Miltenyi Biotec

MACSPlex EV Kit IO

human

For up to 24 tests — Order no. 130-122-209
For up to 96 tests — Order no. 130-108-813

Miltenyi Biotec

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140-004-948.07

Content

1. Description

This product is for research use only.

Components

For up to 24 tests:

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

For up to 96 tests:

- 1.5 mL MACSPlex EV IO Capture Beads, human
- 0.5 mL MACSPlex EV Detection Reagent CD9, human
- 0.5 mL MACSPlex EV Detection Reagent CD63, human
- 0.5 mL MACSPlex EV Detection Reagent CD81, human
- 2×100 mL MACSPlex Buffer
- 1.5 mL MACSPlex EV IO Setup Beads, human
- 1 MACSPlex Filter Plate for 96 tests
- 2 adhesive foils for 96 tests

140-004-948.07

- ▲ Do not substitute or mix kit components with those from other kits or lots.
- ▲ MACSPlex EV 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 Capture Beads and MACSPlex EV 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 Capture Beads, MACSPlex EV Detection Reagents, and MACSPlex EV 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 MACSPlex EV Detection Reagents. MACSPlex EV Detection Reagents can also be combined to create a cocktail comprising of MACSPlex 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.

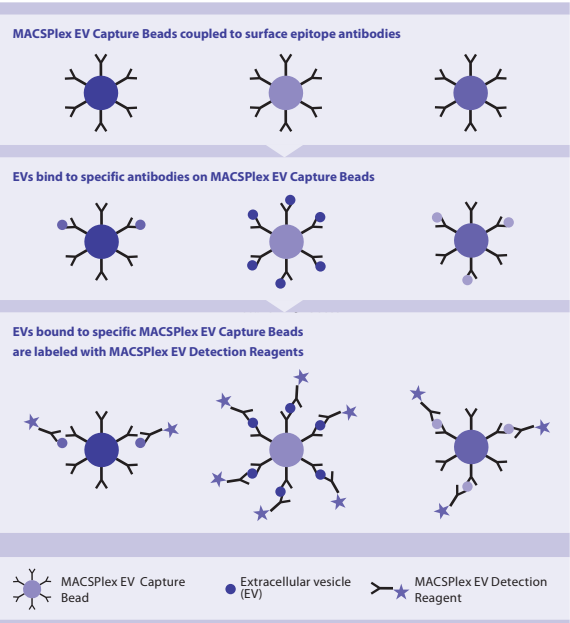

Figure 1: Principle of MACSPlex EV Kits.

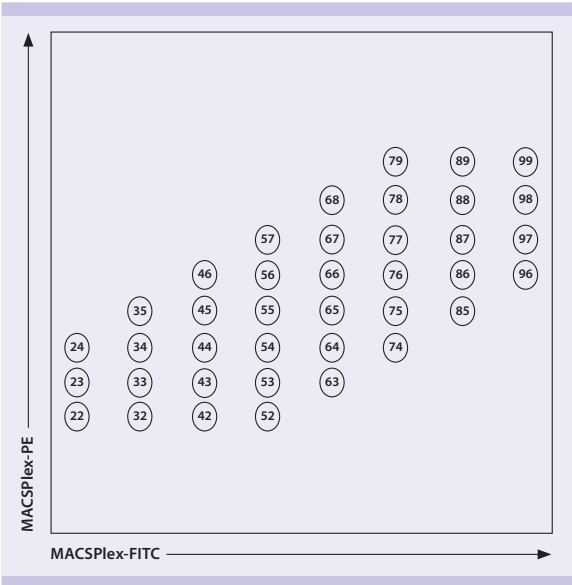

Figure 2: Detection of MACSPlex EV Capture Bead populations in a MACSPlex-FITC (B1) versus MACSPlex-PE (B2) dot plot.

1.2 MACSPlex EV Kit

The protocol of the MACSPlex EV Kit can be performed in the delivered filter plates or as single reactions in tubes. Using the filter plate, the washing can be performed on a vacuum manifold or by using a centrifuge with an adapter for microtiter plates. In most instances, an one hour incubation time is sufficient to bind and stain EVs on the MACSPlex EV 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 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.

▲ For the filter plate supplied with the 96 tests format (# 130-108-813), a centrifuge with an adapter for microtiter plates can be used instead of a vacuum manifold: Put the MACSPlex Filter Plate on top of a conventional 96 flat–bottom microtiter plate without lid and place both into the adapter. Centrifuge at 300×g for 3 minutes at room temperature.

▲ **Note:** Do not overdry.

▲ It is recommended to use a cocktail of the three MACSPlex EV 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 MACSPlex EV Detection Reagent for each reaction, i.e., 15 µL MACSPlex EV 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 MACSPlex EV 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 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, human (# 130-110-913), EV Isolation Kit CD63, human (# 130-110-918), or EV Isolation Kit CD81, human (# 130-110-914) for EV pre-enrichment from plasma without ultracentrifugation. Please note that the EV Isolation Kit Pan, human is not compatible with the MACSPlex EV Kit, human.

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

Microtiter plate format

- Orbital shaker for 96-well plates (frequency 450 rpm)
- Vacuum manifold or centrifuge with adapters to accommodate microtiter plates
- (Optional) Multichannel pipettor

No.	Antibody	Isotype
22	CD3	mouse IgG2a
23	CD4	mouse IgG2a
24	CD19	mouse IgG1
32	CD8	mouse IgG2a
33	HLA-DRDPDQ	recombinant human IgG1
34	CD56	recombinant human IgG1
35	CD105	recombinant human IgG1
42	CD2	mouse IgG2b
43	CD1c	mouse IgG2a
44	CD25	mouse IgG1
45	CD49e	recombinant human IgG1
46	ROR1	mouse IgG1κ
52	CD209	mouse IgG1
53	CD9	mouse IgG1
54	SSEA-4	recombinant human IgG1
55	HLA-ABC	recombinant human IgG1
56	CD63	mouse IgG1κ
57	CD40	mouse IgG1κ
63	CD62P	recombinant human IgG1
64	CD11c	mouse IgG2b
65	CD81	recombinant human IgG1
66	MCSP	mouse IgG1
67	CD146	mouse IgG1
68	CD41b	recombinant human IgG1
74	CD42a	recombinant human IgG1
75	CD24	mouse IgG1
76	CD86	mouse IgG1
77	CD44	mouse IgG1
78	CD326	mouse IgG1
79	CD133/1	mouse IgG1κ
85	CD29	mouse IgG1κ
86	CD69	mouse IgG1κ
87	CD142	mouse IgG1κ
88	CD45	mouse IgG2a
89	CD31	mouse IgG1
96	REA Control	recombinant human IgG1
97	CD20	mouse IgG1
98	CD14	mouse IgG2a
99	mIgG1 control	mouse IgG1

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

2. Protocols for assay performance

▲ Avoid air bubbles.

2.1 Protocol overviews

2.1.1 Short protocol for MACSPlex Filter Plate

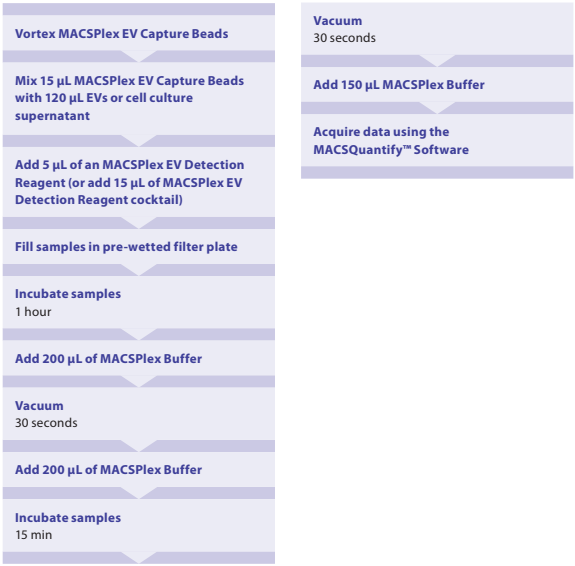

Figure 3: Experimental overview for the short protocol filter plate.

2.1.2 Short protocol for 1.5 mL reagent tubes

Figure 4: Experimental overview for the short protocol tube.

2.1.3 Overnight protocol for MACSPlex Filter Plate

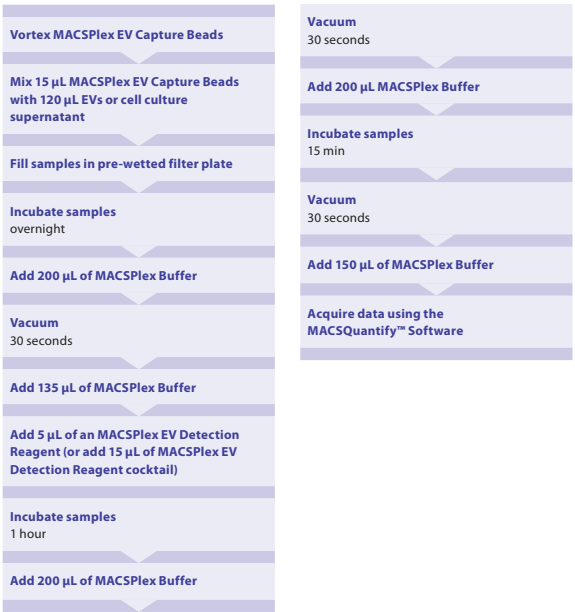

Figure 5: Experimental overview for the overnight protocol filter plate.

2.1.4 Overnight protocol for 1.5 mL reagent tubes

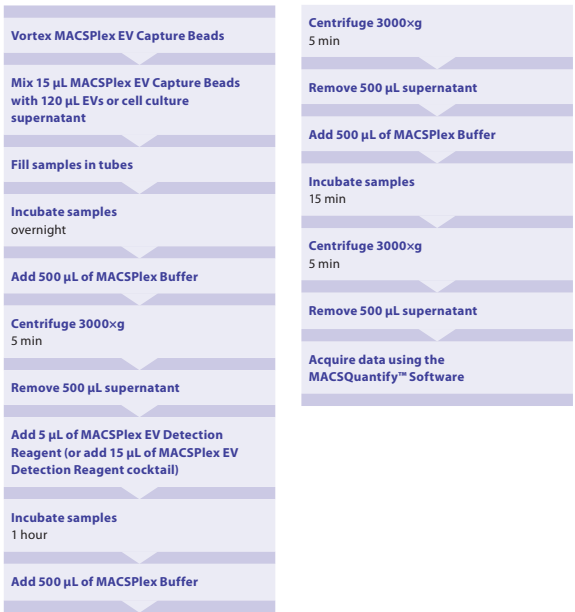

Figure 6: Experimental overview for the overnight protocol tube.

2.2 Sample preparation

The protocol of the MACSPlex EV Kit 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, human (# 130-110-918). For details refer to the data sheet.

Optionally, the EV Isolation Kit CD9, human (# 130-110-913) or the EV Isolation Kit CD81, human (# 130-110-914) can be used. Note that the EV Isolation Kit Pan, human is not compatible with the MACSPlex EV Kit, human.

2.2.3 EV isolation from cell culture supernatant

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

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

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

2.3.1 Short protocol for the assay using the MACSPlex Filter Plate

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

▲ Place the MACSPlex Filter Plate on a non-absorbent surface during loading steps and incubation, i.e., remove any paper towel from the surface to prevent the wells from running dry. Ensure that residual drops under the plate are completely removed to prevent liquid transfer by placing the plate briefly on a paper towel.

▲ Cover unused wells of the filter plate for later use with the adhesive foil provided with the kit.

▲ Use multichannel pipettor.

1. Pre-wet required wells of the MACSPlex Filter Plate with 200 µL of MACSPlex Buffer per well and aspirate off using a vacuum manifold designed to accommodate the filter plate (max. –60 mbar) until the wells are drained or centrifuge 300×g at room temperature for 3 minutes.
2. Place the filter plate briefly on a paper towel to remove residual liquid.

Add to each well of the MACSPlex Filter Plate 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 Capture Beads by vortexing for at least 30 seconds and transfer 15 µL of MACSPlex EV Capture Beads to each well.
4. Add 5 µL of MACSPlex EV Detection Reagent CD9, CD63, or CD81 or 15 µL of detection cocktail to each well and mix by pipetting up and down.

5. Incubate filter plate for 1 hour at room temperature protected from light on an orbital shaker (450 rpm).
6. Add 200 µL of MACSPlex Buffer to each well.
7. Put the filter plate on a vacuum manifold and apply vacuum (max. –60 mbar) for about 30 seconds until wells are drained. Switch off the vacuum and ventilate the vacuum manifold or centrifuge 300×g at room temperature for 3 minutes.
8. Add 200 µL of MACSPlex Buffer to each well.
9. Incubate filter plate for 15 minutes at room temperature protected from light on an orbital shaker (450 rpm).
10. Put the filter plate on a vacuum manifold and apply vacuum (max. –60 mbar) for about 30 seconds until wells are drained. Switch off the vacuum and ventilate the vacuum manifold or centrifuge 300×g at room temperature for 3 minutes.
11. Add 150 µL of MACSPlex Buffer to each well. Resuspend carefully.

2.3.2 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 Capture Beads by vortexing for at least 30 seconds and transfer 15 µL of MACSPlex EV Capture Beads to each tube.
4. Add 5 µL of MACSPlex EV 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.

2.3.3 Overnight protocol for the assay using the MACSPlex Filter Plate

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

▲ Place the MACSPlex Filter Plate on a non-absorbent surface during loading steps and incubation, i.e., remove any paper towel from the surface to prevent the wells from running dry. Ensure that residual drops under the plate are completely removed to prevent liquid transfer by placing the plate briefly on a paper towel.

▲ Cover unused wells of the filter plate for later use with the adhesive foil provided with the kit.

▲ Use multichannel pipettor.

1. Pre-wet required wells of the MACSPlex Filter Plate with 200 µL of MACSPlex Buffer per well and aspirate off using a vacuum manifold designed to accommodate the filter plate (max. –60 mbar) until the wells are drained or centrifuge 300×g at room temperature for 3 minutes.
2. Place the filter plate briefly on a paper towel to remove residual liquid.
3. Add to each well of the MACSPlex Filter Plate 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.

4. Resuspend MACSPlex EV Capture Beads by vortexing for at least 30 seconds and transfer 15 µL of MACSPlex EV Capture Beads to each well.
5. Incubate filter plate overnight at room temperature protected from light on an orbital shaker (450 rpm).
6. Add 200 µL of MACSPlex Buffer to each well.
7. Put the filter plate on a vacuum manifold and apply vacuum (max. –60 mbar) for about 30 seconds until wells are drained. Switch off the vacuum and ventilate the vacuum manifold or centrifuge 300×g at room temperature for 3 minutes.
8. Add 135 µL of MACSPlex Buffer to each well.
9. Add 5 µL of MACSPlex EV Detection Reagent CD9, CD63, or CD81 or 15 µL of detection cocktail to each well and mix by pipetting up and down.
10. Incubate filter plate for 1 hour at room temperature protected from light on an orbital shaker (450 rpm).
11. Add 200 µL of MACSPlex Buffer to each well.
12. Put the filter plate on a vacuum manifold and apply vacuum (max. –60 mbar) for about 30 seconds until wells are drained. Switch off the vacuum and ventilate the vacuum manifold or centrifuge 300×g at room temperature for 3 minutes.
13. Add 200 µL of MACSPlex Buffer to each well.
14. Incubate filter plate for 15 minutes at room temperature protected from light on an orbital shaker (450 rpm).

15. Put the filter plate on a vacuum manifold and apply vacuum (max. –60 mbar) for about 30 seconds until wells are drained. Switch off the vacuum and ventilate the vacuum manifold or centrifuge 300×g at room temperature for 3 minutes.
16. Add 150 µL of MACSPlex Buffer to each well. Resuspend carefully.

2.3.4 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 Capture Beads by vortexing for at least 30 seconds and transfer 15 µL of MACSPlex EV 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 MACSPlex EV 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 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 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 X, MACSQuant Analyzer 10, or MACSQuant Analyzer 16, it is recommended to first use MACSPlex EV 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 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 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/130-122-209.

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 Capture Beads CD9, CD63, and CD81. Use the mean of the median signal intensity of the MACSPlex

EV 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 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 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 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.

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

- **Low counts in samples:**
Mix MACSPlex EV 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 Capture Beads can settle down. Vortex MACSPlex EV Capture Beads briefly at the latest after pipetting of samples. Mix MACSPlex EV 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 EV Kit" available at www.miltenyibiotec.com/130-122-209.

- **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 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.

- **Filter plate will not vacuum:**
Vacuum pressure is insufficient. Increase vacuum pressure.

- **Plate leakage:**
Vacuum pressure is too high. Adjust vacuum pressure to maximal –60 mbar. Place the MACSPlex Filter Plate on a non-absorbent surface during filling steps and incubation, i.e., remove any paper towel from the surface, to prevent the wells from running dry. Ensure that residual drops under the plate are completely removed to prevent leakiness of the wells, by placing the plate briefly on a paper towel, after each washing step. Avoid touching the plate filter with the tip of the pipette when adding reagents to the wells.

- **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 Capture Beads. Signal intensities on the MACSPlex EV 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 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 Setup Beads and to control proper recognition of all bead populations. Instead of an EV sample, 150 µL of MACSPlex EV 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.

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