Miltenyi pan-tetraspanin bead protocol

Pre-clearing cell culture supernatant

1) remove cells, cell debris and larger vesicles by serial centrifugations at 300g 10', 2000g 20' and 12500g 20' (go on to the next step with only the supernatant of the respective centrifugation)
2) filter the supernatant with 0,22 µm filter

Magnetic labelling

1) for 2 ml of cell supernatant add 25 µl Exosome Isolation MicroBeads
2) incubate at room temperature for 1h

Separation

Use reagents that are preheated to room temperature!

1) Place a µ Column into a µMACS separator
2) Prepare the column by applying 100 µl of Equilibration Buffer (practically 1% Triton)
3) Rinse the coulmn by adding 3x100 µl Isolation Buffer (or NaCl)
4) Apply magnetically labeled sample onto the column and let it run through
5) Wash column with 4x200 µl of Isolation Buffer
6) remove any residual drop at the end of the column with a pipette tip before eluating the sample

Eluation

1) take the column out of the magnetic field and put it into an Eppendorf tube
2) eluate the beads with 100 µl of Eluation Buffer by squeezing the plunger into the column

Exosome FACS measurement from cell supernatant

Exosome separation with Miltenyi's magnetic bead kit from THP_1 cells.

For optimal yield of the magnetic bead-based separation, we have to preclear the cell supernatant (it claims to work without these steps, but if the result matters, than I suggest to do the preclearing).

- The day before the isolation, put the cells into serum-free medium (to avoid the serum-derived vesicle contamination, and get vesicles without a protein layer around them)
- As THP_1 is a suspension cell, we have to get rid of the cells by 300 g 10' centrifugation.
- Apply 2000 g force for 30' (to get rid of the APO fraction), than discard the pellet and filter the supernatant through 0,8 µm filter
- 12500 g 40' (to get rid of the MV fraction), discard the pellet and filter the supernatant at 0,2 µm
- after the filtration we divided the EXO-containing supernatant sample into 3 groups:
    - 1 ml sample + 9 ml EV-free FBS
    - 1 ml sample + 9 ml NaCl buffer

  } 2 hour incubation at 37 °C
    - 8 ml sample for 100.000 g 70' ultracentrifugation for positive control
- Put 50 µl of magnetic beads into the samples (exept for the positive control) and incubate at room temperature (RT) for 3 hours while mixing.
- Isolate the magnetic bead according to the Miltenyi protocol:
    - put the column onto the magnet, than wash it with 100 µl Equilibration Buffer
    - wash it with 3 x 100 µl Eluation Buffer
    - pour in the sample (while it is dripping, wash 35 µl latex-sulphate bead in NaCl by centrifugation of 2000 g 10')
    - wash it with 4 x 200 µl of Eluation Buffer
    - get it out of the magnetic field
    - eluate the beads into a clear Eppendorf with plunging 100 µl Eluation Buffer through it
- Put 8-8 µl washed latex-bead into the eluated magnetic bead-containing fluid in a plain-bottom tube and mix it at 4 °C overnight. Also make a negative control by binding BSA to 8 µl latex-bead and a positive control by incubating the ultracentrifugated sample with 8 µl latex bead.
- The next day, put glicin into the tubes until it becomes ~100 mM concentration. Incubate at room temperature for 30'
- get the solution to ~2% with BSA, and incubate it at 4 °C for 2 hours

- wash it with ~0,5 ml NaCl by centrifugating at 2000 g for 10’
- resuspend the beads in 40 µl NaCl, than divide into 4 FACS tubes.

FACS staining:

1) unstained
2) CD63-PerCP/Cy5.5 – CD9-APC
3) CD81-PerCP/Cy5.5 – Hsp70-A488
4) CD105-FITC – CD107a-PerCP/Cy5.5

- staining gets place in 50 µl total volume, of which 10 µl is the bead-binded sample. Stain volume is concentration-dependant, but basically 1-1µl (exept for APC, of which we need 2 µl). The rest volume is NaCl.
- Incubate for 20’ in dark, RT
- wash out the unbinded stain in 2x800 µl NaCl centrifugating at 2000 g 5’
- measure in FACS