Miltenyi Biotec

MACSPlex EV Kit IO

mouse

For up to 24 tests Order no. 130-122-211

For up to 96 tests Order no. 130-122-213

Miltenyi Biotec

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1. Description

This product is for research use only.

Components

For up to 24 tests:

- 0.4 mL MACSPlex EV IO Capture Beads, mouse
- 140 µL EV IO Detection Reagent CD9, mouse
- 140 µL EV IO Detection Reagent CD63, mouse
- 140 µL EV IO Detection Reagent CD81, mouse
- 100 mL MACSPlex Buffer
- 1.5 mL MACSPlex EV IO Setup Beads, mouse

For up to 96 tests:

- 1.5 mL MACSPlex EV IO Capture Beads, mouse
- 0.5 mL EV IO Detection Reagent CD9, mouse
- 0.5 mL EV IO Detection Reagent CD63, mouse
- 0.5 mL EV IO Detection Reagent CD81, mouse
- 2×100 mL MACSPlex Buffer
- 1.5 mL MACSPlex EV IO Setup Beads, mouse

▲ Do not substitute or mix kit components with those from other kits or lots.

▲ EV IO Detection Reagents CD9, CD63, and CD81 can be combined to create a detection cocktail.

Size	Up to 24 tests or up to 96 tests
Product format	MACSPlex EV IO Capture Beads and MACSPlex EV IO Setup Beads are supplied in buffer containing stabilizer and 0.05% sodium azide. MACSPlex Buffer contains stabilizer and 0.09% sodium azide.
Storage	Store MACSPlex EV IO Capture Beads, EV IO Detection Reagents, and MACSPlex EV IO Setup Beads protected from light at +2 to +8 °C. Do not freeze. Store MACSPlex Buffer at room temperature. The expiration dates are indicated on the vial labels.

1.1 Principle of MACSPlex EV Kits

MACSPlex EV Kits allow detection of 37 extracellular vesicle (EV) surface epitopes plus two isotype controls. MACSPlex EV Kits comprise a cocktail of various fluorescently labeled bead populations, each coated with a specific antibody binding the respective surface epitope.

The 39 bead populations can be distinguished by different fluorescence intensities detected in the FITC and PE channel of flow cytometers (B1 and B2 channel of MACSQuant Analyzers).

EVs, like exosomes, are incubated with the antibody-coated MACSPlex EV Capture Beads. Subsequently or in parallel, EVs bound to the MACSPlex EV Capture Beads are labeled with EV Detection Reagents. EV Detection Reagents can also be combined to create a cocktail comprising of EV Detection Reagent for CD9, CD63, and CD81. Consequently, sandwich complexes are formed between the MACSPlex EV Capture Bead, EV, and the detection reagent (figure 1). These complexes can be analyzed based on the fluorescence characteristics of both the MACSPlex EV Capture Bead and the detection reagent. Positive signals indicate the presence of the respective surface epitope within the EV population (figure 2).

It is also possible to compare different EV samples using the MACSPlex EV Kit allowing semi-quantitative analysis of differential surface epitopes.

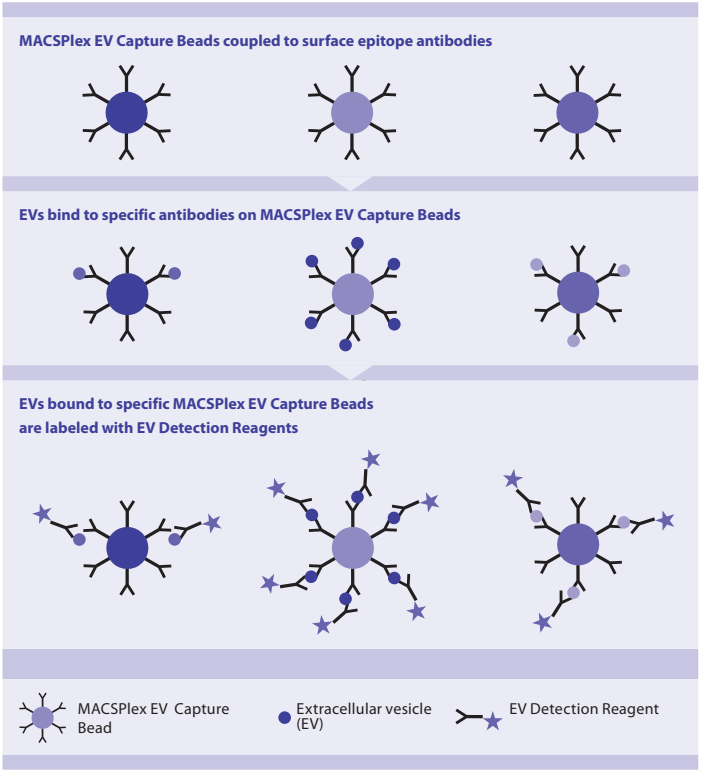

Figure 1: Principle of MACSPlex EV Kits.

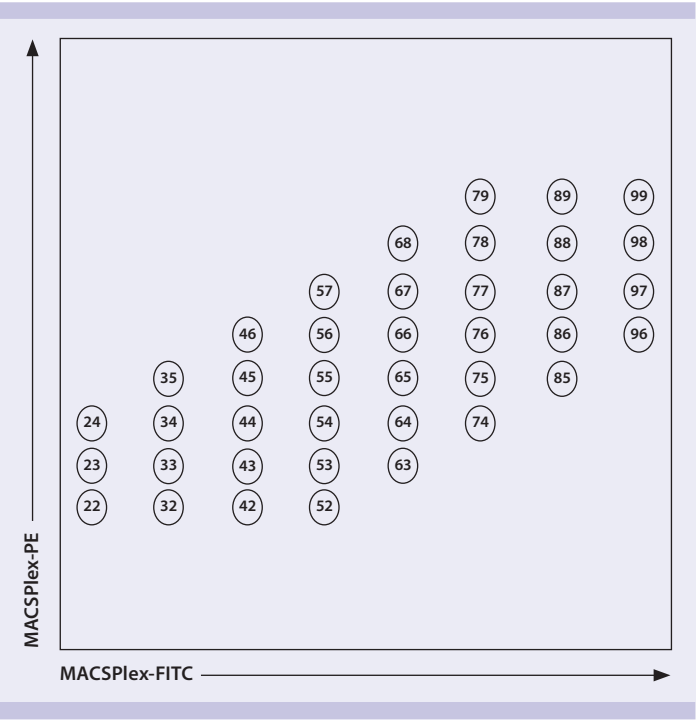

Figure 2: Detection of MACSPlex EV Capture Bead populations in a MACSPlex-FITC (B1) versus MACSPlex-PE (B2) dot plot.

1.2 MACSPlex EV Kit IO

The protocol of the MACSPlex EV Kit IO can be performed in tubes. In most instances, an one hour incubation time is sufficient to bind and stain EVs on the MACSPlex EV IO Capture Beads. For samples comprising low amounts of EVs like supernatants of some cell types, it is recommended to prolong the incubation time to overnight to increase sensitivity.

- For the overnight protocols, staining is performed after the initial EV binding to the MACSPlex EV IO Capture Beads.
- A negative control using only buffer is strongly recommended to determine non-specific signals. The negative control is subtracted from the sample signals to correct potential non-specific signals.
- It is recommended to use a cocktail of the three EV IO Detection Reagents CD9, CD63, and CD81 for a broad EV staining. For each experiment, a master mix can be set up using 5 µL of each EV IO Detection Reagent for each reaction, i.e., 15 µL EV IO Detection Reagent cocktail per well.
  - **Note:** Storage of master mixes is not recommended.
- To detect other surface proteins on the EV samples, APC-conjugated antibodies can be used instead of the EV IO Detection Reagent. Titrate the optimal amount of detection antibody. It is recommended to use 5 µL with a concentration of 0.1 µg/µL or 0.5 µg APC-conjugated antibody per reaction.

1.3 Applications

The MACSPlex EV Kit IO has been developed for the simultaneous flow cytometric detection of 37 surface epitopes that are known to be present on different EVs plus two isotype control beads (table 1).

1.4 Reagent and instrument requirements

- MACSQuant X (# 130-105-100), MACSQuant Analyzer 10 (# 130-096-343), MACSQuant Analyzer 16 (# 130-109-803), or other flow cytometer equipped with blue (488 nm) and red (640 nm) lasers able to discriminate FITC, PE, and APC fluorescence.
  - **Note:** The MACSQuant VYB cannot be used.
- MACS® Chill 96 Rack (# 130-094-459) when using MACSQuant Analyzer 10 or MACSQuant Analyzer 16.
- MACSQuant Calibration Beads (# 130-093-607) when using MACSQuant X, MACSQuant Analyzer 10, or MACSQuant Analyzer 16.
- Disposable pipette tips.
- (Optional) EV Isolation Kit CD9, mouse (# 130-117-042), EV Isolation Kit CD63, mouse (# 130-117-041) or EV Isolation Kit CD81, mouse (# 130-117-040) for EV pre-enrichment from plasma without ultracentrifugation. Please note that the EV Isolation Kit Pan, mouse is not compatible with the MACSPlex EV Kit IO, mouse.

Tube format

- MACSmix™ Tube Rotator (# 130-090-753) or an orbital shaker for tubes (450 rpm)
- Polypropylene or polystyrene reagent tubes
- 96-well round bottom plate

No.	Antibody	Isotype
22	CD3	recombinant human IgG1
23	CD4	recombinant human IgG1
24	CD19	recombinant human IgG1
32	CD8a	recombinant human IgG1
33	MHC Class II	recombinant human IgG1
34	CD49b	recombinant human IgG1
35	CD105	rat IgG2aκ
42	CD2	recombinant human IgG1
43	CD11b	recombinant human IgG1
44	CD25	recombinant human IgG1
45	CD49e	rat IgG2aκ
46	CD140a	recombinant human IgG1
52	CD66a	recombinant human IgG1
53	CD9	rat IgG2aκ
54	CD205	recombinant human IgG1
55	H-2 (MHC Class I)	recombinant human IgG1
56	CD63	recombinant human IgG1
57	CD40	rat IgG2a
63	CD62P	recombinant human IgG1
64	CD11c	recombinant human IgG1
65	CD81	hamster IgGκ
66	AN2	recombinant human IgG1
67	CD146	recombinant human IgG1
68	CD41	rat IgG1κ
74	CD61	hamster IgGκ
75	CD24	recombinant human IgG1
76	CD86	rat IgG2bκ
77	CD44	recombinant human IgG1
78	CD326	rat IgG1
79	Prominin-1	rat IgG1
85	CD29	hamster IgGλ
86	CD69	recombinant human IgG1
87	rat IgG1 Control	rat IgG1
88	CD45	recombinant human IgG1
89	CD31	recombinant human IgG1
96	REA Control	recombinant human IgG1
97	CD20	recombinant human IgG1
98	CD115	recombinant human IgG1
99	EphA2	recombinant human IgG1

Table 1: Overview of surface marker antibodies used for the MACSPlex EV Kit IO, mouse.

2. Protocols for assay performance

▲ Avoid air bubbles.

2.1 Protocol overviews

2.1.1 Short protocol for 1.5 mL reagent tubes

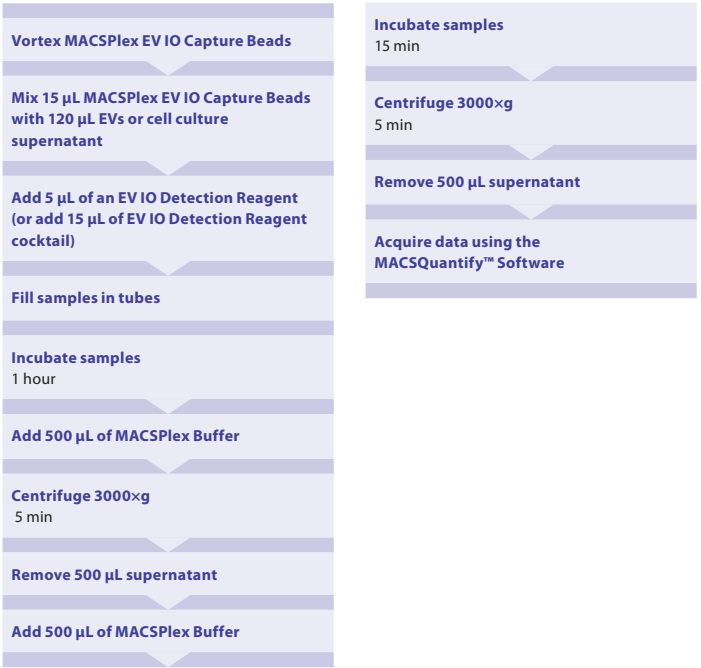

Figure 3: Experimental overview for the short protocol tube.

2.1.2 Overnight protocol for 1.5 mL reagent tubes

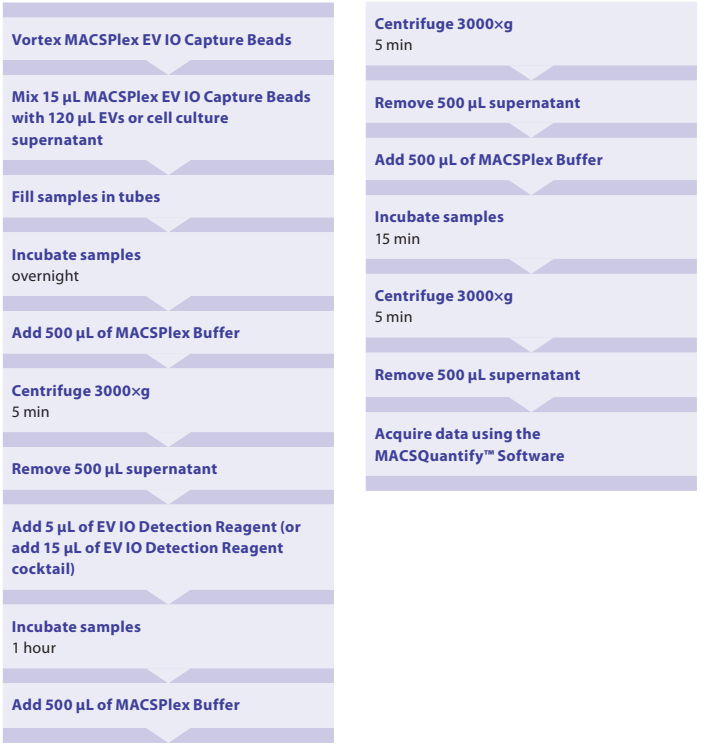

Figure 4: Experimental overview for the overnight protocol tube.

2.2 Sample preparation

The protocol of the MACSPlex EV Kit IO can be performed on pre-cleared cell culture supernatant, ascites, or urine directly. For plasma, serum, or cell culture supernatant of cells scarcely secreting EVs, it is recommended to isolate EVs beforehand as described below.

▲ Other methods for EV concentration or isolation like precipitation methods, density gradients, or immunoaffinity purification can also be used.

2.2.1 Pre-clearing cell culture supernatant

1. Incubate the cells of interest in serum-free medium for 12–72 hours depending on the cell line. Adjust the incubation conditions to an apoptosis rate of less than 5%.
2. Remove cells, cell debris, and larger vesicles by serial centrifugations at 300×g for 10 minutes, 2,000×g for 30 minutes, and 10,000×g for 45 minutes.
3. Filter the supernatant through a 0.22 µm membrane.

2.2.2 EV isolation using MicroBeads

▲ The isolation does not require ultracentrifugation.

For pre-enrichment of EVs from plasma it is recommended to use the EV Isolation Kit CD63, mouse (# 130-117-041). For details refer to the data sheet.

Optionally, the EV Isolation Kit CD9, mouse (# 130-117-042) or the EV Isolation Kit CD81, mouse (# 130-117-040) can be used. Note that the EV Isolation Kit Pan, mouse is not compatible with the MACSPlex EV Kit IO, mouse.

2.2.3 EV isolation from cell culture supernatant (modified according to reference 1)

1. Isolate EVs by ultracentrifugation of the pre-cleared supernatant (refer to 2.1.1) at 100,000×g for 2 hours. Resuspend and pool the pellets in a volume of PBS equivalent to supernatant volume and repeat the ultracentrifugation step.
2. Resuspend the EV pellet in 1/2000 of the original supernatant volume of PBS and determine the EV concentration indirectly by quantifying the protein concentration.
3. Store the EVs at –20 °C or –80 °C.

2.2.4 EV isolation from plasma (modified according to reference 1)

1. Collect blood into EDTA or citrate tubes.
2. Separate plasma by centrifugation at 1,000×g for 10 minutes.
3. Dilute plasma with an equal volume of PBS.
4. Remove cells and cellular debris by serial centrifugations at 2,000×g for 30 minutes and 10,000×g for 45 minutes.
5. Isolate the EVs by ultracentrifugation of the supernatant at 100,000×g for 2 hours. Resuspend and pool the pellets in PBS equal to plasma volume of step 3.
6. Filter the resuspended pellet through a 0.22 µm membrane.
7. Repeat the ultracentrifugation step and resuspend the EV pellet in 1/250 to 1/500 of the initial volume of PBS. Determine the EV concentration indirectly by quantifying the protein concentration.
8. Store the EVs at –20 °C or –80 °C.

2.3 Protocols

▲ EV IO Detection Reagents CD9, CD63, and CD81 can be combined to create a detection cocktail.

2.3.1 Short protocol for the assay using 1.5 mL tubes

▲ Be sure to determine the EV concentration indirectly by quantifying the protein concentration.

1. Label reagent tubes for the blank control and samples.

2. Add to each 1.5 mL tube either
   - 120 µL of buffer (blank control) or
   - 120 µL of pre-cleared cell culture supernatant or
   - 120 µL eluate from EVs isolated with MicroBeads or
   - isolated EVs (4–20 µg protein) from each sample diluted to 120 µL using MACSPlex Buffer.
3. Resuspend MACSPlex EV IO Capture Beads by vortexing for at least 30 seconds and transfer 15 µL of MACSPlex EV IO Capture Beads to each tube.
4. Add 5 µL of EV IO Detection Reagent CD9, CD63, or CD81 or 15 µL of detection cocktail to each tube and and mix by pipetting up and down.
5. Incubate tubes for 1 hour at room temperature protected from light using a MACSmix Tube Rotator on permanent run (12 rpm) or an orbital shaker (450 rpm).
6. Add 500 µL of MACSPlex Buffer to each tube.
7. Centrifuge at room temperature at 3000×g for 5 minutes.
8. Aspirate 500 µL of the supernatant carefully, leaving about 150 µL in the tube.
9. Add 500 µL of MACSPlex Buffer to each tube.
10. Incubate tubes for 15 minutes at room temperature protected from light using a MACSmix Tube Rotator on permanent run (12 rpm) or an orbital shaker (450 rpm).
11. Centrifuge at room temperature at 3000×g for 5 minutes.
12. Carefully aspirate 500 µL of the supernatant, leaving about 150 µL in the tube.

13. Resuspend sample by pipetting up and down.
14. Transfer the samples to a 96-well round bottom plate.

2.3.2 Overnight protocol for the assay using 1.5 mL tubes

▲ Be sure to determine the EV concentration indirectly by quantifying the protein concentration.

1. Label reagent tubes for the blank control and samples.
2. Add to each 1.5 mL tube either
   - 120 µL of buffer (blank control) or
   - 120 µL of pre-cleared cell culture supernatant or
   - 120 µL eluate from EVs isolated with MicroBeads or
   - isolated EVs (4–20 µg protein) from each sample diluted to 120 µL using the MACSPlex Buffer.
3. Resuspend MACSPlex EV IO Capture Beads by vortexing for at least 30 seconds and transfer 15 µL of MACSPlex EV IO Capture Beads to each tube.
4. Incubate tubes overnight at room temperature protected from light using a MACSmix Tube Rotator on permanent run (12 rpm) or an orbital shaker (450 rpm).
5. Add 500 µL of MACSPlex Buffer to each tube.
6. Centrifuge at room temperature at 3000×g for 5 minutes.
7. Carefully aspirate 500 µL of the supernatant, leaving about 135 µL in the tube.
8. Add 5 µL of EV IO Detection Reagent CD9, CD63, or CD81 or 15 µL of detection cocktail to each tube and mix by pipetting up and down.

9. Incubate tubes for 1 hour at room temperature protected from light using a MACSmix Tube Rotator on permanent run (12 rpm) or an orbital shaker (450 rpm).
10. Add 500 µL of MACSPlex Buffer to each tube.
11. Centrifuge at room temperature at 3000×g for 5 minutes.
12. Carefully aspirate 500 µL of the supernatant, leaving about 150 µL in the tube.
13. Add 500 µL of MACSPlex Buffer to each tube.
14. Incubate tubes for 15 minutes at room temperature protected from light using a MACSmix Tube Rotator on permanent run (12 rpm) or an orbital shaker (450 rpm).
15. Centrifuge at room temperature at 3000×g for 5 minutes.
16. Carefully aspirate 500 µL of the supernatant, leaving about 150 µL in the tube.
17. Resupend MACSPlex EV IO Capture Beads by pipetting up and down and transfer the samples to a 96-well round bottom plate.

3. Flow cytometer setup

The kit includes MACSPlex EV IO Setup Beads for setup of flow cytometers.

3.1 Setup of the MACSQuant Instrument

Calibrate the MACSQuant Analyzer using MACSQuant Calibration Beads (# 130-093-607). For details, refer to the data sheet of the MACSQuant Calibration Beads.

After successful finishing of the calibration, the MACSQuant Instrument is ready for measurement. All necessary setup steps are performed automatically during calibration. When running an acquisition on the MACSQuant Analyzer, MACSQuant Analyzer 10, or MACSQuant Analyzer 16, it is recommended to first use MACSPlex EV IO Setup Beads to ensure proper recognition of all bead populations.

▲ The kit is not suitable for use with the MACSQuant VYB.

3.2 Setup of other flow cytometers and data acquisition

The analysis of the MACSPlex EV Kit IO requires a flow cytometer with blue (e.g. 488 nm) and red (e.g. 640 nm) lasers, which are capable of detecting FITC, PE, and APC. MACSPlex EV IO Setup Beads are included in the kit for setting up these instruments.

For details refer to the application note “General instructions for data aquisition und analysis with the MACSPlex EV Kit” available at www.miltenyibiotec.com/130-122-211.

4. Flow cytometric data analysis

4.1 Calculation of relative qualification of EV surface markers

The analysis results in a table listing the median signal intensity for all EV surface markers. The data analysis consists of the following steps:

- Background subtraction
- Optional (steps 3–4):
  Data normalization (Calculation of normalization factor and normalization of detected signals)

  ▲ **Note:** MACSPlex data sets can be normalized if samples with considerably different amounts of EVs have been used. High signals might outperform lower signals detected in a sample with less EVs. Thereby, comparing the marker profiles can be hampered. Normalizing the signal intensities, e.g., according to the signals for the tetraspanin markers CD9, CD63, and CD81 can ease the comparison of such profiles by adapting the different signal ranges (steps 3–4).
- Determination of relative EV surface marker levels

1. Subtract the median signal intensity of each bead obtained from the control sample (buffer only) from the signal intensities of the respective beads incubated with the sample.

   ▲ **Note:** Negative signal intensities can occur due to variation of background signals. It is recommended to mark these signals as non detected.

2. Repeat step 1 for all samples to be analyzed. Then either follow optional steps for data normalization or proceed directly to step 5.

3. (Optional) Calculation of normalization factor:
   For each sample calculate the median signal intensity of the signals detected for the MACSPlex EV IO Capture Beads CD9, CD63, and CD81. Use the mean of the median signal intensity of the MACSPlex EV IO Capture Beads CD9, CD63, and CD81 as the normalization factor for each sample.

   ▲ **Note:** When isolating with MicroBeads consider that the respective marker will be blocked, affecting the signal intensity on the MACSPlex EV IO Capture Beads. The signal intensity of that specific marker cannot be used for normalization (steps 3–4).

4. (Optional) Normalization of detected signals:
   Divide the signal intensities of all beads by the normalization factor of the respective sample. The mean of the MACSPlex EV IO Capture Beads is thereby set to 1 or 100%.

5. Determine the relative EV marker level by calculating the ratio of the signal intensities of each of the two samples to be compared.

5. Performance

The assay sensitivity, specificity, and reproducibility of the MACSPlex EV Kit IO was tested on EVs from cell culture supernatant of cancer cell lines as well as on plasma.

6. Troubleshooting

The following section offers solutions for problems that might be encountered when using the MACSPlex EV Kit IO.

- **Variation between replicate samples:**
  MACSPlex EV IO Capture Beads can settle down. Vortex the MACSPlex EV IO Capture Beads briefly at the latest after pipetting of four samples.

- **Low counts in samples:**
  Mix MACSPlex EV IO Capture Beads sufficiently before pipetting. Ensure that the instrument is calibrated for the relevant 96-well plate to avoid aspiration of air. Avoid aspiration of beads during washing steps. Do not wash or resuspend beads in volumes higher than recommended. Make sure to centrifuge the samples at 3000×g when working with 1.5 mL reagent tubes.

- **Not all expected populations are detected:**
  At least one population could not be detected. Check the liquid level in the wells before starting the measurement. MACSPlex EV IO Capture Beads can settle down. Vortex MACSPlex EV IO Capture Beads briefly at the latest after pipetting of samples. Mix MACSPlex EV IO Capture Beads sufficiently before pipetting. Avoid aspiration of beads during washing steps. Do not wash or resuspend beads in volumes higher than recommended. Make sure to centrifuge the samples at 3000×g when working with 1.5 mL reagent tubes. EVs comprise several surface epitopes and one EV can bind to more than one bead. Thereby, two or more beads can be crosslinked via one or more EVs. High EV concentrations increase the likelihood of such crosslinking events and the most prominent surface markers will preferentially link the respective beads. Only single beads are used for data acquisition and doublets or aggregates are excluded. For prominent surface markers the number of single beads can drop in case of high EV concentrations. It is recommended to repeat the experiment with diluted EV samples (4–20 µg protein diluted in 120 µL of MACSPlex Buffer). Data files have to be analyzed manually. Refer to the application note "General instructions for data aquisition und analysis with the MACSPlex Exosome Kit" available at www.miltenyibiotec.com/130-122-211.

- **Low counts for some bead populations:**
  EVs comprise several surface epitopes and one EV can bind to more than one bead. Thereby, two or more beads can be crosslinked via one or more EVs. High EV concentrations increase the likelihood of such crosslinking events and the most prominent surface markers will preferentially link the respective beads. Only single beads are used for data acquisition and doublets or aggregates are excluded. For prominent surface markers the number of single beads can drop in case of high EV concentrations. It is recommended to repeat the experiment with diluted EV samples (4–20 µg protein diluted in 120 µL of MACSPlex Buffer).

- **High background in buffer control sample:**
  Antibodies can stick non-specifically to MACSPlex EV IO Capture Beads. Sufficient washing is required to avoid increased background signal intensities.

- **High background on isotype control:**
  High concentration of EVs or contaminations, e.g., from cell culture medium can give rise to non-specific binding of EVs to the beads. It is recommended to repeat the experiment with diluted EV samples or to try isolated EVs instead of cell culture supernatant.

- **Little or no detection of EVs in sample:**
  When isolating with MiroBeads consider that the respective marker will be blocked, affecting the signal intensity on the MACSPlex EV IO Capture Beads. Signal intensities on the MACSPlex EV IO Capture Beads mainly depend on the EV concentration. Low signal intensities can be indicative for low EV concentration. Concentrating the EVs, e.g., by isolation from larger volumes or extended culture times to increase EV yield could improve signal intensities. Prolonged incubation times, e.g., overnight usually enhances EV binding and can be used to improve signal intensities. Fluorescent dyes are susceptible to photo bleaching. Avoid prolonged exposure of the fluorescent sample to direct light. Make sure to mix the samples with the reagents during incubation. MACSPlex EV IO Capture Beads tend to sediment and EVs binding might be insufficient.

- **Beads not in region or gate:**
  Ensure proper calibration of the MACSQuant Instrument. It is recommended to use the MACSPlex EV IO Setup Beads and to control proper recognition of all bead populations. Instead of an EV sample, 150 µL of MACSPlex EV IO Setup Beads can be used to control proper bead recognition. Samples containing organic solvents or samples of high viscosity should be diluted or dialyzed, respectively.

- **High variation in samples:**
  Pipette may not be calibrated. Washing was not uniform. Samples may have contained high particulate matter or other interfering substances. Plate agitation was insufficient. Cross-well contamination could have happened. Change pipette tips for each well when touching the reagent.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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