**Small EV purification by Standard Ultracentrifugation+/-Density Gradient**

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*\*\*\*Note, this is not the Cushion density gradient method that is now standard practice in Weaver lab and EVPA Core. It is an alternative. The UC prep is a good way to get started for beginners, before trying density gradient methods.*

*Simple Ultracentrifugation Prep*

1. 80% confluent cells were cultured for 48 h in Opti-MEM or serum-free.
2. Spin down the culture medium at 300 X g for 10 min to pellet cells.
3. Spin down the supernatant from step 2 at 2000 X g for 20 min to pellet dead cells and cell debris.
4. Spin down the supernatant from step 3 at 10,000 x g for 30 min to pellet large EVs
5. Spin down the supernatant from step 4 at 100,000 x g for 70 min to 18 h (we do 18 h) to sediment small EVs (UC-Exo).
* Optima XPN Ultracentrifuge (Beckman Coulter)
* Type 45 Ti rotor (339160, Beckman Coulter)
* 70 ml, 38 x 102 mm bottles (355622, Beckman Coulter)
1. If further purification is desired skip to step 7. If no further purification is desired, resuspend the UC-Exo pellet in 3 ml PBS and spin down at 100,000 x g for 6h. Resuspend the pellet using a desired volume of PBS to prepare UC-Exo.
* Optima MAX Tabletop Ultracentrifuge (Beckman Coulter) or a compatible table top ultracentrifuge
* TLA 100.3 rotor (349481, Beckman Coulter)
* 3.5 ml, 13 x 51 mm tubes (349622 or 349623, Beckman Coulter)
* Alternatively, you can wash UC-Exo using 70 ml, 38 x 102 nm bottles and Type 45 Ti rotor equipped in Optima XPN Ultracentrifuge. Make sure total volume of wash should be at least 50 ml.

Density Gradient purification

1. For further purification by density gradient, resuspend the pellet from step 5 in 1 ml dilution buffer (0.25 M sucrose/10 mM Tric, pH 7.5), mix with 2 ml OptiPrep™ (60% (w/v) aqueous iodixanol, Axis-Shield PoC) to resuspend UC-Exo in 40% (w/v) OptiPrep, and load it on the bottom of a 14 ml, 14 x 95 mm tube (344060, Beckman Coulter).
2. Make discontinuous gradient by loading 3 ml of 20%, 10%, and 5% solutions of iodixanol from the top of the 40% solution in the 14 ml tube. *Load each solution* ***very carefully*** *to make discontinuous gradient!!!*).

***Preparation of discontinuous iodixanol gradient***

Dilute OptiPrep™ (60% (w/v) aqueous iodixanol, Axis-Shield PoC) with 0.25 M sucrose/10 mM Tris, pH 7.5 and prepare 20% (w/v), 10% (w/v), and 5% (w/v) solutions of iodixanol.

1. Spin at 100,000 x g using a swinging bucket rotor for 18 h to make continuous gradient.
* Optima XPN Ultracentrifuge (Beckman Coulter)
* SW 40 swinging rotor (331302, Beckman Coulter)
1. Collect 12 fractions (1ml each) from the top of the 14 ml tube and dilute each fraction in PBS (1ml fraction + 2 ml PBS).
2. Spin down at 100,000 x g for 3 h
* Optima MAX Tabletop Ultracentrifuge (Beckman Coulter) or a compatible table top ultracentrifuge
* TLA 100.3 rotor (349481, Beckman Coulter) or TLA 110 rotor (366735, Beckman Coulter)
* 3.0 ml, 13 x 51 mm tubes (349622 or 349623, Beckman Coulter) for TLA 100.3 rotor
* 3.2 ml, 13 x 56 mm tubes (362305 or 362333, Beckman Coulter) for TLA 110 rotor
1. Resuspend pellets in PBS (50 ul or larger volume depending on how many small EVs the cells secrete or how concentrated small EVs are required for further experiments).
2. Small EVs are expected to be observed in fraction 6 and/or 7 if fractions were numbered from the top.

**Small EV identification**

1. Western blots using exosomal markers such as CD63, flotillin, TSG101, Hsp70, etc.
2. Western blots using negative markers for exosomes such as ER markers (e.g. calnexin), Golgi markers (e.g. GM130), etc.
3. Electron microscopy
4. Nanoparticle tracking analysis such as Nanosight, ZetaView, etc.
5. See <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4275645/> , https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6322352/ and <http://www.nature.com/nmeth/journal/v14/n3/full/nmeth.4185.html?foxtrotcallback=true> for more information of minimal experimental requirements for definition of extracellular vesicles.