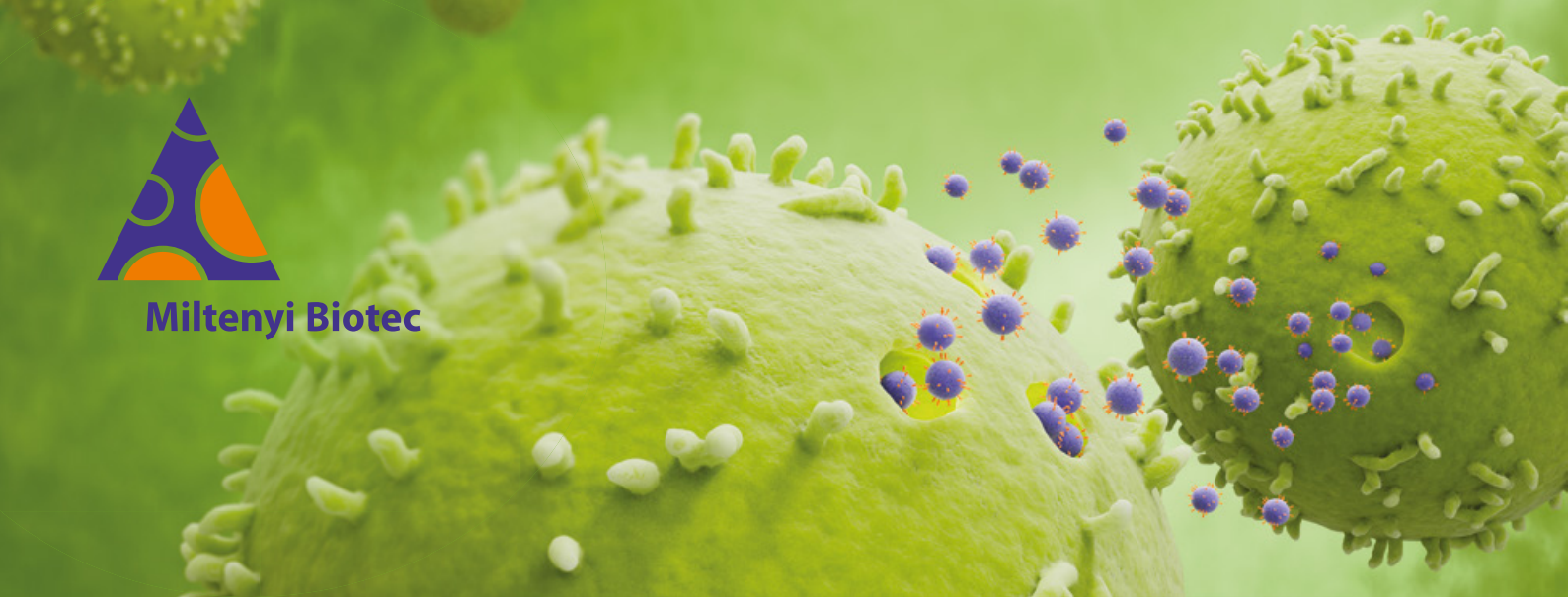

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Customization of MACSPlex Exosome Kit enables comprehensive, tailored EV analysis

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Background

Living cells release extracellular vesicles (EVs) such as exosomes in an evolutionary conserved manner. EVs play an important role in intercellular communication and therefore have a high potential for clinical translation. However, due to their small size and their heterogeneity, it has been challenging to analyze them by conventional flow cytometry. Here, we used Miltenyi Biotec's MACSPlex Exosome Kit, a new multiplex bead-based flow cytometry assay facilitating comprehensive EV analysis, to analyze the expression of a self-defined EV surface marker. To that end, we tested this kit regarding its compatibility with customized adaptations. This application note is based on reference 1.

Methods

MACSPlex Exosome Kit

The MACSPlex Exosome Kit enables the detection of 37 surface epitopes potentially present on EVs. The assay relies on a cocktail of different fluorescently labeled bead populations, the so-called MACSPlex Exosome Capture Beads, which can be distinguished by flow cytometry.
Each of these MACSPlex Exosome Capture Bead populations is coupled to an antibody specifically recognizing one of the 37 exosomal surface epitopes. EVs that bind to the MACSPlex Capture Beads are then stained with a detection reagent, e.g., a cocktail of APC-conjugated antibodies against the tetraspanins CD9, CD63, and CD81, which are commonly found on exosomes. Ultimately, this results in the formation of complexes consisting of i) MACSPlex Exosome Capture Bead, ii) EVs, and iii) APC-conjugated antibodies. Fluorescence characteristics of both the MACSPlex Exosome Capture Beads and the APC-conjugated antibodies allow for flow cytometric analysis of the epitopes expressed on the EVs (fig. 1).

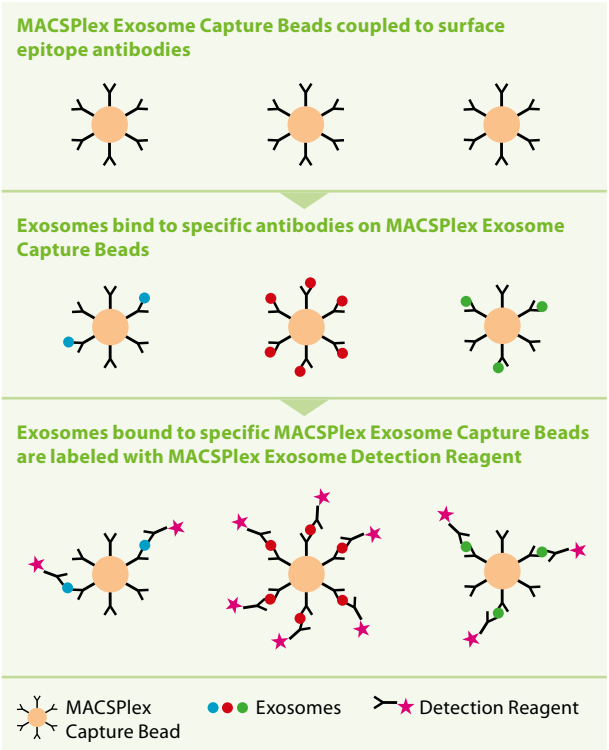

Figure 1: Principle of MACSPlex Exosome Kit. MACSPlex Capture Beads contain a cocktail of various fluorescently labeled bead populations, each coated with a specific antibody reacting with one of the exosome types within the sample. Finally, the detection reagent will generate a signal that is detectable by flow cytometry.

Generation of fluorescently labeled EVs HEK293T cells were transduced using a lentiviral construct enabling expression of a fusion protein consisting of the common EV marker CD63 and the fluorescent protein Cerulean. As a result, this cell line released fluorescent EVs.

Compatibility of the Cerulean fluorescence with the MACSPlex Exosome Kit was tested i) by analyzing spillover into the fluorescence channels needed for the standard protocol of the MACSPlex assay and ii) by comparing the sets of EV markers identified by the MACSPlex Exosome Capture Beads in the presence or absence of the APC-conjugated CD9, CD63, and CD81 antibodies.

Analysis of FOLR1 expression on EVs

FOLR1 surface expression on EVs derived from human cell lines PANC-1 and IGROV1 was assessed by flow cytometry after staining EVs with APC-conjugated anti-human FOLR1 monoclonal antibodies. All flow cytometry analyses were performed using a MACSQuant® Analyzer 10.

Results

Assay compatibility with fluorescently labeled EVs

In our studies, we frequently use EVs constitutively labeled with a fluorescent protein, such as Cerulean, to analyze cellular EV uptake or release, for example. As the MACSPlex Exosome Kit is based on a cocktail of APC-conjugated antibodies for the detection of EVs, we tested whether the fluorescence from CD63-Cerulean–labeled EVs interferes with APC fluorescence.

The Cerulean signals were detectable without notable fluorescence spillover from or to the APC channel. Detected EV surface markers were comparable between unstained and tetraspanin-APC–stained CD63-Cerulean EVs (fig. 2). This suggests that Cerulean-labeled EVs, which are detectable in the channel for the VioBlue® Dye, are fully compatible with this assay.

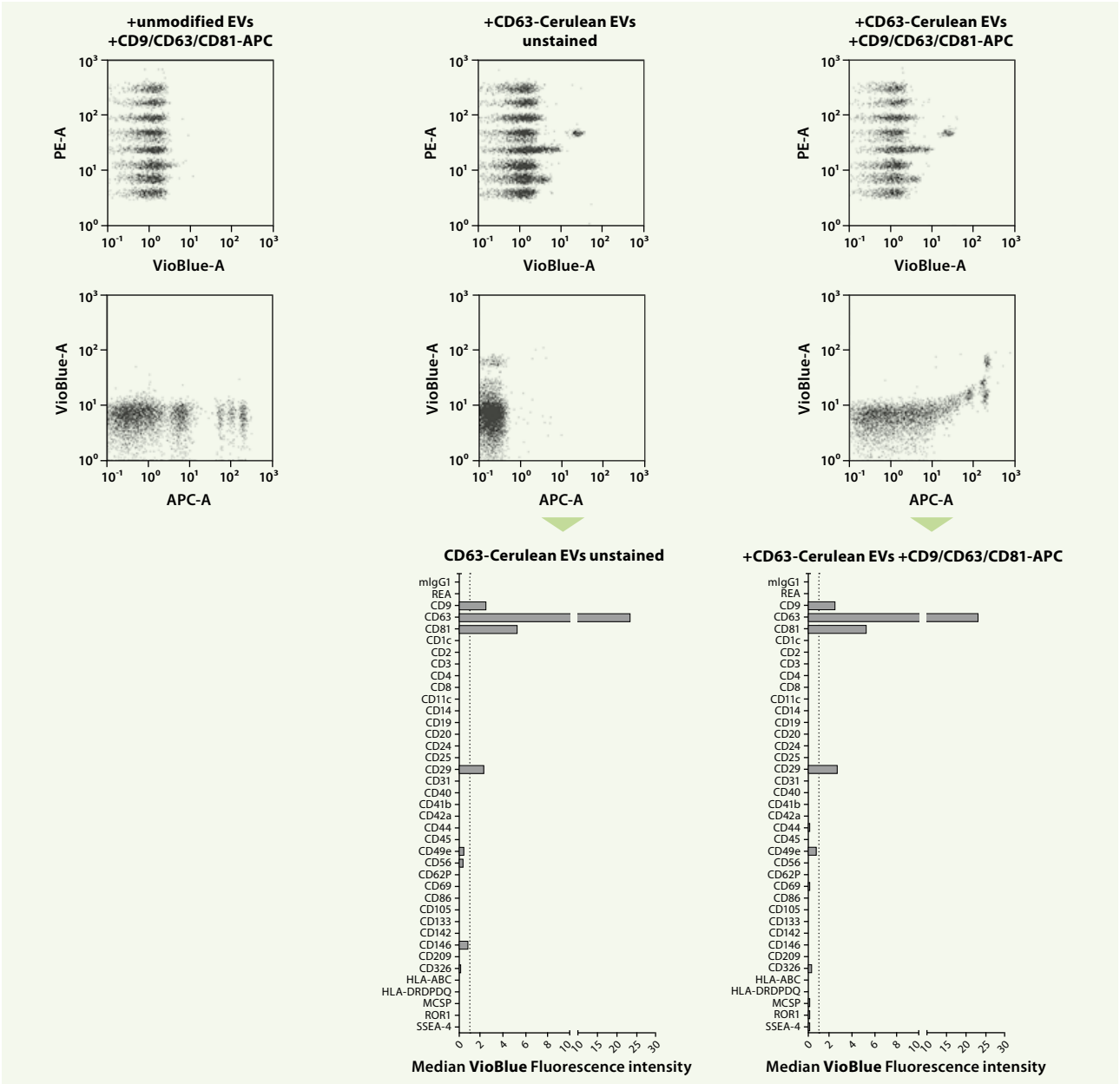

Figure 2: MACSPlex Exosome Kit is compatible with EVs fluorescently labeled with CD63-Cerulean. CD63-Cerulean EVs were isolated from HEK293T cells. Dot plots show capture bead signals in APC and VioBlue Dye channels after incubation with unmodified or CD63-Cerulean EVs that were either left unstained or stained with CD9/CD63/CD81-APC. The epitopes on the bar chart indicate the specificities of the MACSPlex Exosome Capture Beads. Bars show the median fluorescence intensity in the VioBlue Dye channel. Data were adapted from reference 1.

Usage of custom detection antibodies to analyze EV surface epitope expression

The MACSPlex Exosome Kit enables the analysis of 37 EV surface epitopes, as defined by the various MACSPlex Exosome Capture Beads. However, whether or not a certain candidate surface marker, not detectable by the kit, is present on EVs from a given sample will likely be a recurring question. Folate receptor alpha (FOLR1) on EVs was reported to be responsible for shuttling folate into the brain[2,3] and represents one such example. Therefore, we tested whether a custom detection antibody can be combined with the kit to examine the presence or absence of FOLR1 on EVs. EVs from the ovarian adenocarcinoma cell line IGROV1 have FOLR1 on their surface and therefore served as a positive control. In contrast, EVs from the pancreatic ductal adenocarcinoma cell line PANC-1, which does not express FOLR1, were used as a negative control. EVs from both cell lines showed robust expression of abundant markers like tetraspanins or the integrins CD29 and CD49e (fig. 3A), when the cocktail of CD9, CD63, and CD81-APC antibodies was used as detection reagent.

When using anti-FOLR1-APC as detection antibody, no signals were detectable for PANC-1 EVs, whereas clear signals were measured on CD9-, CD63-, CD81-, and CD326-positive EVs from IGROV1 cells (fig. 3B). This indicates that EVs derived from IGROV1 cells, but not PANC-1 cells, express FOLR1 on their surface, and that those EVs co-express the tetraspanins and CD326. Our data also show that a custom detection antibody is compatible with the MACSPlex Exosome Kit and that it can serve as a method to identify surface markers not detected by the kit.

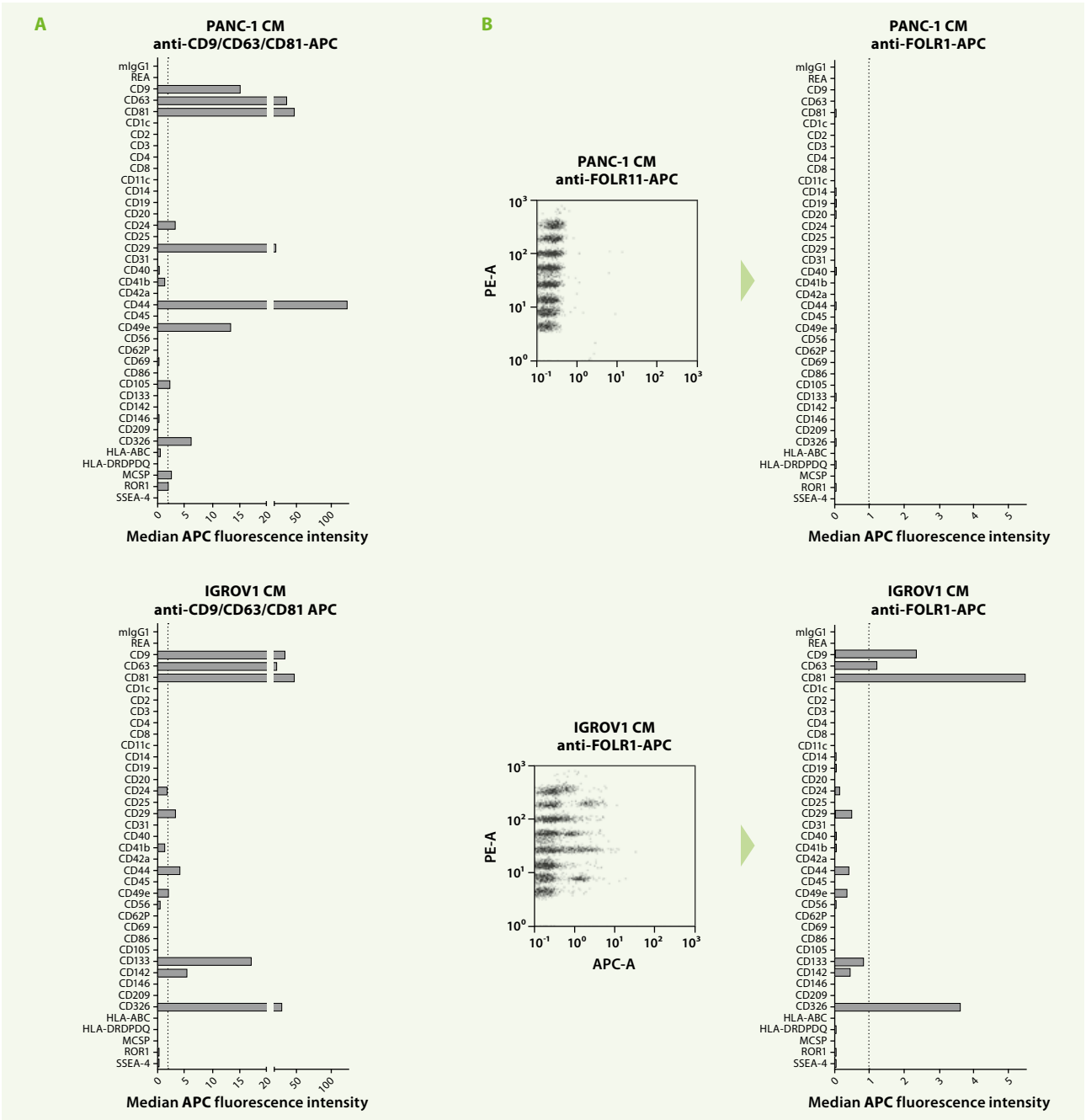

Figure 3: Compatibility of the MACSPlex Exosome Kit with anti-FOLR1-APC as detection antibody. (A) EVs in conditioned medium (CM) from cultured PANC-1 and IGROV1 cells were analyzed using the MACSPlex Exosome Kit and stained with the CD9/CD63/CD81-APC detection cocktail contained in the kit. (B) The same samples as in (A) were stained with APC-conjugated anti-FOLR1 antibodies instead of the detection cocktail. The epitopes on the bar chart indicate the specificities of the MACSPlex Exosome Capture Beads. Bars show the median fluorescence intensity of APC. Data were adapted from reference 1.

However, this antibody needs to fulfill certain criteria:

- Expression of the candidate surface antigen must be abundant enough to be detected above background levels on the instrument used.
- Ideally, the custom detection antibody is available as an APC- or AlexaFluor® 647–conjugate.
- The background signal from the detection antibody control (MACSPlex Capture Beads + antibody) has to be lower than the true positive signal (MACSPlex Capture Beads + antibody + EVs).
- The candidate surface marker must be co-expressed with one of the 37 EV surface markers detected by the MACSPlex Capture Beads.

Conclusion

- The MACSPlex Exosome Kit can be readily adapted to individual needs without affecting its robustness and semi-quantitative detection of EV surface signatures.
- EVs labeled with a fluorescent protein, such as Cerulean, fused to an EV surface marker, can be used with the MACSPlex Exosome Kit instead of, or in combination with, a detection cocktail of APC-conjugated antibodies.
- Furthermore, custom detection antibodies can be used instead of the tetraspanin-APC detection antibodies contained in the kit to investigate the expression of a specific EV surface marker of interest.

References

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