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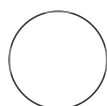
**Protocol status:** Working  
 We use this protocol and it's working

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## Medium fractionation and EV preparation

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



### ABSTRACT

This protocol describes the characterization of the extracellular alpha-synuclein and DNAJC5







### MATERIALS


For specific information about reagents and materials to perform an EV preparation, refer to a previous paper published by our lab [www.bio-protocol.org/e3706](http://www.bio-protocol.org/e3706)



## Medium fractionation

- 1 Centrifuge the conditioned medium at 1,500 x g for  00:20:00 at  4 °C in a Sorvall RC 6+  20m  
centrifuge with fixed angle rotor F14S-6X250y FiberLite
- 2 Pour the supernatant into a new container without disturbing the pellet.
- 3 Centrifuge the supernatant at 10,000 x g for  00:30:00 at  4 °C in a Sorvall RC 6+  30m  
centrifuge with fixed angle rotor F14S-6X250y FiberLite
- 4 The supernatant fractions at each step were collected and treated with methanol/chloroform to precipitate proteins which were then collected by centrifugation
- 5 Pellet fractions were resuspended in sample buffer to achieve a 20-fold concentration.
- 6 The sedimented fractions at each step were also collected and resuspended in sample buffer.
- 7 All the fractions were analyzed by immunoblot.

## Extracellular vesicle (EV) preparation



- 8 Centrifuge the conditioned medium at 1,500 x g for  00:20:00 at  4 °C in a Sorvall RC 6+ centrifuge with fixed angle rotor F14S-6X250y FiberLite 20m
- 9 Pour the supernatant into a new container without disturbing the pellet.
- 10 Centrifuge the supernatant at 10,000 x g for  00:30:00 at  4 °C in a Sorvall RC 6+ centrifuge with fixed angle rotor F14S-6X250y FiberLite 30m
- 11 Pour the supernatant into a new container without disturbing the pellet.
- 12 Add 35 ml of the collected supernatant into a single 38.5 ml ultra-clear tube. Repeat this step 5 times to fill a total of six 38.5 ml ultra-clear tubes.
- 13 Centrifuge at ~100,000 x g (29,500 RPM) for  01:30:00 using a SW32 Ti rotor at  4 °C at maximum acceleration and brake. 1h 30m
- 14 Aspirate the supernatant, put the tubes upside down on paper towel to dry any residue of medium. Aspirate the residue by vacuum system if needed.
- 15 Resuspend the pellet by adding 500 uL of PBS (pH 7.4) buffer to the bottom of each tube, put the tubes on a compatible rack, and rigorously shake the tubes on orbital shaker in cold room for 30m

 00:30:00

- 16** Combine the resuspend pellet (~500  $\mu$ L x 6 tubes = 3mL; +1mL PBS) and centrifuge again at 120,000 x g (36,500 RPM) x  01:00:00 at  4 °C in SW-55 rotor

1h

- 17** Resuspend the pellet again in 200 $\mu$ L PBS Buffer and add 1mL of 60% (1.8M) sucrose buffer (20mM Tris 7.4, 150 mM NaCl) and vortex to mix the sample evenly. The final sucrose concentration should be above 50% measured by refractometer.

- 18** Aliquots of 40% (5 ml) and 10% (2 ml) sucrose buffer were sequentially overlaid above the sample. The tubes were then centrifuged at 150,000 $\times$ g for  16:00:00 at  4 °C in an SW41 Ti swinging-bucket rotor (Beckman Coulter)

16h

- 19** After centrifugation, 0.5 ml fractions were collected from top to bottom and samples were analyzed by SDS-PAGE and immunoblot.