Miltenyi Biotec

Antibody panel building guide

Introduction

To obtain meaningful data in multicolor flow cytometry, it is important to keep certain parameters in mind when creating the antibody panel. This guideline describes the basic workflow and provides some rules for designing and validating complex antibody panels.

Identify antigens and their characteristics

To appropriately match fluorochromes with the respective antigens, it is crucial to categorize the antigens according to their expression patterns and importance.

Primary antigens

- Necessary for panel
- Generally distinct and strong expression
- Expression will not change between samples. These antigens are typically lineage markers like CD3, CD4, CD8, CD14 etc.
- Primary antigens are typically labeled with dim fluorochromes.

Secondary antigens

- Further antigens for immunophenotyping
- Expression levels can vary between samples.
- Broad range of expression levels, from low to high
- Second-tier markers (e.g. CD25 for Treg cells)
- Brighter fluorochromes are reserved for secondary antigens.

Tertiary antigens

- "Wish list" or experimental markers
- Marker expression very weak or even unknown
- Reagent availability might be limiting.
- Brightest fluorochromes are reserved for tertiary antigens.

Assign antigens to fluorochromes

As outlined before, match the markers/antigens with a suitable fluorochrome and follow these further guidelines:

1. Check availability of fluorochrome conjugates using the Antibody Panel Builder on the Miltenyi Biotec website, for example.

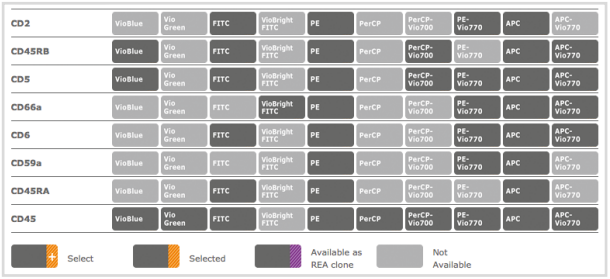


Figure 1: The Antibody Panel Builder available at **www.miltenyibiotec.com/panel**

2. Assign the antigens with the weakest or unknown expression level to the brightest fluorochromes, using the stain index ladder.

Fluorochrome	Brightness based on SI
APC	1
VioBright™ FITC	1
PE	1
PE-Vio® 770	2
FITC	3
PerCP-Vio® 700	3
APC-Vio 770	4
VioBlue®	4
PerCP	5
VioGreen™	5

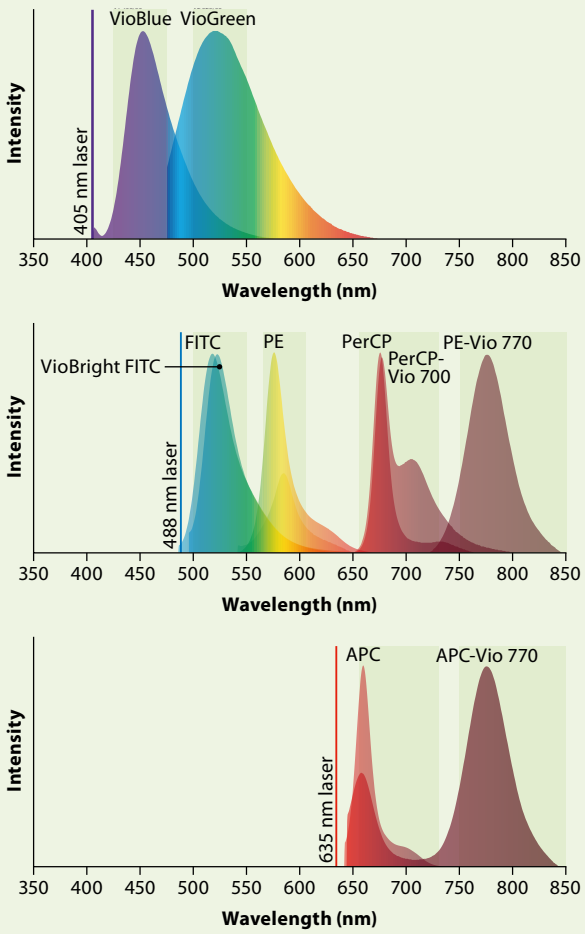
Table 1: Relative brightness of fluorochromes. The fluorochromes are ranked based on their stain index (SI). Rank 1 is assigned to brightest fluorochromes and rank 5 to dim fluorochromes.

3. Assign the remaining antigens to the other fluorochromes.
4. Avoid placing strongly expressed antigens in fluorescence channels that emit a lot of fluorescence spillover into a channel where a weakly expressed antigen is measured.
   To estimate the spillover, compare the fluorochrome spectra (table 3, fig. 2) with the filter configuration of your instrument.

Lasers	Channel	Filter (nm)	Dye or parameter
Violet 405 nm	V1	450/50	CFP, VioBlue
	V2*	525/50	Pacific Orange™, VioGreen
Blue 488 nm	B1	525/50	GFP, FITC
	B2	585/40	PE
	B3	655–730	PI, PerCP, PE-Cy™ 5.5, PerCP-Vio 700
		750 LP	PE-Cy7, PE-Vio 770
Red 635 nm	R1	655–730	APC
	R2	750 LP	APC-Cy7, APC-Vio 770
Blue 488 nm	FSC	488/10	Size
	SSC	488/10	Granularity

Table 2: Filter and laser configuration of the MACSQuant® Analyzer 10.

A



B

Fluorochrome	Emission$_{max}$	Excitation$_{max}$	Channel
VioBlue	452 nm	400 nm	V1 450/50
VioGreen	520 nm	388 nm	V2 525/50
FITC	517 nm	490 nm	B1 525/50
VioBright FITC	522 nm	494 nm	B1 525/50
PE	575 nm	565 nm	B2 585/40
PerCP	675 nm	482 nm	B3 655–730
PerCP-Vio 700	676 nm	482 nm	B3 655–730
PE-Vio 770	776 nm	565 nm	B4 750 LP
APC	660 nm	652 nm	R1 655–730
APC-Vio 770	775 nm	652 nm	R2 750 LP

Figure 2: (A)Emission spectra of selected fluorochromes. Wavelengths of lasers and bandwidths of filters used for excitation and detection on the MACSQuant Analyzer 10 are indicated by colored lines and shaded areas, respectively. (B) Details on selected fluorochromes. Wavelengths of maximal emission and excitation as well as channels used for detection of the fluorochromes on the MACSQuant® Analyzer 10 are specified.

5. When using tandem conjugates, please keep their characteristics in mind. A tandem conjugate consists of two covalently linked fluorochromes. One of the fluorochromes serves as the "donor" and the other one as "acceptor" of energy. Upon excitation the "acceptor" fluorochrome emits a signal that is detected during flow cytometry analysis. Due to their structure, tandem conjugates are prone to damage upon exposure to heat, light, and fixation. This can result in decoupling of donor and acceptor dyes, leading to fluorescence emission from the donor fluorochrome (e.g. PE when using PE-Vio 770) into the corresponding channel (in case of PE-Vio 770 into the B2 channel). This would result in an increased background fluorescence in this channel, which can be detrimental when analyzing very dim cell populations. To minimize this background fluorescence, make sure to store the tandem conjugate protected from light. The higher the level of degradation, the stronger the background fluorescence.

Depending on the intended analysis it might not be possible to follow all rules exactly. Some compromises however can still result in very good antibody panels.

Test antibody panels

It is advisable to design several panels with differing antibody/fluorochrome combinations. Cell samples should then be stained with these panels, measured, and analyzed. The panels which enable the best analysis of the critical markers, such as weakly expressed antigens, should be selected.

Important controls

To determine and eventually increase the specificity of an antibody staining, certain controls are needed. The most common controls include:

Compensation controls

- Beads or cells stained with a single fluorochrome
- Included in every experiment

Fluorescence minus one (FMO) controls

- For panel validation and gating

Specificity controls

- Dead cell exclusion
- Fc receptor (FcR) blocking
- Tandem Signal Enhancer to increase binding specificity of tandem dyes
- Isotype controls

Experiment-specific controls

- Stimulated vs. unstimulated cells
- Healthy donor vs. patient

Miltenyi Biotec

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

