

Isolation of extracellular vesicles from primary osteoblasts.



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Extracellular vesicles (EVs) are lipid membrane-bound structures released by cells that facilitate intercellular communication. Principally classified by size and formation, microvesicles are released from the plasma membrane, whereas exosomes are small membrane-bound vesicles (<150nm) of endocytic origin [1]. This HubLE Method describes the protocol for isolation of EVs from primary osteoblasts by differential ultracentrifugation [2,3].

Materials

- Primary osteoblasts [See Tip No. 1]
- Standard FBS ('Unspun') [See Tip No. 2]
- EV-depleted ('Spun') FBS [See Tip No. 2]
- Osteogenesis α MEM unspun (os α MEM unspun) and spun (os α MEM-spun) [See Tip No. 3]
- 1X Phosphate Buffered Saline (PBS)
- Ultracentrifuge [See Tip No. 4]
- Ultracentrifuge tubes [See Tip No. 5]
- Sucrose (30%)

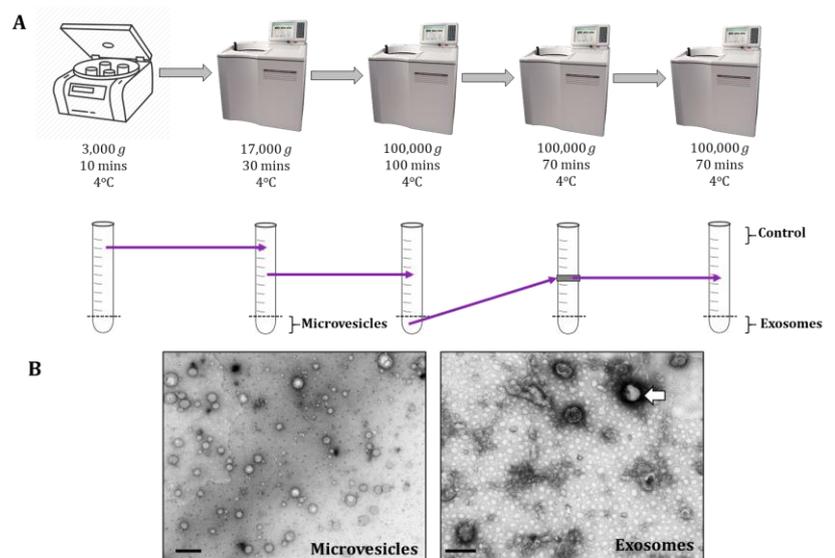


Fig. 1. (A) Schematic representation of the EV isolation protocol. Purple arrows denote transfer of supernatant or pellet to fresh ultracentrifuge tubes. The grey rectangle represents the sucrose cushion. **(B)** Transmission electron images of microvesicles (scale: 500nm) and exosomes (white arrow, scale: 200nm).

Method

All procedures should be performed under sterile conditions.

1. Seed primary osteoblasts in os α MEM-unspun at 1×10^6 cells per 75cm^3 flask. Incubate at 37°C with 5% CO_2 [See Tip No. 1].
2. For EVs from mature, mineralising osteoblasts, culture for 17-19 days with half medium changes of os α MEM-unspun every 2-3 days.
3. For the final 48-72 hours of culture, remove medium, wash with PBS and add os α MEM-spun [See Tip No. 6].
4. On the day of isolation, transfer the spent os α MEM-spun media to a collection tube and centrifuge at $3,000 \times g$ for 10 minutes at 4°C to remove cell debris [See Tip No. 3].
5. Carefully transfer the supernatant to fresh ultracentrifuge tubes and place the tubes within the ultracentrifuge buckets [See Tip No. 5].

6. Ultracentrifuge at $17,000 \times g$ at 4°C for 30 minutes with maximal acceleration and low deceleration to pellet microvesicles [See Tip No. 7].
7. Carefully transfer the supernatant to a second set of ultracentrifuge tubes and return to the ultracentrifuge buckets.
8. Resuspend the microvesicle-containing pellet in PBS to a final volume of 1ml, transfer to a 1.5ml collection tube and store at -80°C until required.
9. Ultracentrifuge the supernatant at $100,000 \times g$ for 100 minutes at 4°C with maximal acceleration and no brake to pellet the exosomes.
10. Carefully remove and discard the supernatant. Pool the exosome pellets (if ultracentrifuging the same treatment) and resuspend the pooled exosome pellet in up to 9ml PBS.

11. Add 4ml of 30% sucrose to fresh ultracentrifuge tubes and layer the re-suspended pooled exosome pellet on top of the sucrose cushion [See Tip No. 8].
12. Ultracentrifuge at 100,000 x g for 70 minutes at 4°C with maximal acceleration and no brake. This will purify the exosomes within the sucrose cushion.
13. Carefully remove and transfer the floating exosome pellet at the sucrose cushion interface to a fresh ultracentrifuge tube (Fig.1A). Bring up to maximum

volume by adding PBS to the ultracentrifuge tube [See Tip No. 6].

14. Ultracentrifuge at 100,000 x g for 70 minutes at 4°C with maximal acceleration and no brake.
15. Transfer the first 1ml of the supernatant to a fresh 1.5ml collection tube and store at -80°C until required [See Tip No. 9].
16. Discard the remaining supernatant and resuspend the exosome-containing pellet in 200µl PBS. Store exosomes at -80°C until use. [See Tip No. 10].

Tips

1. **Primary osteoblasts:** cells can be obtained from neonatal rats (2-3 days) or mice (3-6 days) and isolated as per Bourne and Orriss' HubLE Method [4]. This method can also be used with common osteoblast cell lines with the initial culture time adjusted as appropriate.
2. **EV-depletion of FBS:** EVs are abundant in FBS and, therefore, need to be depleted to ensure that any EVs isolated from primary cultures are, in fact, osteoblast-derived. To deplete FBS of EVs, ultracentrifuge FBS at 100,000 x g overnight (~18 hours) at 4°C with maximal acceleration and no brake. Transfer the supernatant (now EV-depleted FBS) to a fresh collection tube and store at -80°C until media preparation.
3. **Osteogenesis αMEM-Un/Spun:** Add 10% unspun or spun FBS and AB/AM (100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin), 50µg/ml ascorbate and 2mM β-glycerophosphate to α-Minimal Essential Media (αMEM) to stimulate osteoblast differentiation and bone formation.
4. **Ultracentrifuge rotor:** There are two ultracentrifuge rotor types: swinging bucket and fixed angle. Rotor types within your institution should be established to determine the maximum volume and speed the rotor can spin. Due to the very high centrifugal force applied, it is essential that the rotor is appropriately and correctly balanced. It may take up to 5 minutes for the ultracentrifuge to run at speed; this is accounted for in the presented spin times.
5. **Ultracentrifugation tubes:** It is essential that the ultracentrifugation tubes to be used are compatible with the instruments and rotors available within your research facilities. For example, 14ml polyallomer tubes are compatible with a SW40-Ti rotor in the Beckman-Coulter Optima™ L-80 ultracentrifuge. Tubes can be re-used up to 5 times. Wash the ultracentrifugation tubes at the end of each spin by removing any additional liquid and pellet, wash in 70% EtOH and air dry. Ensure that ultracentrifugation tubes are not over-filled to prevent interference with the rotor lid seal.
6. **48-72 hour osαMEM-spun conditioning:** This method cultures osteoblasts to mineralisation using standard conditions reported in the literature [5] but switches to EV-depleted conditions for the final 48-72 hours of culture without affecting osteoblast function. The total volume a polyallomer tube and ultracentrifuge rotor can carry will determine the appropriate volume of osαMEM-spun to add for the final ≤72 hours of culture. For example, a SW40-Ti rotor in the Beckman-Coulter Optima™ L-80 ultracentrifuge holds a maximum of 75ml over 6 polyallomer tubes.
7. **EV pelleting:** The pellet at each ultracentrifugation step is often very difficult to see; therefore, a very small volume of the supernatant can be left at the bottom of the tube to prevent pellet disruption.
8. **Sucrose cushion:** The sucrose cushion allows the separation of morphologically intact particles by density and acts as an additional purification step.
9. **Final spin supernatant:** Sampling the supernatant after the final ultracentrifugation step acts as quality control. No EVs should be in this fraction due to their sedimentation.
10. **Quantification and characterisation of EVs:** Isolated EVs can be characterized according to their morphology (cup-shaped, Fig.1B) and immunological (CD9, CD63 & CD81) characteristics. EV concentration can be estimated through nanoparticle tracking analysis or protein-based assays (e.g., Bradford and/or micro-BCA)[1]. EV yields from primary mineralized neonatal mouse osteoblasts are typically very low (≤100µg/ml).

References

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