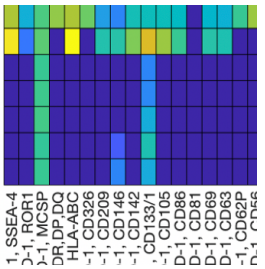
Sep 19, 2020

Multiplexed assay for detection of cell culture EV surface membrane proteins



In 1 collection

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

Abstract

Protocol for using Miltenyi Biotec's human MACSplex Exosome Kit to assay one cell-line derived EV sample with up to 3 additional detection antibodies, at two EV count titration points (1E9 and 1E8 per LM10, NanoSight, NTA.). The optimisation of this protocol was done using bead kits released between 2017-2020.

Materials

MATERIALS

Low Protein Binding Collection Tubes (2.0 mL) **Thermo Fisher Catalog #**88379

MACSPlex Exosome Kit human **Miltenyi Biotec**

AcroPrep Advance Filter Plates for Aqueous Filtration - 350 µL 0.2 µm Supor membrane (10/pkg)

Before start

- The protocol and attached planning template spreadsheet have been designed for an experiment assaying one cell-line derived EV sample with up to 3 additional detection antibodies, at two EV count titration points (1E9 and 1E8 per LM10, NanoSight, NTA.) Modifications of the spreadsheet and protocol may be necessary to use as a guide to assay multiple samples, human sample-derived EVs, or use additional numbers of detection antibodies.

Experiment planning

1 Determine which antibodies to use to detect EV surface membrane proteins in addition to the included CD9, CD63 and CD81 antibodies. All additional antibodies must be either APC or AF647 conjugated. Ensure you know the concentration of the antibodies, and if you are using an antibody conjugated in-lab, avoid preparations that have unbound dye.

2 Calculate the particle concentration of your EV sample, and the total particle count.

3 Use this template document to input your sample information and generate a plate map to visualize your experiment and the wells you will fill in the 96-well plate to be analyzed by the flow cytometer. Check the "How much volume of your sample is needed for this assay (µL)" section of the sheet to ensure your selections for EV count titration points are reasonable. If you have a very concentrated sample (~5E11part./mL or greater) you should increase the upper EV titration point to increase the fluorescent signal of the assay. If your sample is so dilute that you cannot incubate 1E9 EVs with each detection antibody consider methods to concentrate your sample as the fluorscent signal may be very weak and lower titration points not possible.

FILE 2020-07-30 - MACSplex Protocol ...

Note

Additionally, this template was designed so that the user is directed to transfer a volume of EVs directly from the stock EV preparation into a tube with MACSplex capture beads and MACSplex buffer. However, for the lower titration point(s) the user may wish to prepare dilutions of their EV stocks in PBS and use equal volumes of EV dilutions for all mixes. For example Tube 1 contains 10 µL of a 1E11 part/mL EV dilution and Tube 2 contains 10 µL of a 1E10 part/mL EV dilution.

Note

This template specifically applies to cell culture supernatant derived EVs. It is not recommended to use 10 µL (~15,000 beads) of beads with EVs derived from human fluids, such as plasma or serum as the bead recovery tends to be lower with biofluid ssamples than for cell culture supernatant EV preparations. A higher volume of beads would therefore be required.

4 Below is an example of how you might modify the template spreadsheet plate map to analyze more than one EV sample. Organize the plate so that the multichanel pipette can be used to transfer one antibody solution to a column or row. The "EV - bead capture" section of the spreadsheet will need to be duplicated and modified to help calculate needed volumes for each EV sample.

	1	2	3	4	5	6	7
A	CD9 1E9 EVs Cell line 1 10 µL capture beads	CD63 1E9 EVs Cell line 1 10 µL capture beads	CD81 1E9 EVs Cell line 1 10 µL capture beads	mAb 4 1E9 EVs Cell line 1 10 µL capture beads	mAb 5 1E9 EVs Cell line 1 10 µL capture beads	mAb 6 1E9 EVs Cell line 1 10 µL capture beads	Setup beads
B	CD9 1E8 EVs Cell line 1 10 µL capture beads	CD63 1E8 EVs Cell line 1 10 µL capture beads	CD81 1E8 EVs Cell line 1 10 µL capture beads	mAb 4 1E8 EVs Cell line 1 10 µL capture beads	mAb 5 1E8 EVs Cell line 1 10 µL capture beads	mAb 6 1E8 EVs Cell line 1 10 µL capture beads	5 µL capture beads
C	CD9 1E9 EVs Cell line 2 10 µL capture beads	CD63 1E9 EVs Cell line 2 10 µL capture beads	CD81 1E9 EVs Cell line 2 10 µL capture beads	mAb 4 1E9 EVs Cell line 2 10 µL capture beads	mAb 5 1E9 EVs Cell line 2 10 µL capture beads	mAb 6 1E9 EVs Cell line 2 10 µL capture beads	
D	CD9 1E8 EVs Cell line 2 10 µL capture beads	CD63 1E8 EVs Cell line 2 10 µL capture beads	CD81 1E8 EVs Cell line 2 10 µL capture beads	mAb 4 1E8 EVs Cell line 2 10 µL capture beads	mAb 5 1E8 EVs Cell line 2 10 µL capture beads	mAb 6 1E8 EVs Cell line 2 10 µL capture beads	
E	CD9 PBS control 10 µL capture beads	CD63 PBS control 10 µL capture beads	CD81 PBS control 10 µL capture beads	mAb 4 PBS control 10 µL capture beads	mAb 5 PBS control 10 µL capture beads	TGF-B1 PBS control 10 µL capture beads	

Day 1: Incubating EVs with capture beads

5 Using the "EV - capture bead mix preparation" section of the template as a guide, determine the volumes of MACSplex buffer, MACSplex capture beads, and EVs that will be mixed together for the overnight bead capture incubation.

The spreadsheet has been designed so that the combined volume of MACSplex buffer and EVs in PBS is 65 µL for every 10 µL of MACSplex capture beads, or 75 µL of volume per detection antibody "test.'

6 Prepare an Eppendorf 2.0 mL LoBind collection tube for each EV sample being assayed, but before use ensure that the planned volume to be transferred to the tube is able to invert when the tube is rotated, otherwise the capture beads may sediment at the bottom of the tube during the overnight incubation and prevent proper interaction of EV proteins with their capture antibodies.

Other high quality low-protein binding tubes can be used if necessary.

7 Using our shared digital inventory, identify which capture bead tube you will use for this experiment and write down the lot number.

Note

CRITICAL STEP: The lot number is important for potential quality control issues that may arise with any of the 39 different bead populations.

8 Transfer the calculated amount of MACSplex buffer to each EV sample collection tube.

9 Spin down and vortex very thoroughly on the highest setting a tube of human MACSPlex Exosome Capture Beads and aliquot the calculated volumes into each prepared EV sample collection tube.

10 Vortex and then add the calculated volume of undiluted or diluted EVs to each labeled tube containing MACSplex buffer and capture beads.

11 Vortex each tube well, then place in a tube rotator, covered with foil, and rotate overnight at RT.

Note

CRITICAL STEP: make sure the volume inside each tube is inverting and falling down the side of the tube when it is rotated so that the large capture beads remain in suspension throughout the night to allow for adequate mixing.

Day 2: Staining captured EVs with detection antibodies

12 Use the "Antibody preparation table" section of the template to calculate the volumes of antibodies and buffer you will need to prepare for the detection antibody staining step of

the assay.

13 Get 1 new Pall 0.2 µm PES filter plate.

14 Using a multichannel pipet, add 150 µL of MACSplex buffer to all sample and control wells.

It is recommended to use the top portion of the vacuum manifold as a plate holder to aid pipetting.

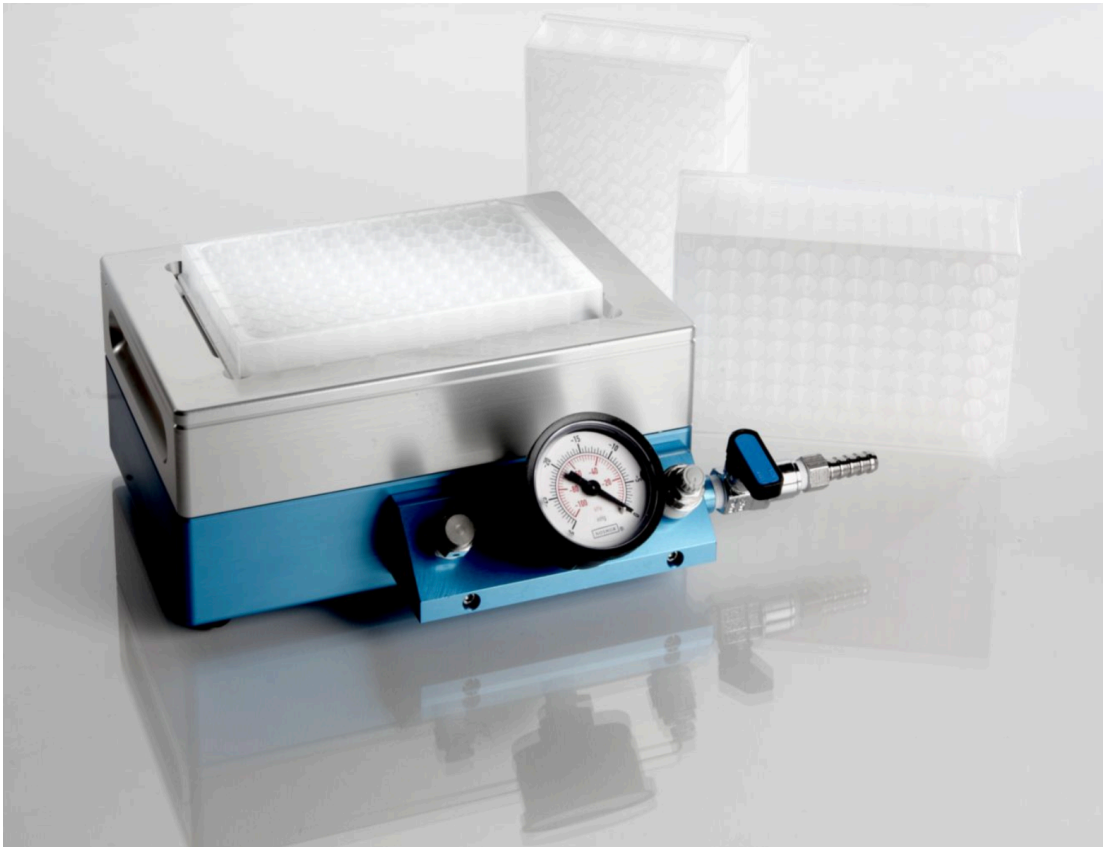

Figure 1: Pall Vacuum Manifold (ID: 5017.) The silver metal top is removable and rests on the blue metal bottom
component. The blue pressure valve can be opened slowly to prevent sudden pressure changes disturbing fluid.
Vacuum should only be applied until sufficient to empty wells of fluid. The silver metal top can be used as a
temporary plate rack for pipetting and mixing samples during washing steps. Figure from Pall's website.

15 Subject the plate to vacuum just until all wells are emptied of buffer.

(release the vacuum pressure gently by pressing the vacuum release rapidly)

Quickly and gently blot the bottom of the filter plate against a clean paper towel.

16 As quickly as possible, add 50 µL of MACSplex buffer to previously wetted wells.

17 Vortex each EV sample tube very thoroughly and add 75 µL to each test well.

75 µL should contain 10 µL of capture beads with EVs bound to them.

Based on the template spreadsheet you should have 10% excess volume so there should be no concern with running out of sample for the last detection well on the filter plate.

(To aspirate all volume at the bottom of the collection tube, the tube may need to be spun down using a bench-top centrifuge and then quickly vortexed or pipetted again.)

18 Add 10 µL of vortexed MACSplex capture beads to the indicated detection antibody control wells (1 per detection antibody.)

19 All detection antibodies should be vortexed gently and spun quickly in a table top centrifuge if possible.

20 Prepare all antibody solutions according to the template spreadsheet. If doing a large experiment make sure to only prepare the antibody solutions immediatley before use.

21 Once antibody solutions are prepared, vacuum the plate until the wells are just empty of liquid. Very quickly and gently blot the bottom of the filter plate against a clean paper towel to remove any drops of buffer on the bottom of the plate.

22 Using a multichanel pipette and reagent well if possible, and dispensing quickly to prevent filter plate membranes from drying out, dispense antibody solutions onto their indicated wells, 200 µL of antibody solution per well. Avoid bubble formation and do not mix initially.

Note

CRITICAL STEP: when using the multichanel pipette it is easy to accidentally draw up unequal volumes of solution. Rock the pipette very firmly on the tips to ensure a strong connection, ensure all tips are level by visual inspection before use, when drawing up fluid ensure all tips are fully placed below the top level of the reagent, and **visually confirm** that each tip has a similar amount of fluid. All the fluid should remain at the bottom of the pipette tip and not drift higher up in the tip, otherwise it will not be dispensed equally and bubbles will form in an attempt to eject residual fluid.

23 Using a multichannel pipet set to 100 µL, mix the volumes in the wells up and down, without contacting the filter membranes at the bottom of the wells to avoid puncturing them. The tip should initially be placed exactly in the middle of the fluid in the wells, then rotated slightly toward the sides of the well to mix beads which may be on the side of the well.

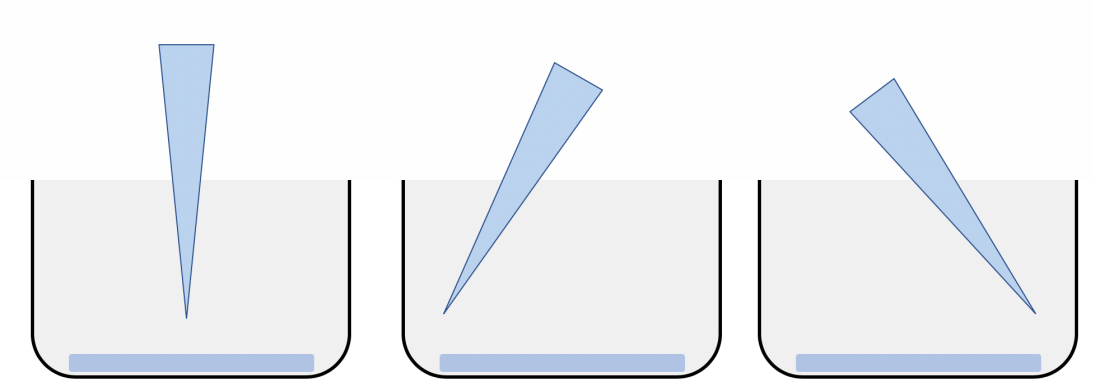

Figure 2: Three ~400 µL wells with a 0.2 µm membrane at the bottom. The triangle represents the optimal
placement of the pipette tip during mixing. One scrape of the membrane does not mean the experiment is
ruined, but hard scrapes can either puncture a hole in the membrane, allowing bead-captured EVs to spill out, or
introduce membrane debris into the sample mixture.

24 Cover the plate with a foil plate sealer and incubate for 2 hours at RT, shaking.

25 After 2 hours, vacuum the plate until fluid just empty, and then add 150 µL of MACSplex buffer to all used wells immediately.

26 Using the 75 µL setting, with the plate positioned on top of the silver metal part of the manifold, reverse pipette all wells with careful tip positioning to avoid bubbles but also not scrape filter membrane.

27 Clear all wells with the vacuum.

28 Immediately added 150 µL buffer to all wells

29 Using the 75 µL setting, with plate positioned on top of manifold, reverse pipette all wells with careful tip positioning to avoid bubbles but also not scrape filter membrane

30 Place on vacuum manifold until all wells are cleared

31 Immediately add 200 µL of buffer to all wells.

This final resuspension volume can be adjusted to produce more or less concentrated final suspensions of the capture beads, depending on the optimal concentration to run samples on the flow cytometer.

32 Using 200 µL setting, with plate on top of metal manifold piece, all wells should be reverse pipetted with tip positioning careful to avoid bubbles but also not scrape filter membrane and then transferred to Axygen racked mini 1.1 mL tubes, or a 96-well plate compatable with the plate reader for the particular clow cytometer being used.

When preparing this final resuspension press the mutlichanel plunger half-way to its stopping point to use a volume of ~100 µL to fully resuspend all capture beads sitting on top of the filter plate membrane.

33 Add 5 µL of capture beads in 200 µL of PBS to a free well on the plate or tube rack for the cytometer, as well as 20 µL of setup beads with 20 µL of PBS (varies largely depending on setup bead concentration) to another free well.

Day 2: running plate on cytometer

34 Run setup beads on the flow cytometer to adjust cytometer settings and gate around a single bead population.

35 Analyze the full volume of each 200 µL stained EV sample, including the non-EV containing capture bead + detection antibody controls to check for nonspecific binding of detection antibody to capture beads.

Data analysis

36 Use MPA_{PASS} software to analyze the multiplexed EV protein expression data. Protocol under development.