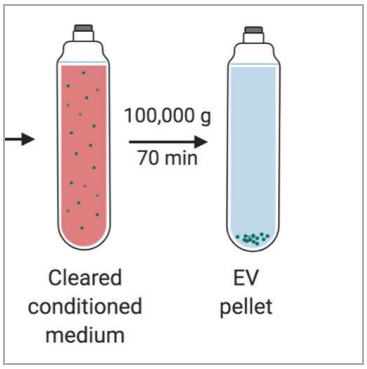


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# Isolation of EVs by ultracentrifugation

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ABSTRACT

How to isolate EVs from culture media using differential centrifugation.

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

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## 1 Preparation of cells

Notes: Cell cultures must be grown to approximately 90% confluency and EVs need to be isolated from a minimum of 200mL media to get a high enough

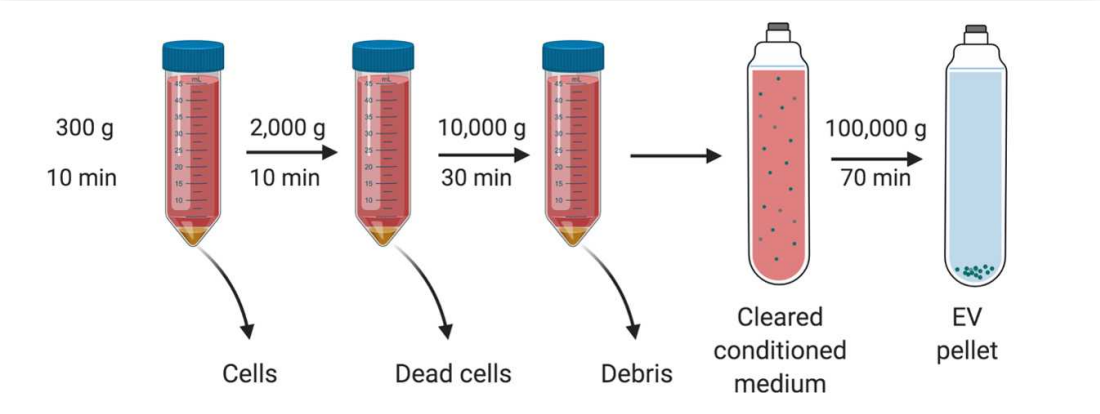
EV yield. As FBS contains natural EVs, EV depleted FBS should be used in the media incubated with cells for 48 hrs prior to isolation. This media is called
collection media (CM). Alternatively, collection media can be FBS free, or FBS can be substituted with ITS (Insulin-Transferrin-Selenium (ITS -G) (100X) Gibco). If using ITS, 500mL collection media will require 5mL of the ITS. ITS only requires a 24hr incubation before collection.

STEP 1: Remove culture media media

2 Wash cells X2 with PBS

3 Collection media (CM) is added to cells for 24/48 hours.

4 DIFFERENTIAL ULTRACENTRIFUGATION




NOTE: The 2000g spin (2nd spin) is for 20 minutes, not for 10 as the image shows. The image needs altering by someone with biorender! The final two spins are done using the ultracentrifuge and ultracentrifuge tubes.

NOTES before starting

How to use the ultracentrifuge: The ultracentrifuge is in the store room on floor 1. It needs to be booked on Clustermarket (Not CeMi; Pharmacology).The ON switch is on the side of the machine. Before going downstairs, remember to bring grease and the little key to help remove

tubes from the rotor.

5 - It can fit 8 tubes with max volume of 25mL per tube.

6 - Cool it before using by switching on the vacuum to 4 degrees

7 - Get the rotor from the fridge (this is really heavy, so be careful!). Always place blue roll underneath so we don't scratch the bottom.

8 - Place in the centrifuge. You can manually give it a wee spin and look to see that the number changes on the screen to see that you have placed in in correctly.

9 - Slide the door shut

10 - Click vacuum. Vacuum is ON when '200' appears

11 - The settings need to be put in twice for weird reason. One whilst cooling and one before the spin. I do this by setting speed -> press enter; time -> enter; temp -> enter. Then press vacuum.

12 - To remove the rotor we need to remove the vacuum. Press vacuum button. When '200' is gone the vacuum is off

13 - Now we can open door and remove rotor, placing it on blue roll

14 - Unscrew the rotor and add a little grease to bottom of circle and bottom of the other screw thing (rubber parts). This stops the lids becoming stuck onto the rotor.

15 - Ensure the lids on the ultracentrifuge tubes are tightly closed for the centrifuge. THE TUBES MUST BE BALANCED by measuring using scales. Ensure you place the paired balanced tubes into the rotor correctly. When putting tubes into the rotor place labels face the outside of the rotor. This means we know roughly where to look for our pellet afterwards (on the side of the label).

16 - Screw lid back onto rotor tightly

17 - Put rotor back in and close lid

18 - Click vacuum and will till ‘200’ appears

19 - MAKE sure ACC = MAX and DECEL = SLOW. This is essential!!

20 - Put in settings again and press go

21 - Always fill in the log book after each run

22 - Notes on the 25mL ultracentrifuge tubes. The ultracentrifuge tubes are cleaned with chemgene and then with deionised water 4 x . Make sure no chemgene left as it will kill all EVs. I usually sit the tubes upside down on blue roll overnight to dry and then put away.

23 - The tubes should always be filled to the line. If not they are more likely to crack. So if you have for example 23mL of EVs, just top this up with 2mL of filtered PBS.

24 - The lids and the plugs all weigh differently so when weighing before centrifugation we HAVE to include these on the scales. Get a beaker and place the tube with supernatant and lid on into the beaker. Zero it.

25 - Get the next tube on see how much needs to be added to get it to be the same weight.

26 - You can use filtered PBS to add to the tube for balancing.You want it to be within 0.01g. This is most important at the 70min highest speed spin. I tend to top up the tube with the lesser volume with filtered PBS, rather than remove from the heavier tube.

27 - Label the tubes on one side before centrifuging. This is handy if you have EVs from different cells, but also helpful for orientating them in the centrifuge.

28 · FIRST SPIN: After the incubation time, CM can either be further processed (this is best) or frozen at -20oC until ready to do EV isolation. It is best to do the first spin (300g for 10mins) before freezing to remove any cells.

CM is first centrifuged in 50mL Falcon tubes for 10 minutes at 300g. As mentioned above, do this step before freezing media.

29 · SECOND SPIN: Supernatant is transferred to new 50mL Falcon tubes and centrifuged for 20 minutes at 2000g.

30 · THIRD SPIN: Supernatant is transferred to 25mL ultracentrifuge tubes (Beckman Coulter, 355654). These are balanced using a set of scales followed by 30 minutes at 10,000g (10,000 RPM), using a fixed angle rotor, to remove cell debris and any large apoptotic bodies.

31 · FOURTH SPIN: The resulting supernatant is transferred to new 25mL ultracentrifuge tubes (Beckman Coulter, 355654). These require balancing again. Then they are centrifuged at 100’000g (41,800 RPM) for 70 minutes. Since this is the last spin, make sure to clean the ultracentrifuge with ethanol to remove the grease around the sides and switch off using switch at the right hand side of machine. Make sure you have filled in the log book.

32 · The supernatant is then discarded. Remove as much media as possible using a tip after removing the bulk of media with stripette. The pellet basically looks like a small smudge on the tube. Sometimes this is visible, sometimes it is not.

33 · EV pellets are re-suspended in 200µL (per tube) X1 filtered PBS. If not doing Size Exclusion Chromatography step then the 200µL resuspension is then divided into 25/50µL aliquots. (we added 1ml across all 8 ultracentrifuge tubes; i.e. 1ml total volume combining 8 pellets). Aliquots are then stored at -80oC and used immediately after thawing. Use low-bind tubes.