HTG Exosome Isolation Preparation Protocol for Library HTG Preparation

Exosome Isolation Protocol

Exosomes are small (approximately 50-150 nm) lipid vesicles secreted into the extracellular matrix. They are found in cell culture conditions secreted into the culture media, and within the human body exosomes are present in blood, urine, CSF, and other biofluids (1). Exosomes are implicated in the release of microRNA (miRNA) from cells as a means of active and passive cell signaling by miRNA, and these miRNAs are an attractive target for study as diagnostic and/or prognostic indicators for a variety of disease states (2,3,4,5)

Owing to their size and the relative abundance of other materials in culture media and/or biofluids (other microvesicles and microparticles, platelets, etc) it is critically important to isolate the exosomes themselves and use as pure a preparation as possible for exosome analysis. This document outlines and discusses several procedures for exosome isolation. At the same time, exosomes were initially hypothesized to carry miRNA because of their co-isolation in differential centrifugation protocols, but this hypothesis is being reevaluated on several fronts (6,7,8,9). Given the current uncertainties, HTG presents this as a collection of exosome isolation strategies—without further analysis or characterization of isolates, HTG cannot guarantee the presence of miRNA and success of downstream EdgeSeq assays.

I. Exosome isolation from cell culture media, using ultracentrifugation (condensed from Théry *et al.* 2006. *[1]*)

Notes:

i) Care should be taken to avoid carrying over exosomal contaminants from serum or other media components which might interfere with investigation of secreted exosomes in culture. This can be addressed by using an exosome extraction media (serum-free media, media containing Bovine Serum Albumin instead of serum, or media conditioned by spinning complete serum-containing media overnight at 100,000 x g and at 4°C, in order to remove exosomes present in serum, followed by sterile-filtering).

ii) It is recommended that enough cells are grown to achieve a 70-ml volume of harvested media, as exosome isolation efficiency decreases with diminishing volumes.

iii) All centrifugation steps should be performed at 4°C. For a table of centrifuge rotors and speed conversions, as well as a general schematic of ultracentrifugation protocol, see Table 1.

1. Cells should be grown under their usual conditions to 70-80% confluency, or 60-70% of maximal concentration for nonadherent cells. At this time, replace media with exosome

extraction media and continue growth for 24-48 hours. (Cells which do poorly in exosome extraction media or become confluent within 48 hours may be harvested at 24 hours.)

2. Collect media from cells, transfer to 50-ml polypropylene tubes, and centrifuge for 10 min at 300 x g and 4°C to remove dead cells and other large debris.

3. Transfer cleared medium to 50-ml centrifuge tubes and centrifuge 20 min at 2,000 x g and 4°C.

4. Pipette off supernatant and transfer to appropriate tubes for the ultracentrifugation rotor to be used. (At this step it is recommended to mark one side of the centrifuge tube with a waterproof marker and orient it with the marking facing up as an aid to locating the pellet following centrifugation.) Centrifuge supernatant for 30 min at 10,000 x g and 4°C.

5. Transfer supernatant to a fresh tube, taking care to avoid disturbing the pellet. Pellet may not be visible, use marked spot as a general indicator of location and leave 0.5 cm of supernatant behind if need be.

6. Centrifuge for at least 70 minutes and up to 3 hours at 100,000 x g and 4°C. (**NOTE: For this step make certain all tubes are at least ¾ full to avoid damage to tubes. If insufficient supernatant is available, top up with PBS.**)

7. Remove supernatant completely, taking care to save pellet. Wash all pellets in 1 ml PBS. At this time if multiple tubes were used, washed exosomes can be pooled. Add PBS to fill tube completely, and spin again at 100,000 x g and 4°C.

8. Remove supernatant completely,

    a. Resuspend exosomes in 50-100µl HTG Lysis Buffer.

    b. Add 1/10th volume of Proteinase K and mix well by pipetting 10 times.

    c. Incubate the mixture at 50 °C for 180 minutes.

    d. Add 25 µl Exosomes lysates to HTG Sample Plate well.

9. (Optional) Remove supernatant completely, and resuspend exosomes in 50-100µl PBS. Exosomes can be used at this time by combining with an equal volume of HTG Biofluids Lysis Buffer, or for a more concentrated preparation, pellet may be directly resuspended into regular HTG Lysis Buffer

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II. Purification of Exosomes from biofluids and other viscous sample types

Notes:

i) Biofluids may be stored in glass for up to 5 days at 4°C prior to proceeding with exosome isolation.

ii) This modified protocol relies on a dilution of the initial sample and increased centrifugation times to account for the additional viscosity in these sample types.

1. Dilute biofluid samples with an equal volume of PBS and transfer to 50-ml tubes. Spin down for 30 minutes at 2,000 x g and 4°C.
2. Transfer supernatant to ultracentrifuge tubes, leaving pellet behind, and centrifuge for 45 min at 12,000 x g and 4°C.
3. Transfer to a new tube and spin again for 2 hours at 110,000 x g and 4°C.
4. Aspirate supernatant and resuspend pellet(s) in 1 ml PBS. Ensure pellets are completely resuspended. Pool all pellets together if there is more than one, adding all pellets for a given sample to a single tube.
5. Fill tube to top with PBS to dilute exosomes, and then filter through a 0.22 µM filter to remove cellular debris. Collect filtrate in a fresh ultracentrifuge tube.
6. Spin down filtered exosomes by centrifuging for 70 min at 110,000 x g and 4°C. Remove supernatant.
7. Resuspend pellet in 1 ml PBS then add PBS to fill tube. Spin down again at 110,000 x g and 4°C to wash pellet. Remove supernatant.
8. Remove supernatant completely,
    a. Resuspend exosomes in 50-100µl HTG Lysis Buffer.
    b. Add 1/10th volume of Proteinase K and mix well by pipetting 10 times.
    c. Incubate the mixture at 50 °C for 180 minutes.
    d. Add 25 µl Exosomes lysates to HTG Sample Plate well.
9. (Optional) Remove supernatant completely and resuspend exosomes in 50-100µl PBS. Exosomes can be used at this time by combining with an equal volume of HTG Biofluids

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Lysis Buffer, or for a more concentrated preparation, pellet may be directly resuspended into regular HTG Lysis Buffer.

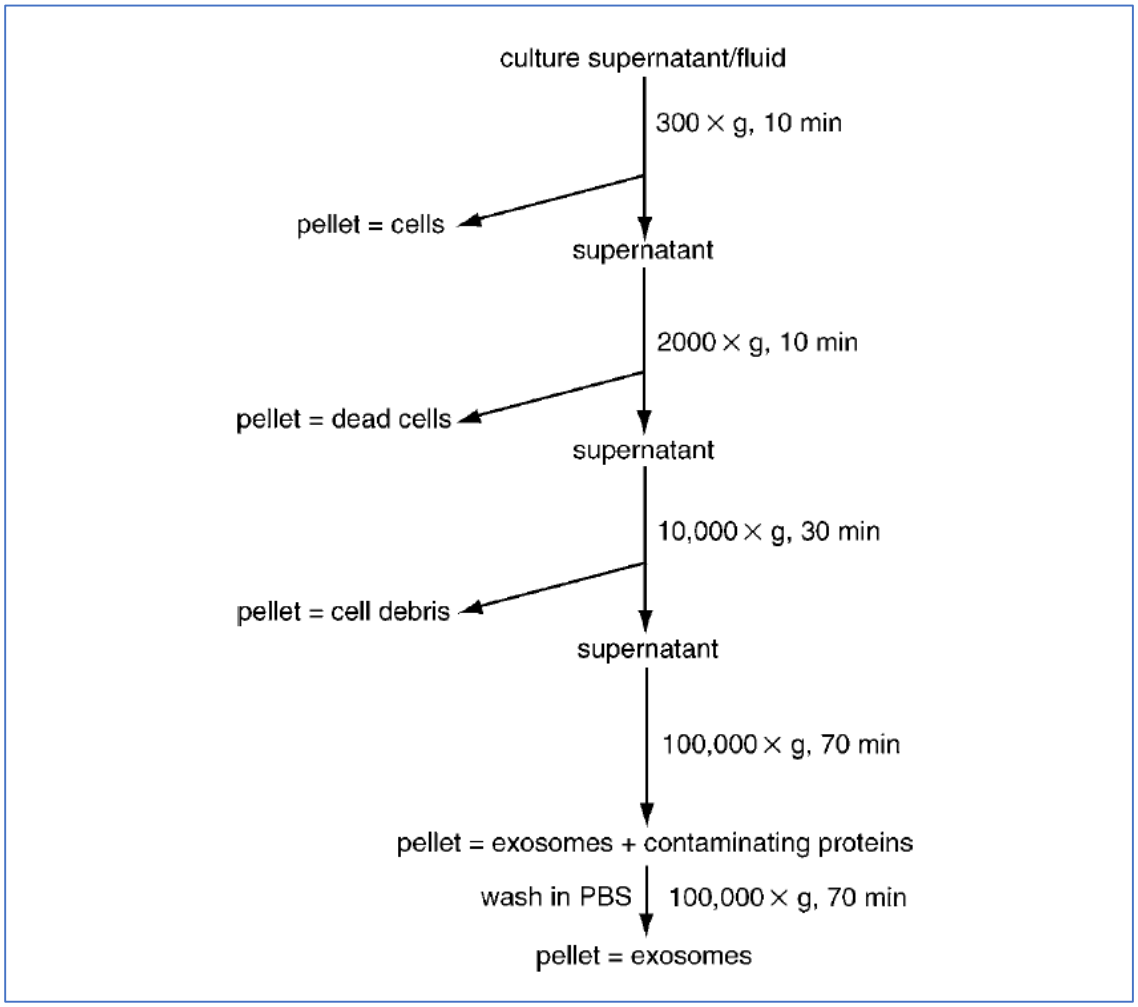

Figure 1: Flow Chart of exosome isolation by ultracentrifugation protocol (Théry *et al.* 2006).

Rotor (Beckman)	Tubes (Beckman)	Max vol/tube (ml)	Max vol/rotor (ml)	rpm for 10,000 × *g*	rpm for 12,000 × *g*	rpm for 100,000 × *g*	rpm for 110,000 × *g*
SW 41 or 40 (swinging bucket)	Polyallomer	12	72	7,500	8,200	24,000	25,000
SW 28 or 32 (swinging bucket)	Polyallomer	30	180	7,500	8,200	24,000	25,000
70 Ti	Polycarbonate bottle	22	180	10,000	11,000	31,000	32,000
45 Ti	Polycarbonate bottle	68	400	9,000	10,000	30,000	31,000
TLA-100.3	Thick-walled	3	18	13,000	15,000	43,000	45,000
TLA-110	polycarbonate	5	40				

Table 1: Ultracentrifuge rotor and speed/ x *g* settings (Théry *et al*. 2006).

III. Other Purification Methods

There are numerous additional protocols for exosome purification including density gradient separation, immunoaffinity, and others which are cited but not discussed here (10,11, 12, 13, 14). These offer their own potential advantages and disadvantages and they may be worthy of consideration as well (15).

More recently, a novel protocol for extracellular vesicle and exosome labeling and capture by nanoscale flow cytometry has also been reported (16). HTG has also had customers report success with several commercial kits, including Thermo Fisher's Total Exosome Isolation kits and Cell Guidance Systems' Exo-Spin™ Exosome Purification Kit. Information on these and other kits may be found online.

Citations

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