Robust EV profiling in various sample types with the MACSPlex Exosome Kit

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Background

Extracellular vesicles (EVs) can be released by almost any cell type and can be found in all body fluids. They are involved in several pathophysiological activities, thus making them good candidates to investigate different types of diseases[1,2]. However, EV analysis by conventional flow cytometry or Western blotting is hampered by EV heterogeneity and their small size. Here, we used Miltenyi Biotec's MACSPlex Exosome Kit, a new multiplex bead-based flow cytometry assay[3], to evaluate the expression of EV surface markers in different types of samples. To that end, a methodological validation of this assay was performed, exploring different parameters in terms of sample preparation and assay execution. This application note is based on reference 4.

Materials and methods

EV analysis with the MACSPlex Exosome Kit

The MACSPlex Exosome Kit, human allows the detection of 37 different epitopes potentially present on the surface of EVs. The assay contains a cocktail of different fluorescently labeled capture bead populations, which can be distinguished by flow cytometry. Each of these capture bead populations is coupled to an antibody specifically targeting one of the 37 surface epitopes. EVs that bind to the capture beads are then stained with a detection reagent, i.e., a cocktail of APC-conjugated antibodies against the tetraspanins CD9, CD63, and CD81. Based on the fluorescence properties of both the capture beads and the APC-conjugated antibodies, a semi-quantitative analysis of EV surface markers is performed by flow cytometry (fig. 1).

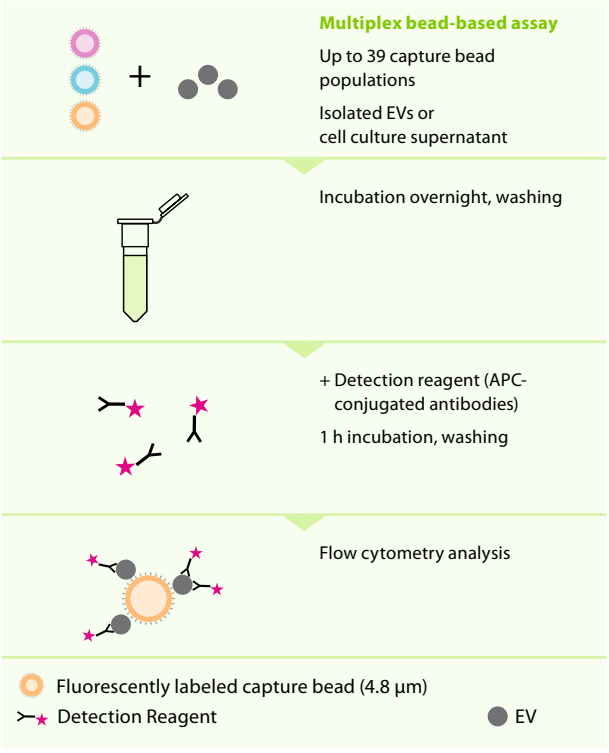

Figure 1: Principle of the MACSPlex Exosome Kit, human. Isolated EVs are incubated overnight with 39 differently labeled capture bead populations each coupled to a different antibody. After incubation with the detection reagent, EVs can be analyzed by flow cytometry.

EV isolation and enumeration

For the analysis of cell culture–derived EVs, EVs were prepared from supernatants of HEK293T cells or immortalized, human bone marrow–derived mesenchymal stromal cells (MSCs), respectively. Several EV preparation protocols were compared; in all of them, cells and larger debris were depleted by two different centrifugation steps (at first 10 min at 500–900×g and second 10–20 min at 2,000×g). Subsequently, unless indicated otherwise, samples were filtered through 0.22 µm filters. EVs were then either concentrated by ultracentrifugation (2× 110,000×g; fig. 2) or by ultrafiltration (300 kDa tangential flow filtration (TFF) followed by 10 kDa spin filtration; fig. 3A, 4, 6). Otherwise EVs were measured directly after 0.22 µm filtration in conditioned medium (fig. 3B). Generally, sample input of EVs per assay was determined based on particle counts assessed by nanoparticle tracking analysis (NTA) with NanoSight® instruments (Malvern, UK). Of note, not all particles as quantified in such NTA analyses are necessarily EVs.

Results

Analysis with the MACSPlex Exosome Kit reflects EV abundance in cell culture supernatants

The MACSPlex Exosome Kit enables a sensitive semi-quantification of EV surface markers in pre-cleared cell culture supernatants. The sensitivity of the assay highly depends on the abundance of EV surface antigens, however, reliable results were obtained when supernatants contained 1×10^6 to 1×10^8 HEK293T-derived particles (fig. 2).

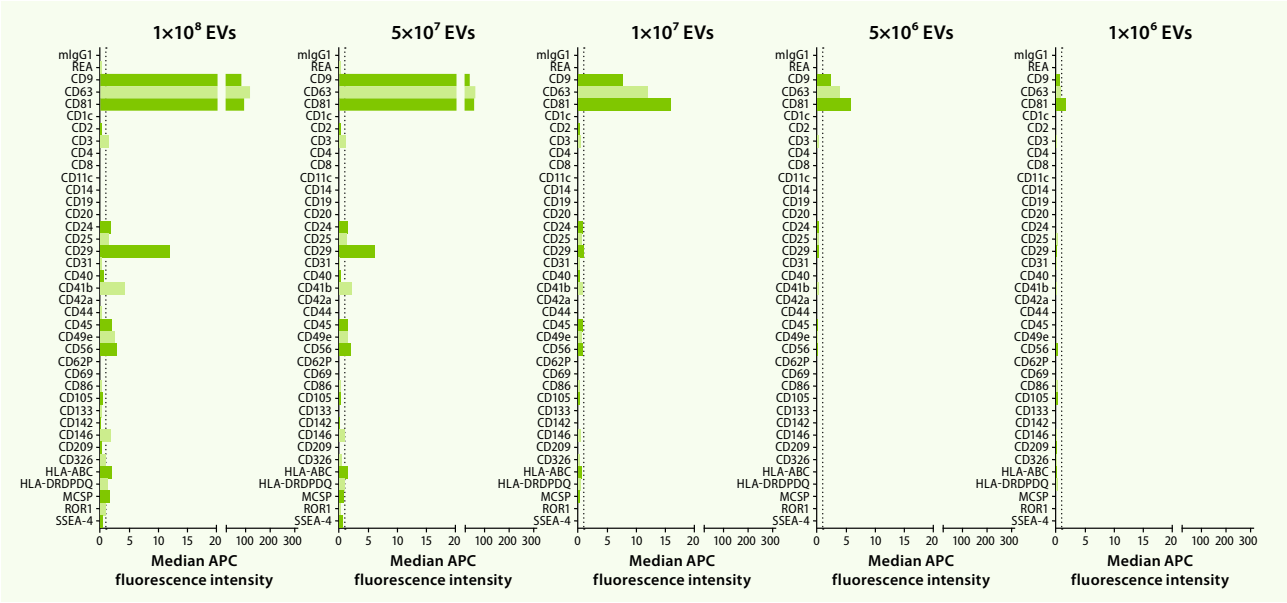

Figure 2: Detection range of the MACSPlex Exosome Kit. EVs were prepared from HEK293T conditioned supernatant. The particle concentration was determined by NTA. Subsequently, the performance of the assay was evaluated for input ranges between 5×10^5 and 5×10^8 particles. EV surface marker compositions after CD9/CD63/CD81-APC detection are shown for the given particle input ranges. Data were adapted from reference 4.

Uncovering EV heterogeneity in supernatants of different cell sources

Furthermore, we evaluated the suitability of the MACSPlex Exosome Kit for the detection of EV heterogeneity within a sample[4] (data not shown) and between samples from different cell sources. Exemplary results showing different EV surface marker profiles for $CD9^+/CD63^+/CD81^+$ EVs derived from HEK293T cells and from an immortalized MSC line are depicted in figure 3A. EV heterogeneity was also analyzed in cell culture supernatants of rare primary hematopoietic stem and progenitor cells (HSPCs) isolated from human umbilical cord blood. EVs captured from supernatants of multipotent (MP), lymphomyeloid (LM), and erythromyeloid (EM) HPSCs were found to differ in their EV surface profiles (fig. 3B).

MACSPlex Exosome Kit is compatible with different protocols for sample preparation and assay execution

EV surface markers were further assessed in both unprocessed cell culture supernatant and in samples following EV enrichment by ultrafiltration or ultracentrifugation. No major qualitative differences were observed within this comparision[4] (data not shown), highlighting the compatibility of the kit with different EV isolation protocols.

The fact that no cross-reaction was recorded between FBS-derived EVs and the antibodies of the kit assures that the assay is compatible with serum-supplemented samples (fig. 4A).

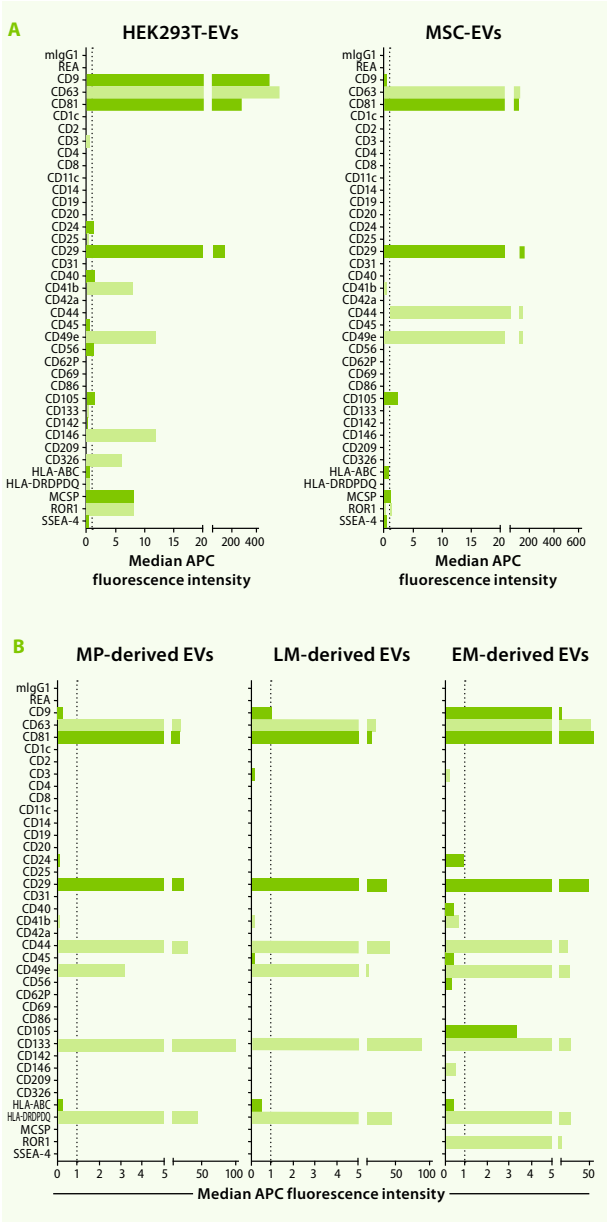

Figure 3: Evaluation of the heterogeneity of independent EV preparations with the MACSPlex Exosome Kit. The EV surface marker composition of different cell types was compared after CD9/CD63/CD81-APC labeling. (A) EVs were isolated from HEK293T cells or immortalized MSCs. The assays were performed with an input amount of 5×10^8 particles. (B) EVs were prepared from rare MP, LM, and EM progenitor cell conditioned media. Media were cleared from cells by low speed centrifugation before analysis. Background signals as estimated in non-conditioned media control samples were subtracted. Data were adapted from reference 4.

In addition, sample stability after one week of storage at 4 °C in the dark was assessed. No critical influence of the time between EV preparation and analysis of the samples within the assay was observed. All samples allowed to record similar EV surface antigen profiles (fig. 4B).

Suitability of the MACSPlex Exosome Kit for EV analysis in human and mouse biological fluids

The applicability of the kit to detect EVs in human cerebrospinal fluid (CSF), plasma, and serum was explored. High and consistent levels of tetraspanins were detected in pre-cleared CSF samples, in non-chromatography–purified plasma, as well as in serum, with no major difference among EV surface marker profiles in the latter two sample types (fig. 5). Furthermore, the suitability of MACSPlex Exosome Kit with samples undergoing

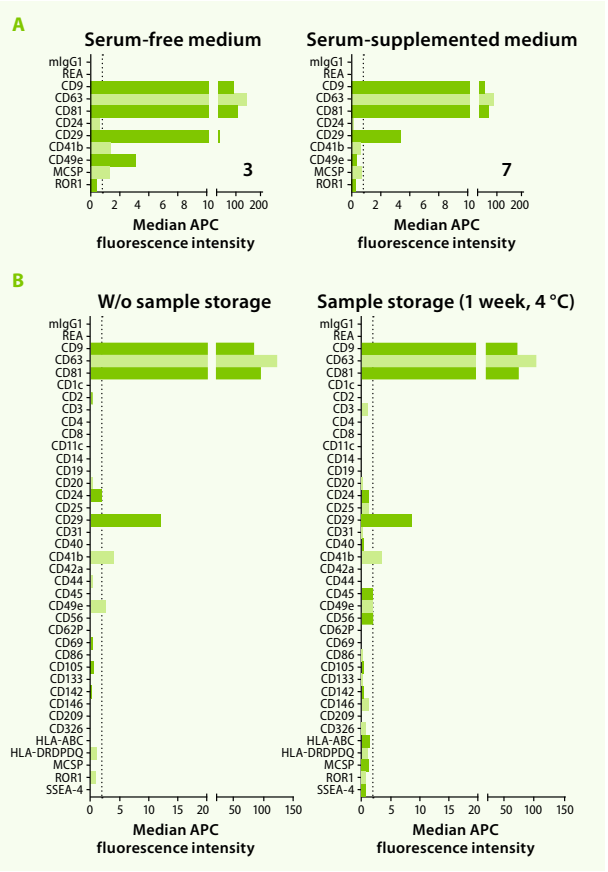

Figure 4: Compatibility of the MACSPlex Exosome Kit with sample preparation and storage. Surface marker composition after CD9/CD63/CD81-APC detection is shown for variable sample conditions. (A) HEK293T cells were either cultured in serum-free (left) or serum-supplemented (10% FBS, right) medium, and EV-containing CM samples were measured directly after pre-clearing by centrifugation and after 0.22 µm filtration. (B) Isolated HEK293T-derived EVs were either analyzed immediately (left) or after sample storage for one week at 4 °C (right). Data were adapted from reference 4.

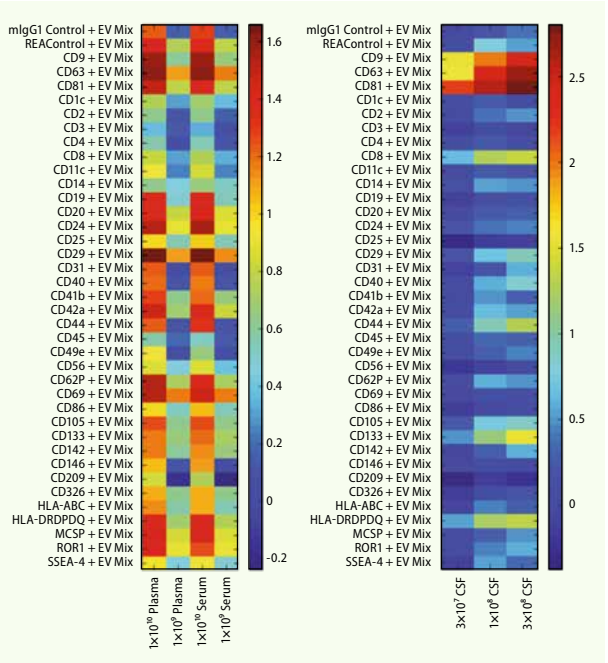

Figure 5: Analysis of human biological fluids with the MACSPlex Exosome Kit. Pre-cleared CSF, non-purified plasma, and serum samples with different input amounts were analyzed. Heatmaps of surface marker detection on $CD9^+/CD63^+/CD81^+$ EVs are shown. Data were adapted from reference 4.

freezing and thawing cycles was confirmed for all biological fluids investigated[4] (data not shown).

Last but not least, it was assessed whether the assay facilitates specific detection of native, non-manipulated human EVs and their surface marker signature in murine blood samples, analyzed 1 min after EV injection (fig. 6).

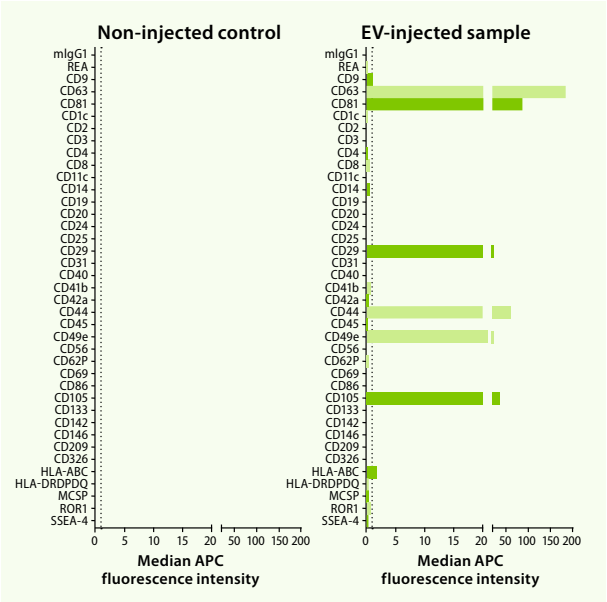

Figure 6: Detection of human EVs in mouse blood with the MACSPlex Exosome Kit. Non-manipulated human EVs from an immortalized human MSC line were injected intravenously into NMRI mice. Doses of 21011 EVs/mouse were injected. Blood was taken 1 min after injection and plasma was analyzed. EV surface marker composition after CD9/CD63/CD81-APC detection is shown for non-injected (left) versus injected (right) blood sample. Data were adapted from reference 4.

Conclusions

- The MACSPlex Exosome Kit facilitates specific and robust semi-quantification of EV surface antigen profiles in different types of samples, including cell culture media, CSF, as well as human and mouse plasma.
- The high sensitivity of the assay allows the detection of EVs in cell culture supernatants of rare cells, e.g., primary HSPCs from umbilical cord blood, which were seeded in low cell numbers.
- The kit is compatible with different protocols to prepare EV samples, particularly with different methods, freeze-thaw processes, and serum-supplemented culture medium.
- The possibility to specifically identify human EVs in the blood of EV-injected mice opens a series of potential applications, including the detection of non-manipulative human vesicles in cancer xenografts.

References

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4. Wiklander, O. P. B. *et al.* (2018) Systematic methodological evaluation of a multiplex bead-based flow cytometry assay for detection of extracellular vesicle surface signatures. Front. Immunol. 9: 1326.

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