoids FcR-mediated non-specific labeling of cells (fig. 3). The following reagents are available from Miltenyi Biotec for use with either human or mouse cells:

- FcR Blocking Reagent, human (#130-059-901)
- FcR Blocking Reagent, mouse (#130-092-575)

An alternative way to avoid binding of the staining antibodies to FcRs is the use of the **genetically engineered REAfinity™ Antibodies** (Miltenyi Biotec antibodies whose clone names start with "REA"). Besides exhibiting a high specificity for the respective antigen, these antibodies carry mutations in the Fc region (fig. 2) and therefore cannot be recognized by FcγRs anymore (fig. 3).
For technical details, see our brochure "REAfinity Antibodies" or go to **www.miltenyibiotec.com/REA**

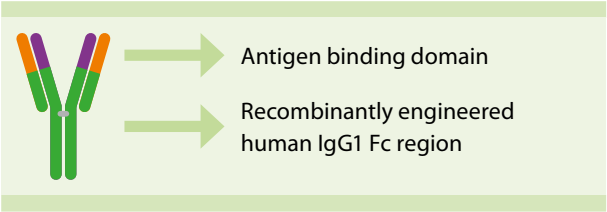

Figure 2: Structure of REAfinity Antibodies. All REAfinity Antibodies have the same recombinantly engineered human IgG1 Fc region, which does not bind to FcγRs.

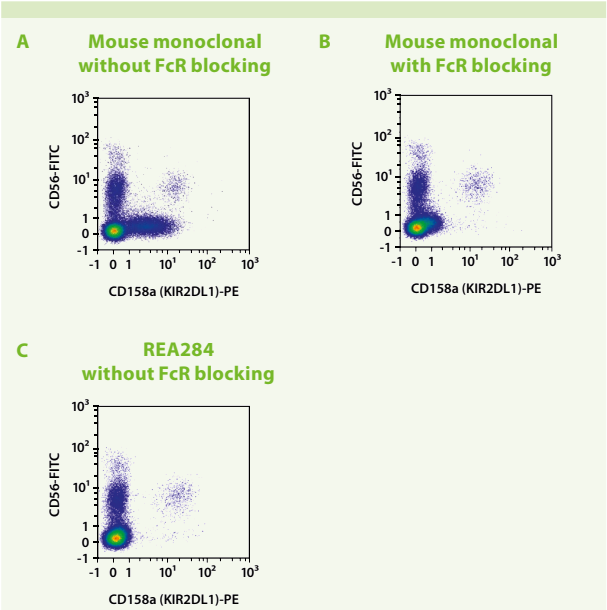

Figure 3: Staining of cells with a mouse monoclonal antibody or a REAfinity Antibody in the absence or presence of FcR blocking reagent. PBMCs were stained with either a PE-conjugated mouse monoclonal antibody (A, B) or a PE-conjugated REAfinity Antibody (C) recognizing CD158a. Cells were also stained with CD56-FITC (# 130-100-746) and analyzed by flow cytometry on the MACSQuant® Analyzer. Staining with the mouse monoclonal antibody was performed either without (A) or with (B) pre-treatment with FcR blocking reagent. No FcR blocking reagent was included prior to staining with the REAfinity Antibody (C). Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide (PI) fluorescence.

Eliminating binding of fluorochromes to monocytes

One of the few known non-specific interactions of fluorochromes with cells is the binding of tandem fluorochromes to human monocytes. The Tandem Signal Enhancer, human minimizes this binding and thus reduces background staining. It also blocks binding to FcRs.

- Tandem Signal Enhancer, human (# 130-092-575 and # 130-099-888)

Controls to account for non-specific antibody-cell interactions

Usually, it is not possible to block non-specific binding of fluorochrome-conjugated antibodies to cells when the nature of the interaction is unknown. However, to evaluate the magnitude of the background signal, different types of controls can be performed:

Isotype control

To examine the non-specific binding of a fluorochrome-conjugated antibody to cells, a separate aliquot of cells is incubated with an antibody of the same antibody class, conjugated to the same fluorochrome as the staining sample (fig. 4). This antibody must not recognize any molecule on the cells used. The resulting positive events are then evaluated as non-specific background labeling by this isotype. It is crucial that the conjugation and other production processes for the isotype control antibody are the same as for the staining antibody. Any difference might alter the binding behavior of the conjugate and thus render a reliable estimation of background labeling impossible. Therefore, isotype control antibodies should be from the same manufacturer as the staining conjugate. The isotype control antibodies from Miltenyi Biotec are carefully matched to the antibody conjugates.

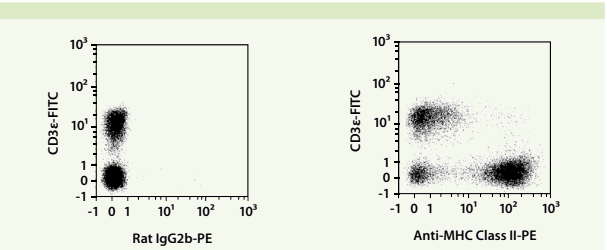

Figure 4: Splenocytes from BALB/c mice were stained with a PE-conjugated Anti-MHC Class II antibody or with the corresponding Rat IgG2b antibody. Flow cytometry was performed with the MACSQuant Analyzer. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence.

To minimize the difference between staining antibody and isotype control, the use of REAfinity Antibodies (fig. 2) and the respective isotype controls is beneficial: The sequences for the constant regions of heavy and light chains are identical on all REA clones; the only difference is the antigen binding site. Thus, this is the most precise isotype control possible and enables the most accurate measurement of non-specific labeling (fig. 5).

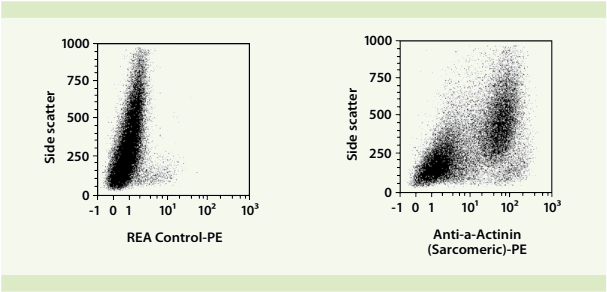

Figure 5: Heart tissue from P1 Wistar rats was dissociated using the Neonatal Heart Dissociation Kit and the gentleMACS™ Dissociator. Neonatal cardiomyocytes were then fixed, permeabilized, and stained with a PE-conjugated Anti-α-Actinin (Sarcomeric) antibody or with the corresponding REA Control antibody. Flow cytometry was performed using the MACSQuant Analyzer. Cell debris was excluded from the analysis based on scatter signals.

Isoclonic control

Similar to the isotype control, the isoclonic control is used to estimate non-specific binding of antibody conjugates to cells. In the isoclonic control sample, the cells are first incubated with an excess amount of unconjugated antibody. Then the conjugated form is added. As the unconjugated antibody saturates the epitopes, no or only very little fluorescent staining should occur in this sample compared to a sample that was incubated without unconjugated antibody. If staining is still detectable, it is probably due to non-specific binding of the conjugate, since the epitopes are blocked by the unconjugated antibody.

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