Methods for isolating extracellular vesicles

Density Gradients

Size Exclusion Chromatography (SEC)

07012022

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Density gradient ultracentrifugation

- Method for isolating particles based on their size or density
- Increase the **purity** of the vesicles
- Loading position
  - Top-loading (Kriszti V proposed that for Condition media)
  - **Bottom-loading** (This for Isolated EV)
- The most common gradient medium
  - Sucrose
  - Ioxidanol gradient or "**Optiprep gradient**"

Optiprep (Iodixanol)

Protocol

1. Prepare gradient solutions

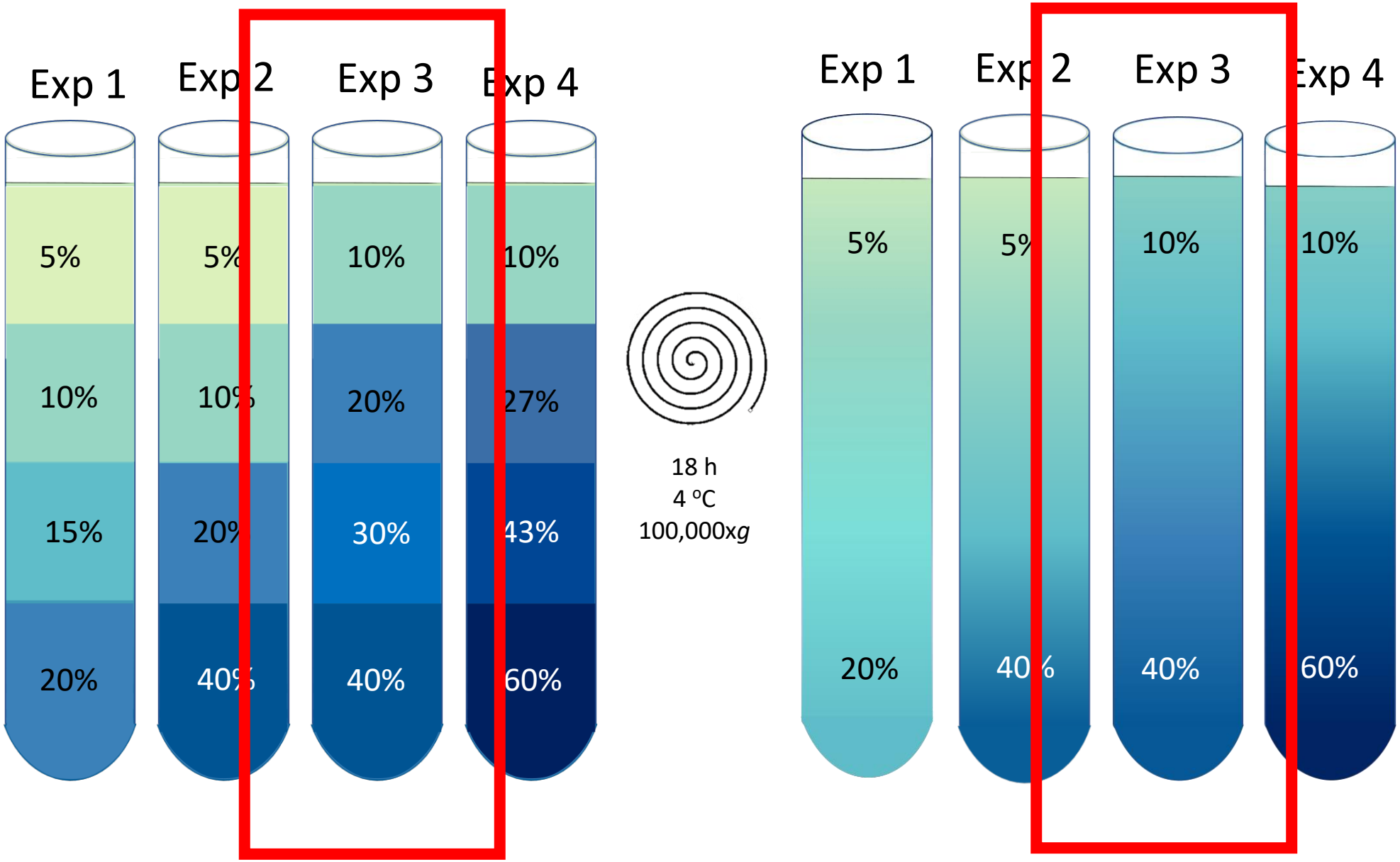
- HM: homogenization medium (stored in cold room)
- WB: working solution buffer (stored in cold room)
- Prepare working solution (WS) by adding 1 volume of WB to 5 volumes of OptiPrep solution.

For 2 gradients, prepare gradient solutions following the next scheme:

Percentage	WS (mL)	HM (mL)
5%	0,25	2,25
10%	0,5	2
20%	1	1,5
40%	2	0,5

Solutions can be stored in cold room protected from light for 2 weeks

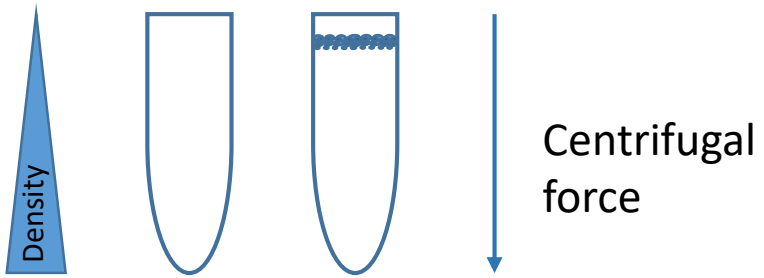
Paulina method : using 12 ml tubes and using whole plasma on TOP down



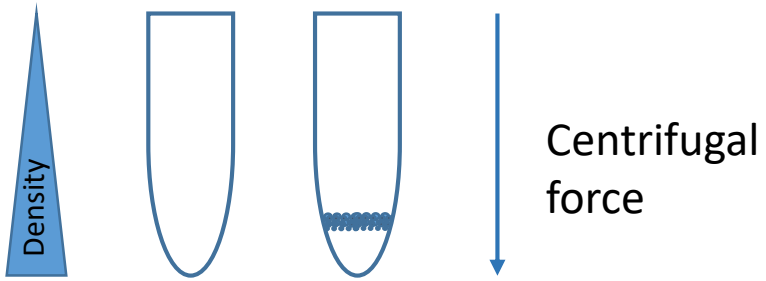
2. Prepare gradient

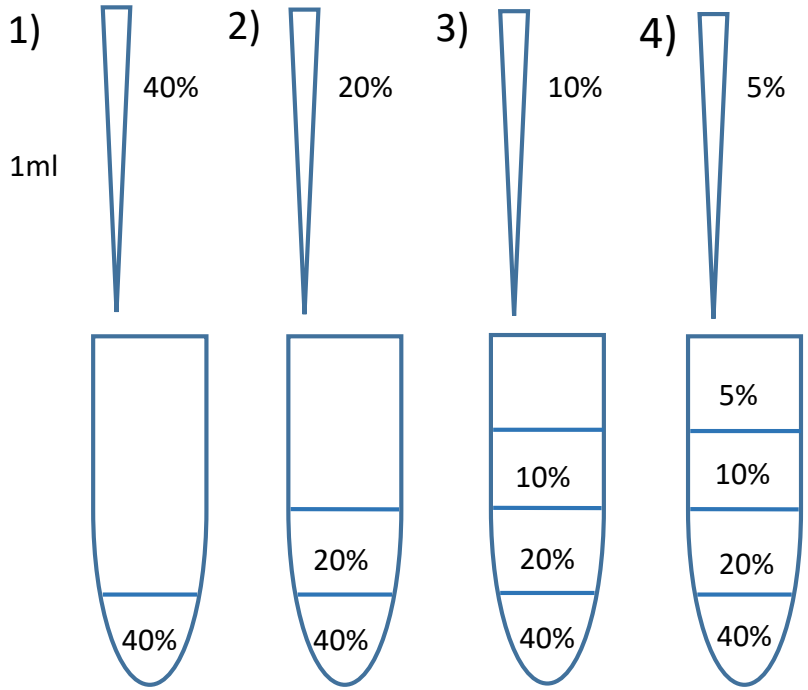
- Sequentially layer the following amounts of gradient solutions on top of each other in a 5 ml tube.
- Put in 1 ml of each concentration, starting first with 40%, after 20%, 10%, 5%.
- Mix the first one (40%) with the sample (bottom up). And slowly drop by drop put the rest.

- Top-loading Density Gradient



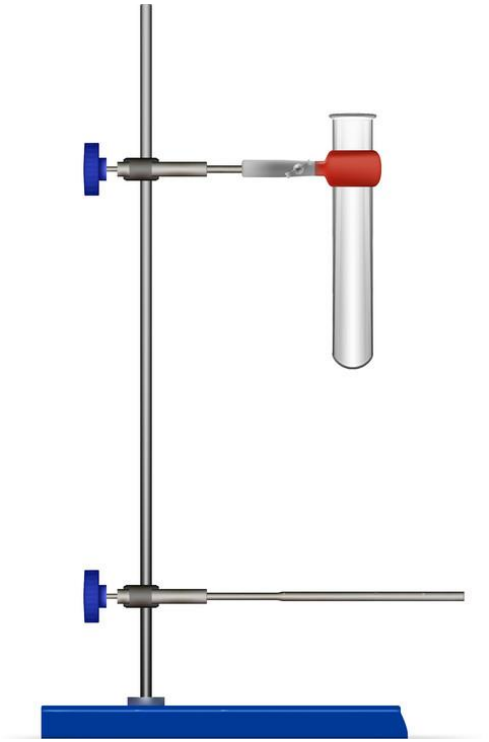
- Bottom-loading Density Gradient

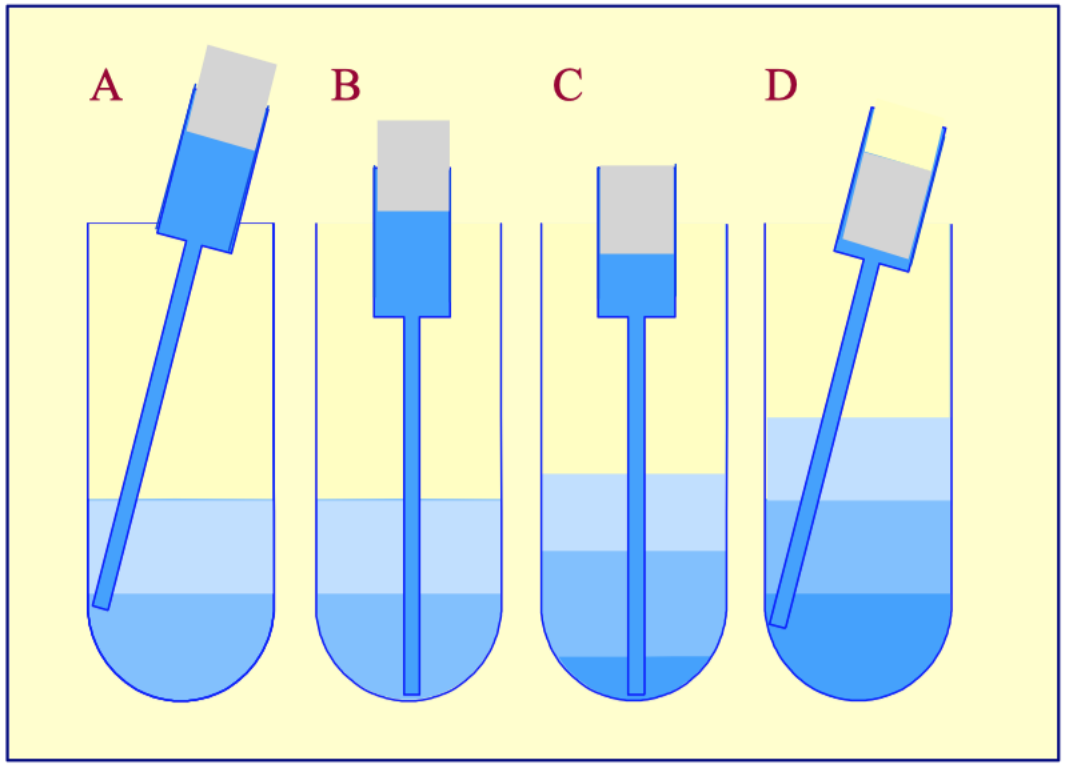




Paulina method : she load first the 10% and then go to higher sucrose concentration

Under layering of solutions





3. Centrifugation

Parameters:

- Time: 18h
- Temperature: 4°C
- 100,000g (acceleration: slowest, deceleration: coast)

4. Collection of fractions and centrifugation

- Collect eight 2ml eppendorfs tubes and measure the weight.
- After centrifugation, carefully collect 500ul fractions and put them into the 2ml eppendorf tubes.
- Measure the weight.

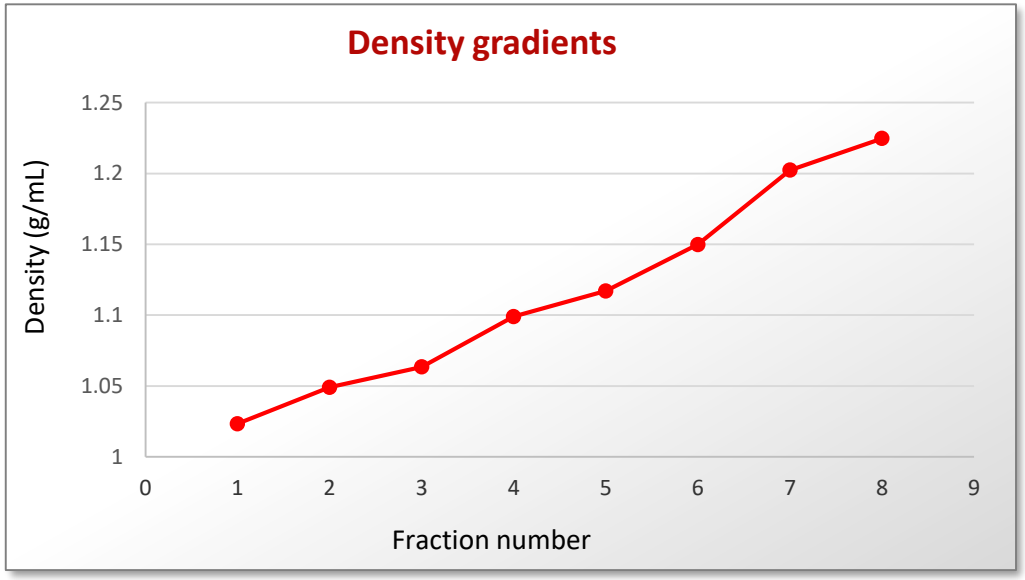
Beckman Coulter MLS-50 Optimax Ultracentrifuge Swinging Bucket Rotor

5. Wash

- Fill up the tubes with PBS or NaCl and wash it.
- (for mEVs spin down at 12,500g, 40min, at 4°C)
- Remove supernatant with syringe and resuspend pellet with 50-100ul of PBS.

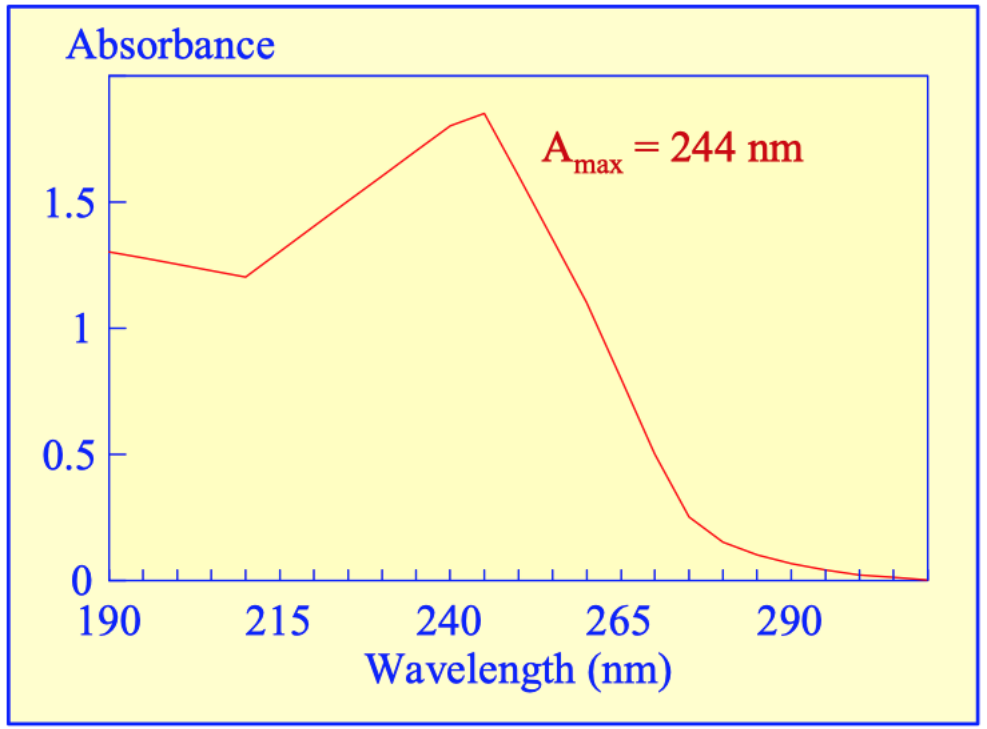
6. Density determination

- Calculate the density calibration graph: filled tube - empty tube x2 (d=m/V)



Density determination by UV spectroscopy

- Prepare a standard curve of iodixanol solutions 5, 10, 20, 30, 40 and 50% iodixanol.
- Dilute the standards and the sample to a ratio of 1:5000
- Measure the UV absorbance at 244 nm



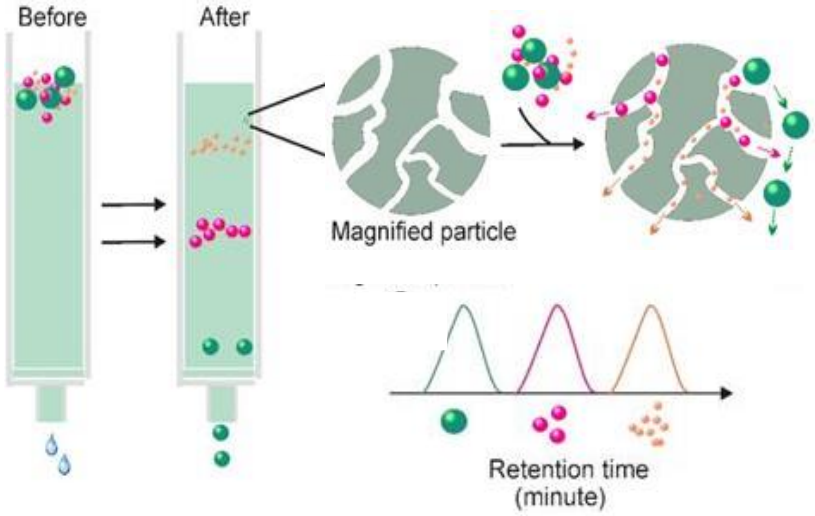
Kriszti V tried also this method but not okay for samples in Optiprep

Size Exclusion Chromatography (SEC)

- Method for separating particles by **size** as they pass through a column consisting of porous resin particles.

- Larger particles are eluted the earliest, as they cannot enter the pores and therefore flow around the resin.

- In contrast, particles smaller than the isolation range (35 nm+ or 70 nm+) enter pores in the resin and are eluted later.



- You can obtain **qEV isolation columns,** optimised for your research, (column size and isolation range) from IZON.

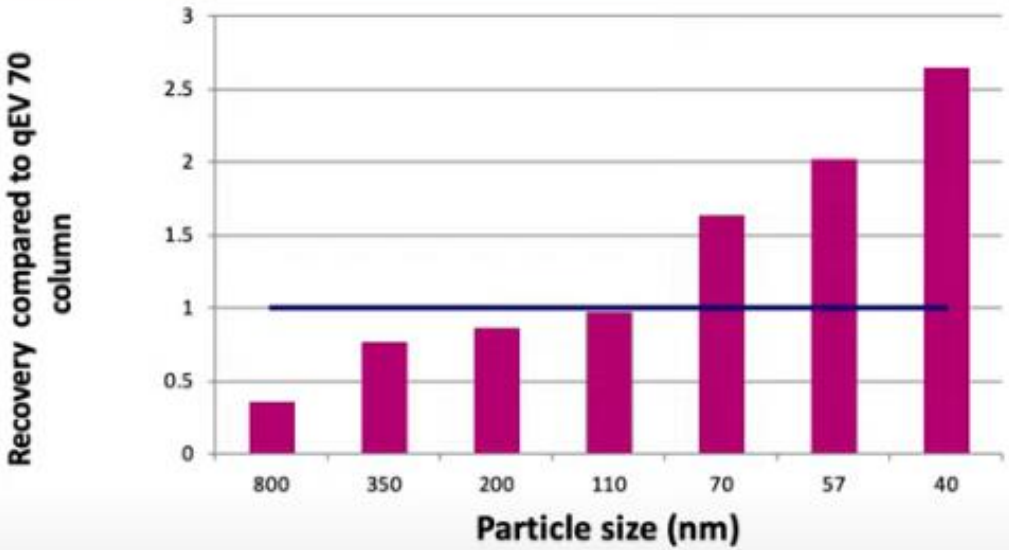
- **Column size** selection is based on the sample loading required. Each column has a recommended sample loading volume for highest purity. (qEV single, qEV original, qEV1, qEV2, qEV10, qEV100)

- Each of the column sizes are available in two isolation ranges (35 nm and 70 nm).

- The 70 nm qEV columns have an optimum particle recovery of 70 nm to 1000nm, while the 35 nm columns have an optimum recovery range of 35 nm to 350 nm.

Andras question : 70nm is used for MV and also small vesicles and why we do not use 35nm for small one

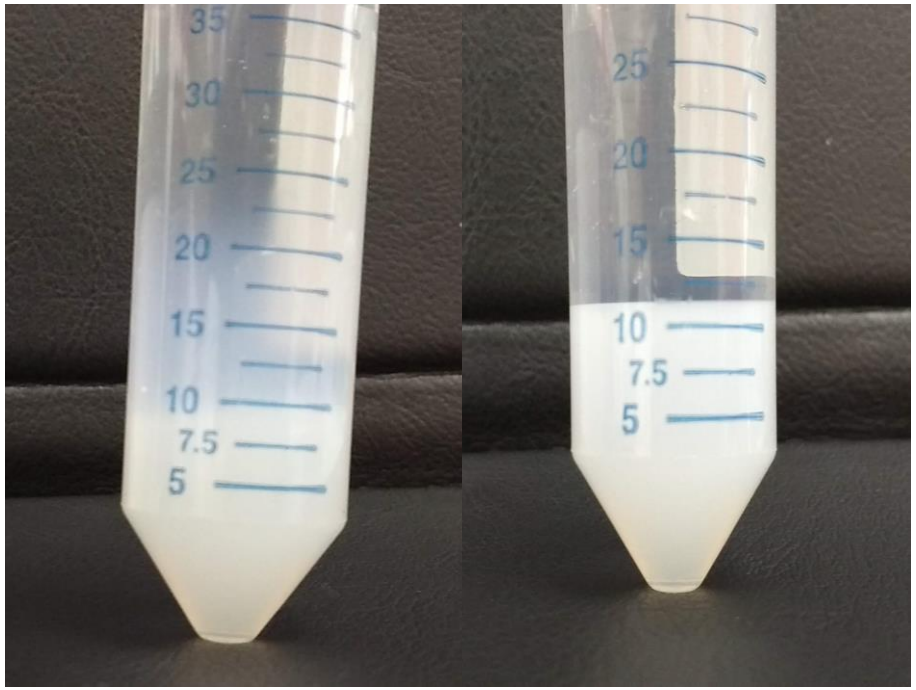
qEV Columns

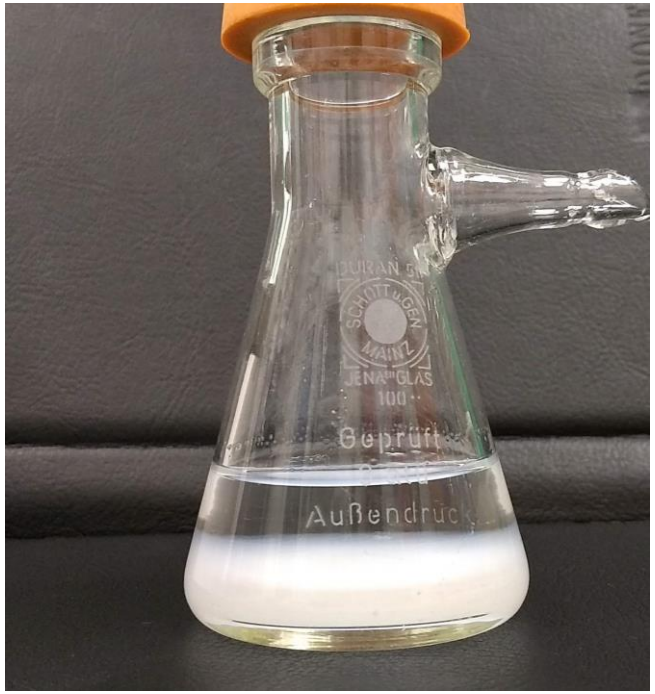


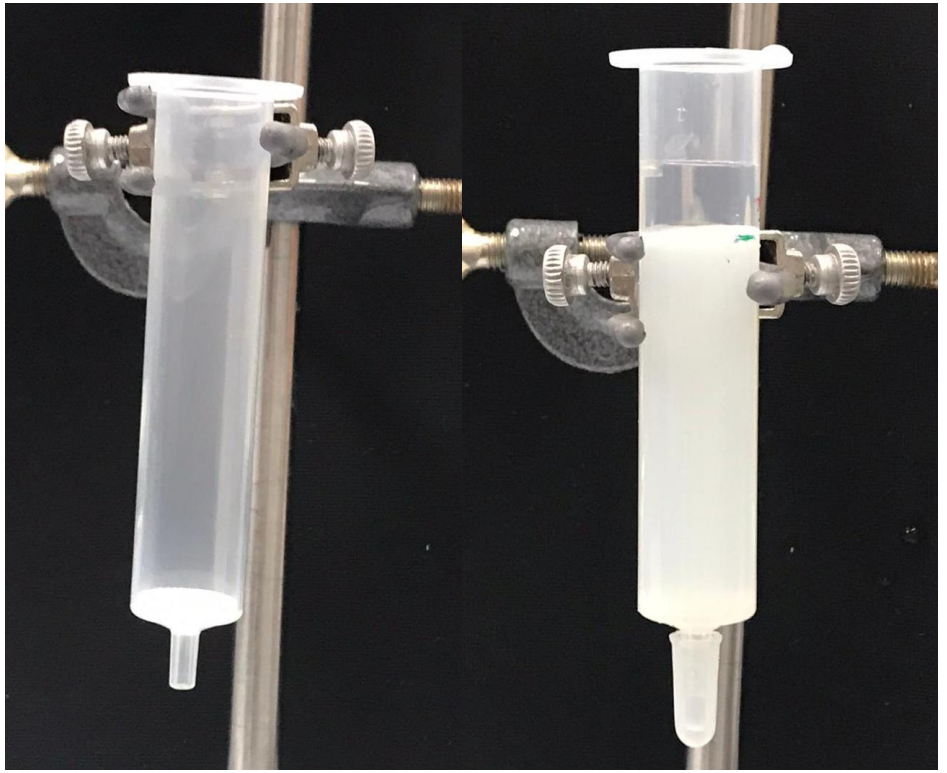
- 5 Column Sizes Available (100 µL, 500ul, 2mL, 10mL and 100 mL Sample Input Volume)
- 2 Media Sizes Available (35 and 70 nm)
- Manufactured According to ISO 13485

IZON

Setting up the column







After setting up the column

1. Check the column
   - Void volume: Dextran Blue 2mg/ml
   - Visual inspection
2. Check the protein profile elution: Albumin .5 mg/ml
   - Colorimetric assay: microBCA or Qubit

Column setup and equilibration

1. Place the column in a holder.
2. Carefully remove the top-cap.
3. Remove the bottom cap and allow the buffer to start running through the column.
4. Flush the column with at least one column volume of sample buffer.

Sample loading

1. Load the prepared centrifuged sample volume.
2. Immediately start collecting the void volume.
3. Allow the sample to run into the column.
4. Top up the column with buffer and continue to collect the void-volume.

Column Flush and Storage

1. After all the desired fractions have been collected, flush the column with at least 1.5 column volumes of buffer.
2. If storing the column for future use, flush with buffer containing a bacteriostatic agent (e.g. 0.05 % w/v sodium azide).
3. Store the column at 4 °C.

Buffer for the samples (all are filtered at 0.2 um with a vacuum filter)

- Cell supernatant: PBS
- Blood plasma: NaCl-Hepes

Column wash

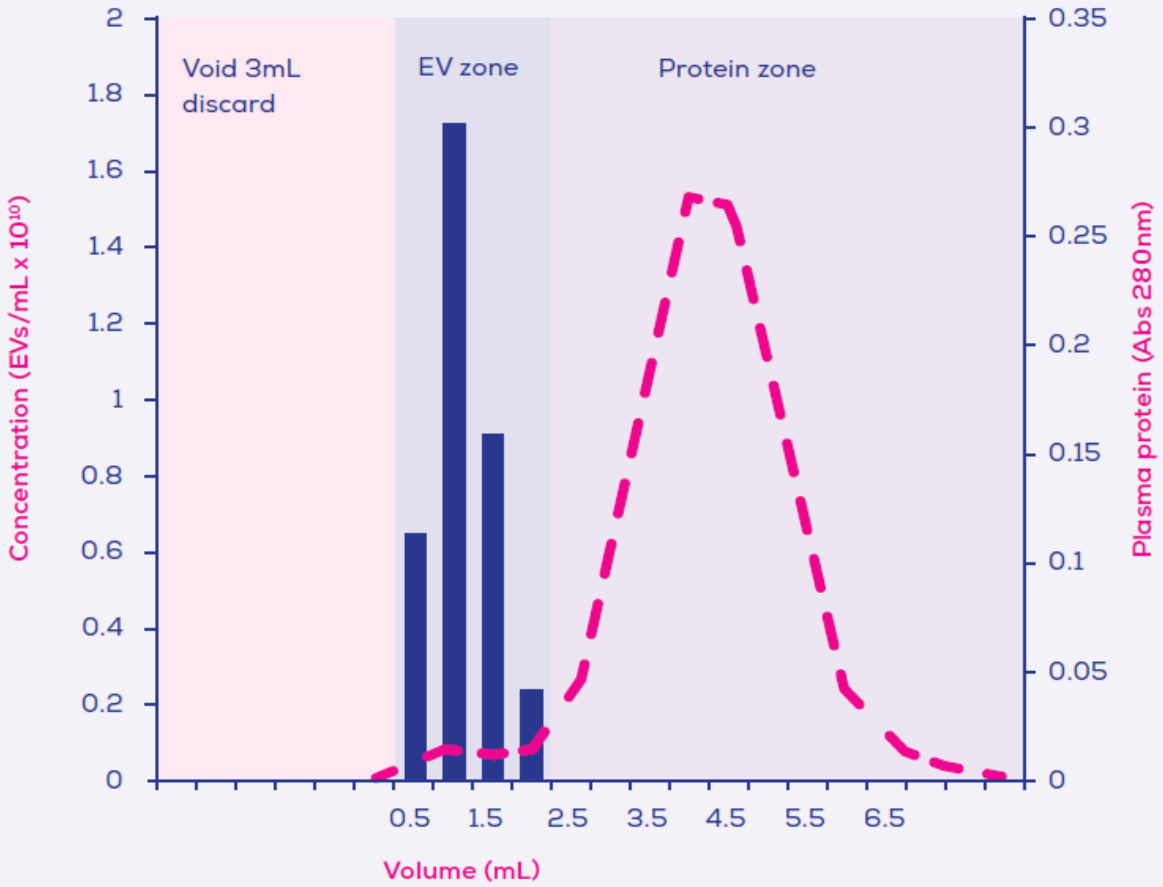
Washing: 0.5M NaOH (10ml) (add lithmus indicator for your ease!)
Buffer: 15 ml
After this, fill the column and close it

Importantly

Work in a sterile hood!
If you don't use the column for more than half a year: add sodium-azide (0.05 % w/v) or wash the column with 70% EtOH
If the column is dried out (cracks in the gel inside) don't use it, but you can regenerate it by washing 2-3 times with buffer

Storing of the columns:
4 °C, standing!

The holder of the SEC columns are to be washed only with washing-up liquid and water. Storing in next to the hood in 604 lab



Typical elution profile for a qEVoriginal-70nm column with 0.5 mL of plasma loaded; proteins elute in a later volume than vesicles. The vesicle concentration was measured using a qNano and protein levels by absorbance at 280nm.

qEV / 35 nm

35 - 350 nm
Optimum Recovery Range

‹110 nm
Higher recovery of EVs smaller than 110nm

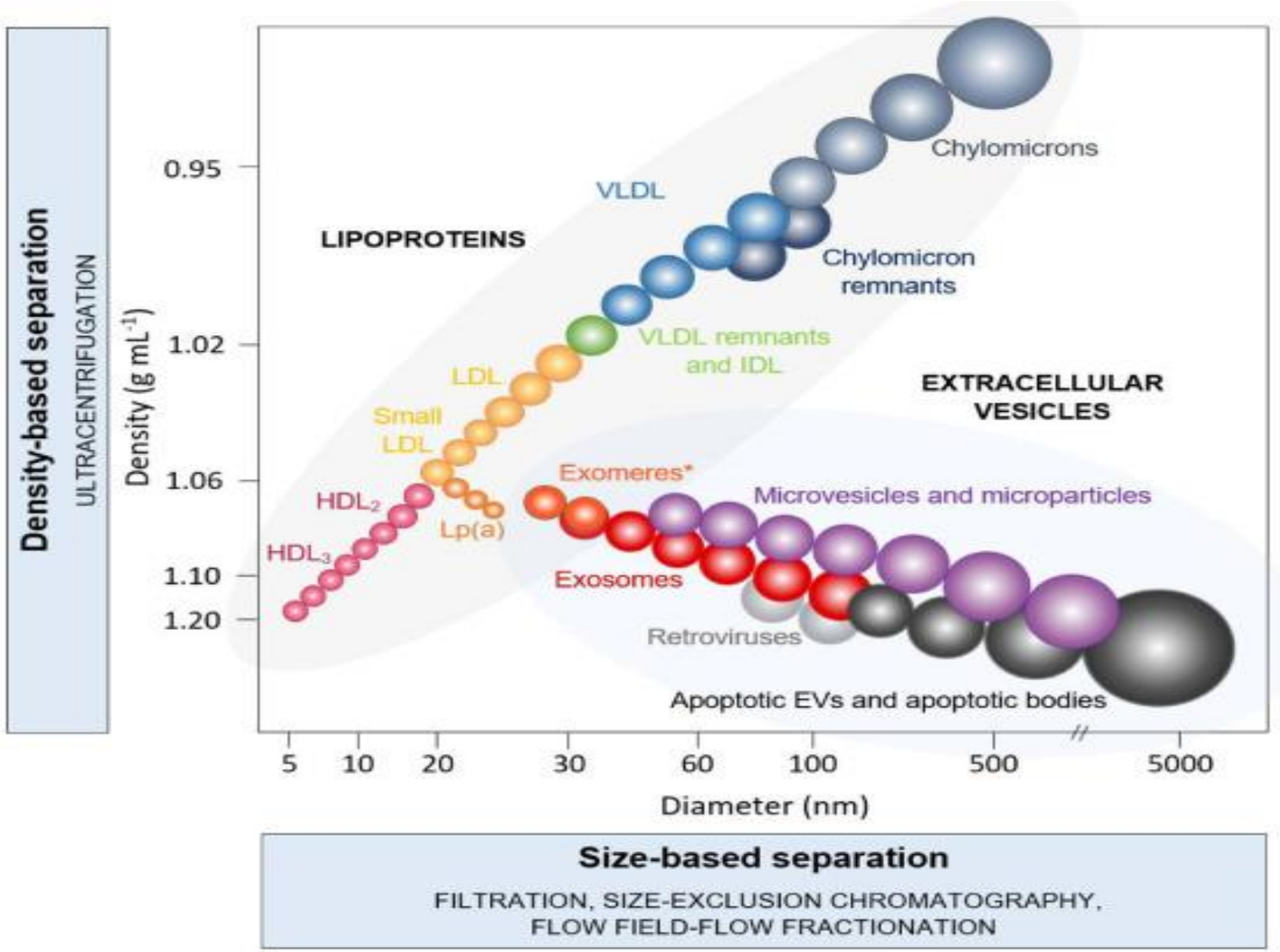
More Lipoprotein Overlap
When working with blood plasma

qEV / 70 nm

70 - 1000 nm
Optimum Recovery Range

›110 nm
Higher recovery of EVs larger than 110nm

Less Lipoprotein Overlap
When working with blood plasma



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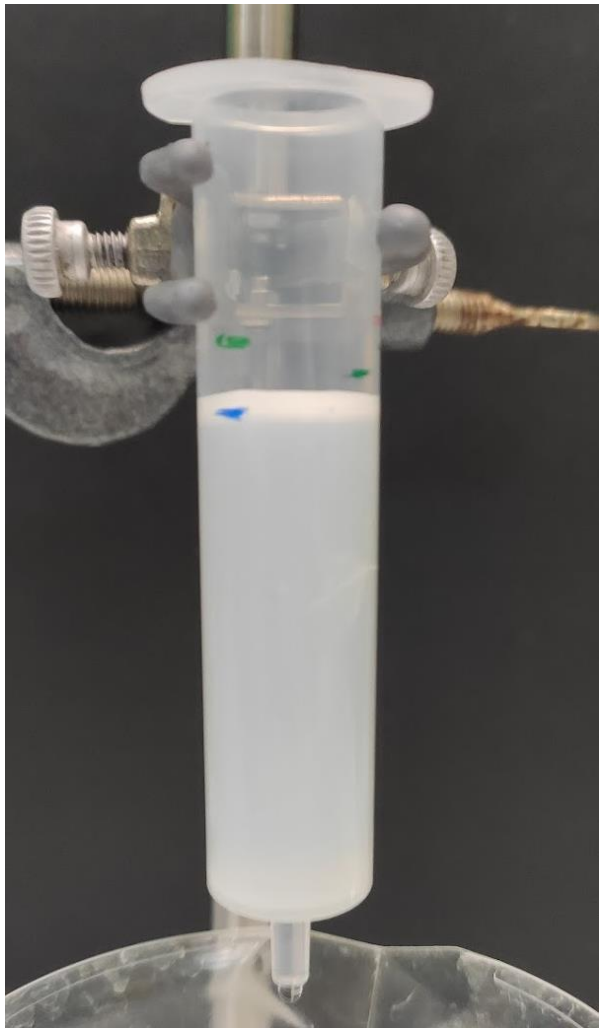

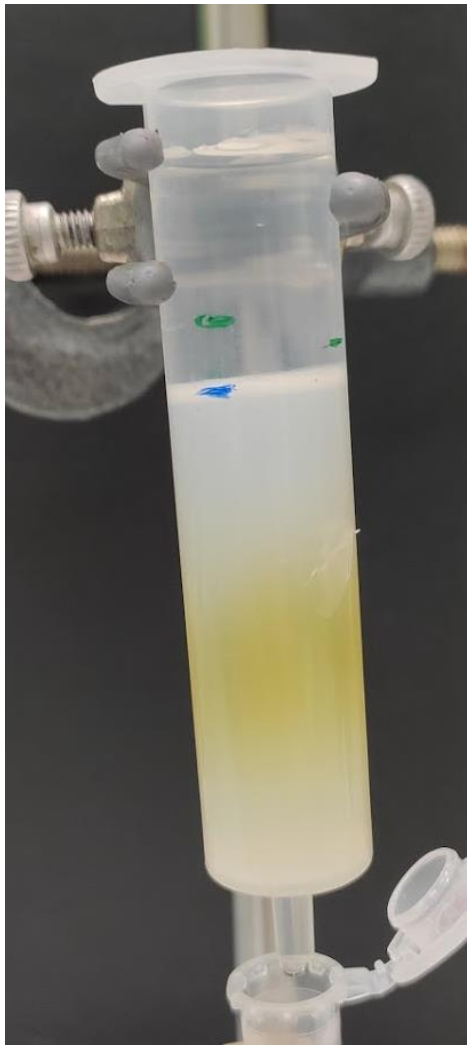

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Kriszti V propose to wash with PBS 2 or 3 times the fractions after Optiprep for Cytoflex and qNANO
And she proposed to do SEC (based on articles) for the fraction with optiprep to clean up before lipid assay or BCA as optiprep can interfere