

## EV Concentrating Protocol after NanoEX isolation

### INTRODUCTION

NanoEX can directly enrich EV by up to 25X, depending on the input volume. If higher concentrating factors are desired, an ultrafiltration (UF) step can be applied.

### PROTOCOL

1. Use 100kD MWCO Polyethersulfone (PES) Concentrator, e.g. Satorius Vivaspin® Polyethersulfone Centrifugal Concentrators (Cat # VS2041).  
**IMPORTANT:**
  - It is important to use 100kD MWCO and PES filter. Based on experimental data, this additional UF concentrating step can recover 85-95% of EV.
  - Higher MWCO, such as 300kD will lead to dramatic EV loss, while lower MWCO, such as 10kD will lead to excessive retention of residual proteins.
  - Non-PES filters may also lead to significant EV loss.
2. Centrifuge NanoEX isolated EV in the concentrator at 500g using a swing bucket centrifuge until desired volume has reached, typically 15-60min.  
**IMPORTANT:**
  - Due to the high purity of NanoEX-isolated EVs, excessive concentration may promote EV aggregation. Based on internal evaluation, it is recommended that the final EV concentration, as measured by NTA particle concentration, does not exceed  $\sim 1 \times 10^{11}$  particles/mL.
3. Because EVs tend to accumulate near the UF membrane after concentration, a two-step recovery strategy (flush + wash) is required to maximize EV recovery.
  - Step 1: Gently flush the UF membrane by repeated pipetting 10-15 times with the concentrated sample in the retention chamber and then carefully transfer the sample to a new tube.
  - Step 2: Flush the filtration membrane with a small amount of buffer (e.g. PBS) by repeated pipetting 10-15 times. Combine the wash buffer with the collected concentrate. For example, after concentrating EV solution from 15ml to 1ml, flush the membrane with 0.5ml of buffer for a final volume of 1.5ml to reach 10X concentration.
4. See **Supporting Data** comparing 3 concentrate extraction methods:
  - 1) **No Flush** – Directly extract the concentrate without flushing the UF membrane.
  - 2) **Flush** – Flush the UF membrane with the concentrate then extract.
  - 3) **Flush + Wash** – Flush the UF membrane with the concentrate plus an additional wash step with buffer.

## SUPPORTING DATA

### Materials:

- HEK293 Conditioned Culture Medium (CCM) with 10% Exosome-depleted FBS

### Methods:

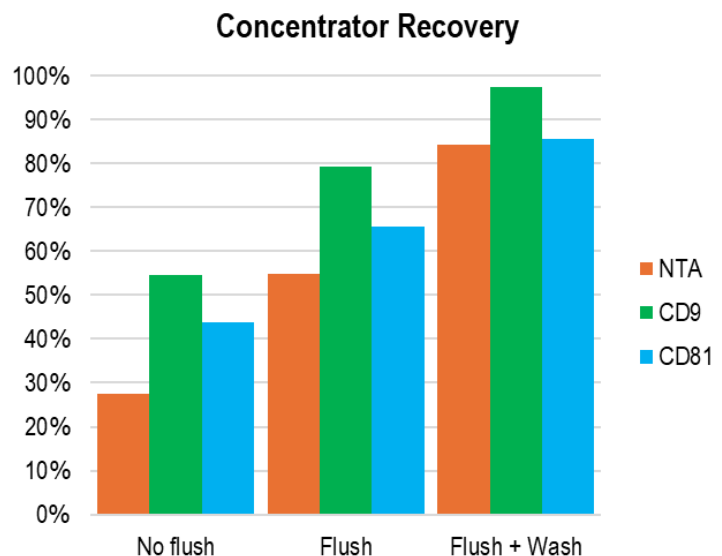
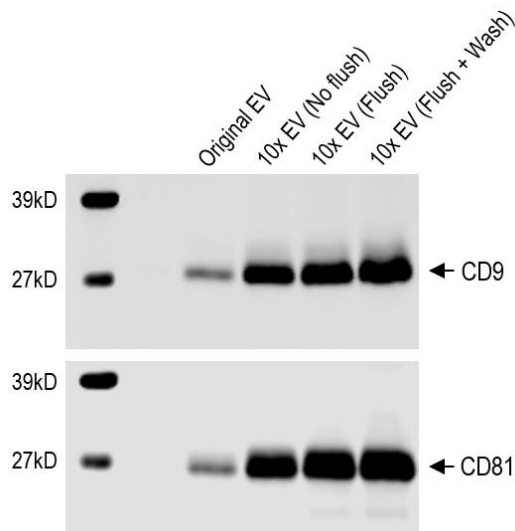
- EV Isolation by NanoEX:
  - Cassette: VF-EXO-10
  - Input volume: 250 ml
  - Output volume: 22 ml
- EV Concentration by ultrafiltration:
  - 100kD MWCO PES Concentrator
  - Input volume: 5 ml
  - Output volume: 0.5 ml
- EV Characterization:
  - Nanoparticle Tracking Analysis (NTA): ZetaView
  - Western Blot: CD9 (Cell Signaling #13174), CD81 (Cell Signaling #56039)

### Results:

- NTA (ZetaView)

Sample	Particle Concentration	Enrichment Factor	Recovery
Original EV	1.00E+10	-	-
10X EV (No flush)	2.74E+10	10	27%
10X EV (Flush)	5.47E+10	10	55%
10X EV (Flush + Wash)	8.42E+10	10	84%

- Western Blot



### Conclusion:

- The two-step recovery method (Flush + wash) is crucial for maximum EV recovery using ultracentrifugation.