Chapter 29

Isolation and Characterization of Exosome from Human Embryonic Stem Cell-Derived C-Myc-Immortalized Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSC) are currently the cell type of choice in many cell therapy trials. The number of therapeutic applications for MSCs registered as product IND submissions with the FDA and initiation of registered clinical trials has increased substantially in recent years, in particular between 2006 and 2012. However, defined mechanisms of action underpinning the therapeutic efficacy of MSCs are lacking, but they are increasingly attributed to MSC trophic secretion rather than their differentiation potential. A promising secreted therapeutic candidate is an extracellular vesicle (EV) known as the exosome. The use of exosomes instead of cells as a therapeutic agent provides several advantages. A critical advantage is the prospect of a conventional pharmaceutical manufacturing process that is highly scalable and amenable to the stringent manufacturing process. For example, MSCs used as producers of therapeutics, and not as therapeutics per se, could be immortalized to generate infinitely expansible clonal lines to enhance the reproducible production of therapeutic exosomes. In this chapter, we will describe the immortalization of MSCs, and the production, isolation, and characterization of exosomes from immortalized MSC.

Key words Mesenchymal stem cells, Immortalization, Exosome

1 Introduction

Mesenchymal stem cells (MSCs) are currently the most used cell type in the field of cell therapy. Between 2006 and 2012, there was a threefold increase in MSC-based product FDA IND submissions [1]. There were also 246 MSC-based clinical trials registered in this same period. These cells are being tested for a wide range of clinical indications with the most common ones being cardiovascular and neurological diseases. Despite the intensive clinical testing of MSCs, the mechanism of action responsible for their therapeutic efficacy remains nebulous. The initial rationale was based on the differentiation potential of MSCs and has become increasingly redundant, particularly in light of new insights from clinical and

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animal studies [2]. Recent demonstrations that MSCs elicit their therapeutic effects through secreted factors have radically revolutionized the field [3–12].

Paracrine secretion by MSCs was first described almost two decades ago when Haynesworth et al. [13] reported that MSCs synthesize and secrete a broad spectrum of growth factors, chemokines and cytokines, that exert significant effects on cells in their vicinity. This was followed by numerous reports that these secreted factors enhance arteriogenesis [14], protect against ischemic renal and limb tissue injury [15], promote neovascularization [16], and increase angiogenesis [17, 18]. Gnecchi et al. demonstrated that intramyocardial injection of culture medium conditioned by MSCs overexpressing the Akt gene reduced infarct size in a rodent model of AMI to the same extent as the Akt-MSC cells themselves [3]. Our group subsequently demonstrated that human embryonic stem cell-derived MSCs (hESC-MSC) secrete more than 200 unique gene products [19] into the culture medium and this conditioned culture medium reduces reperfusion injury in a pig model of myocardial ischemia/reperfusion [6]. Fractionation studies of the conditioned medium led to the identification of exosomes as the active therapeutic agent in the MSC secretome [7]. Since then MSC exosomes have been implicated in the therapeutic efficacy of MSCs against graft-versus-host disease [20, 21], cerebral ischemia [22], liver fibrosis [23], hypoxic pulmonary hypertension [24], acute kidney injury [25], and acute liver injury [26].

The exosome is an extracellular vesicle with a diameter of 50–100 nm, which can be differentiated from other extracellular vesicles through several biophysical and biochemical parameters, e.g., flotation density of 1.1–1.19 g/mL, the presence of tetraspanin proteins, Alix, and TSG101 as summarized by Thery et al. [27]. Exosomes were first reported to be secreted by sheep reticulocytes in 1983 as a means to discard unwanted protein [28]. Exosomes are now known to be secreted by many cell types and to exert a wide spectrum of functional activities that have been implicated in both therapeutic and pathological processes [2]. The discovery that the cardioprotective activity of MSCs was mediated by exosomes introduced a new perspective to current MSC stem cell-based therapies, and engenders novel approaches in the development of cell-free tissue repair.

Exosome-based therapy offers tremendous advantages over cell-based therapy. It is nonviable; therefore it is safer, easier to store, transport, and administer (*see review* [2]). However, MSCs have limited expansion capability. In the long term, large-scale production of MSC exosomes could be sustained only by constant replenishment with new sources of MSCs either from new donors or, in the case of hES-MSC, repeated derivation from hESC. Such replenishment is not only costly as each new source will have to be

tested and validated, but also the batch quality and reproducibility of the exosome production could also be compromised.

We had previously proposed MYC immortalization of hESC-MSC to generate infinitely expansible clonal cell lines for exosome production as a means to overcome the obstacle of limited cell supply [29]. This approach virtually ensures that MSC exosomes will be generated from the same MSC source and therefore, minimize batch-to-batch variation. Here we provide detailed protocols on how to immortalize MSCs, produce, purify, and characterize their exosomes, thus enabling researchers to produce sufficient MSC exosomes to evaluate their therapeutic efficacy.

2 Materials

2.1 Generation of Lentivirus Particles Carrying MYC

1. DMEM High Glucose.
2. OPTI-MEM I.
3. Sodium pyruvate.
4. MEM Non-essential amino acids (MEM NEAA).
5. Penicillin-Streptomycin-Glutamine (PSG).
6. Fetal bovine serum, ESC qualified (FBS).
7. Phosphate buffered saline, pH 7.4 (PBS).
8. Trypsin, 0.05 % EDTA.
9. Lipofectamine.
10. 0.1 % Gelatin in water.
11. HEK293T cells.
12. Plasmids: pMDLg/pRRE, pCMV-VSV-G, pRSV-Rev, pLVX-MYC-puro.
13. Amicon Ultra Centrifugal Filters Ultracel 100 K.
14. Corning T175 flasks.
15. 0.45 μm Minisart Syringe Filter.
16. 50 mL Precise Syringe.

2.2 MYC Immortalization of Human Embryonic Stem Cell-Derived Mesenchymal Stem Cells

1. DMEM High Glucose.
2. Sodium pyruvate.
3. MEM Non-essential amino acids (MEM NEAA).
4. Penicillin-Streptomycin-Glutamine (PSG).
5. Fetal bovine serum, ESC qualified (FBS).
6. Phosphate buffered saline, pH 7.4 (PBS).
7. Trypsin, 0.05 % EDTA.
8. Hexadimethrine bromide (Polybrene).

9. Puromycin dihydrochloride.
10. 0.1 % Gelatin in water.
11. Dimethyl sulfoxide (DMSO).
12. HuES9.E1 cells.
13. *MYC* lentivirus particles.
14. Corning T25, T75, 175 flasks.
15. 6, 12, 48 Well Cell Culture Cluster.
16. Pyrex cloning cylinder.

2.3 Mesenchymal Stem Cell Expansion

1. hES-MSC Medium: DMEM supplemented with 10 % FBS, 1× PSG, 1 mM sodium pyruvate, 1× MEM NEAA.
2. Dulbecco's Phosphate Buffered Saline without Calcium and Magnesium Chloride (PBS-).
3. Serum-Free Medium: DMEM without phenol red supplemented with 1× insulin, transferrin, and selenium (ITS-X), 5 ng/mL FGF2, 5 ng/mL PDGFAB, and 1× β-mercaptoethanol, 1× NEAA, 1× l Glutamine, 1 mM Sodium pyruvate.
4. Virus collection medium: DMEM supplemented with 1 % FBS, 1× PSG, 1 mM sodium pyruvate, 1× MEM NEAA.
5. Trypsin, 0.05 % EDTA.
6. 0.1 % Gelatin in Water.
7. Cell Stack.

2.4 HPLC Isolation of Mesenchymal Stem Cell Exosome

1. 1× Phosphate buffered saline (PBS) for tangential flow filtration (TFF): 137 mM sodium chloride, 2.7 mM potassium chloride, 1.8 mM potassium phosphate monobasic, 10 mM disodium hydrogen phosphate, pH 7.4.
2. Size exclusion chromatography (SEC) buffer preparation:
   (a) Measure 5.52 g sodium dihydrogen phosphate (Mr = 137.99) and 11.94 g sodium chloride (Mr = 58.44) separately into a weighing boat.
   (b) Transfer and dissolve all salts into a 2000 mL volumetric flask containing ~1900 mL ultrapure water. Mix well.
   (c) Adjust the pH to 7.2 using ~7 mL of 4 M NaOH and top up to the final volume of 2000 mL using ultrapure water.
   (d) Remove particulate matter by vacuum filtration through a 0.1 μm 47 mm PESU membrane filter (Sartorius) into a clean, labeled 2000 mL glass bottle.
   (e) Degas the buffer in a sonicator for 5 min.
3. Sartoflow Slice 200 Benchtop crossflow filtration system.
4. ÄKTA explorer 100 system with UNICORN software.
5. Shimadzu HPLC system with Class VP and ASTRA V (for Light Scattering Detector) software.

6. Multi-Angle Light Scattering (MALS) Detector.
7. Quasi Elastic Light Scattering (QELS) Detector.
8. Refractive Index (RI) Detector.
9. ≥99.5 % Sodium chloride.
10. >99 % Sodium dihydrogen phosphate.
11. ≥98 % Sodium hydroxide.
12. ≥99% Disodium hydrogen phosphate.
13. Ultrapure water (18.2 MΩ).
14. ≥99.5% Potassium chloride.
15. ≥99.5 % Potassium phosphate monobasic.
16. Custom Size Exclusion Chromatography Column (TSKgel G3000SW; 13 μm, 600 mm × 7.5 mm, Tosoh).
17. HPLC Size Exclusion Chromatography Column (TSKgel G3000SWxl; 5 μm, 300 mm × 7.8 mm, Tosoh).
18. PESU membrane filter; 0.1 μm.
19. Minisart® NML Syringe Filters; 0.2 μm.
20. Vivaspin 20; 30,000 kDa MWCO PES.
21. Hydrosart® ultrafiltration membrane; 100 kDa MWCO, 0.1 m^2.

2.5 Exosome Protein Quantification

1. 96 Well flat bottom transparent microplate.
2. Bio-rad Protein Assay Kit.

2.6 Sucrose Density Gradient Assay

1. 1 M Tris–HCl, pH 7.4.
2. Sucrose.
3. 4 mL ultracentrifuge tube.
4. SW60Ti rotor.

2.7 NanoSight

1. NanoSight LM10.
2. NanoSight syringe pump.
3. NTA 2.3 analytical software.
4. 0.22 μm syringe filter.

3 Methods

3.1 Generation of Lentivirus Particles Carrying MYC

3.1.1 Lentivirus Production

1. Coat the entire surface of a T175 flask with 5 mL of gelatin for 20 min at room temperature (RT) and aspirate to dryness.
2. *Day 1*: Seed 1.2×10^7 HEK293T cells in the coated T175 flask with 35 mL hES-MSC medium. Incubate at 37 °C, 5 % CO_2 overnight.
3. *Day 2*: Prepare two tubes of OPTI-MEM I, 8 mL per tube

Label tube 1 as "Plasmids" and add the following and vortex mix.

(a) pMDLg/pRRE 27 μg.

(b) pCMV-VSV-G 9 μg.

(c) pRSV-Rev 9 μg.

(d) pLVX-MYC-puro 27 μg.

Label tube 2 as "Lipofectamine" and add 108 μL lipofectamine, invert gently, and incubate the mixture at RT for 5 min.

4. Add tube 1 to tube 2, invert gently. Incubate at RT for 20 min.
5. Flask seeded with HEK293T cells should be 80–90 % confluent. Remove spent medium and wash cells twice with PBS.
6. Add the contents of the combined tubes 1 and 2 gently onto the cells, pour evenly across entire flask surface. Incubate the flask at 37 °C, 5 % CO_2 for 6 h.
7. Remove transfection medium, replace with 24 mL virus collection medium. Run medium down the walls of the dish slowly to prevent cells from dislodging.
8. *Days 4 and 5* (*see* **Note 1**): 48 h after transfection, collect the first harvest of 24 mL virus-rich medium. Replace with fresh 24 mL virus collection medium.
9. Centrifuge virus-rich medium at 300×*g* for 10 min at 4 °C, filter supernatant through a 0.45 μm syringe filter. Store at 4 °C.
10. 72 h after transfection, collect the second harvest of 24 mL virus-rich medium.
11. Centrifuge virus-rich medium at 300×*g* for 10 min at 4 °C, filter supernatant through a 0.45 μm syringe filter. Combine both harvests to obtain 48 mL of virus-rich medium.
12. Prewet 100 kDa Amicon Ultra Centrifugal Filters by adding 10 mL PBS and centrifuge at 4000×*g* for 1 min.
13. Remove filtrate, load filter with 10 mL virus-rich medium, centrifuge at 4000×*g* for 15 min. Repeat this process until the 48 mL of medium is reduced to 600 μL.
14. Split virus concentrate into 60 μL aliquots. Use immediately for infection or store at −80 °C.
15. Determine virus concentration using the Lenti-X™ qRT-PCR Titration Kit according to the manufacturer's instructions. Viral titer should be at least 10^{11} copies per mL.

3.1.2 MYC Immortalization of hES-MSC

1. *Day 1* Target cell infection:

(a) Coat the surface of a 6-well plate with gelatin for 20 min at RT, aspirate and allow to dry.

(b) Seed HuES9.E1 cells at 2.5×10^5 cells per well in hES-MSC medium, incubate at 37 °C, 5 % CO_2.

2. *Day 2:*

(a) 24 h after seeding HuES9.E1 cells, wells should be 70–80 % confluent.

(b) If using a frozen virus aliquot, remove a vial of virus from −80 °C and thaw on ice. Calculate the volume of virus concentrate required to infect HuES9.E1 cells at an MOI of 5. Add this to 1.5 mL of hES-MSC medium.

(c) To this, add 1.5 μL of polybrene (4 mg/mL in water) and mix by pipetting (*see* **Note 2**).

(d) Remove spent medium from HuES9.E1 cells, add virus-containing medium drop by drop distributing the medium evenly over the cell culture.

(e) Use one well of cells as an uninfected control. Replace the medium with 1.5 mL of fresh medium containing 1.5 μL of polybrene.

(f) Incubate at 37 °C, 5 % CO_2 overnight.

3. *Day 3*: Transfer cells from each of the wells into T75 flasks. Incubate in fresh hES-MSC medium at 37 °C, 5 % CO_2 for 24 h.

4. Antibiotic selection and population expansion.

5. Prepare selection medium by diluting 10 mg/mL puromycin in hES-MSC medium to obtain 1 μg/mL concentration.

6. Replace spent medium with selection medium for both infected and uninfected cells.

7. Change selection medium once every 2 days until complete cell death is observed with the uninfected cells.

8. Puromycin-resistant MYC-transfected HuES9.E1 cells (E1MYC) should be observable after 3–4 days of selection. Replace selection medium with fresh medium.

9. Expand the population from a T75 flask to a T175 flask. Freeze cells in freezing medium (10 % DMSO, 20 % FBS, and 70 % hES-MSC) at −80 °C overnight. Transfer cells to −150 °C thereafter.

3.1.3 Clonal Selection

1. Seed 10^4 cells in a gelatin-coated T175 flask.

2. Once visible, round, colonies have formed, isolate colonies by trypsinization in cloning cylinders. Transfer each colony to a single well in a 48-well plate.

3. Expand colonies from a 12-well to 48-well plate, to T25 and finally to T175 flasks. Label cells as P1 and freeze cells as per (Subheading 3.1.2, **step 9** above).

3.2 Passaging hES-MSCs

1. Upon reaching 80 % confluence, passage E1MYCs at 1:3 or 1:4 split ratio (*see* **Note 3**).
2. Aspirate spent medium from T175 flask and rinse cells with 10 mL PBS (−).
3. Aspirate PBS (−), add 3.0 mL Trypsin per T175 flask, and rotate flask gently to ensure liquid covers the entire cell surface.
4. Return flask to the incubator, for 3–5 min.
5. Remove flask and gently tap sides to dislodge cells.
6. Add 6–7 mL of hES-MSC media to neutralize Trypsin, and gently wash the sides of the flask.
7. Transfer cell suspension into 15 mL tube. Wash flask again with 3–5 mL media.
8. Pool cell suspension, and centrifuge at 800 × *g* for 5 min.
9. Aspirate supernatant, resuspend pellet in 4 mL hES-MSC media and then transfer to a new T175 flask with 30 mL fresh hES-MSC media.
10. Replace spent medium with fresh medium every 2 days.
11. It is always recommended to maintain E1MYC between 25 and 80 % confluence or ~15–50,000 cells per cm^2.

3.3 Expansion of E1MYC Using a Cell Stack

1. Start this procedure with ten T175 flasks of 90–100 % confluent E1MYC.
2. Gelatinize the cell stack (500 mL gelatin/stack) for 30 min.
3. Warm hES-MSC medium and Trypsin in a water bath for at least 20 min.
4. Aspirate spent medium from flask and wash once with 10 mL PBS (−).
5. Aspirate PBS (−), add 3.0 mL Trypsin per T175 flask, and rotate flask gently to ensure liquid covers the entire cell surface.
6. Return flasks to the incubator for 3–5 min.
7. Remove flasks and gently tap sides to dislodge cells.
8. Add 6–7 mL of media to neutralize Trypsin and gently wash the sides of the flask.
9. Transfer cell suspension into a 15 mL tube. Wash flask again with 3–5 mL of medium.
10. Pool cell suspension and centrifuge at 800 × *g* for 5 min.
11. Aspirate the supernatant and resuspend the pellet in 4 mL hES-MSC medium.
12. Pool pellets (from 10× T175 cm^2 flasks) into 50 mL hES-MSC medium and then transfer to 1 L growth medium.

13. Pour out gelatin from cell stack.
14. Add 1 L medium with cells into the cell stack. Distribute evenly.
15. Transfer the cell stack to 37 °C incubator and check for adherence 24 h later.

3.4 Conditioning of E1MYC

1. Start this procedure when E1MYC are 80 % confluent in a cell stack.
2. Pour out spent medium and wash once with 500 mL PBS (+) per cell stack.
3. Add 1 L serum-free medium (*see* Subheading 2.3, **item 3**) per stack.
4. 24 h later, pour out medium and wash once with 500 mL PBS (+).
5. Add 1 L serum-free medium per cell stack.
6. Return the cell stack to incubator for 72 h.
7. Carefully pour out conditioned medium into a sterile bottle.
8. Pass through a 0.22 μm filter before further processing.

3.5 HPLC Purification of Mesenchymal Stem Cell Exosomes

3.5.1 Tangential Flow Filtration

1. Clamp the ultrafiltration membrane to the holding device at a torque of 20 Nm as per the manufacturer's recommendations.
2. Connect the above assembly to a peristaltic pump, pressure sensors at the feed, attach retentate and filtrate streams to the corresponding tubing.
3. Flush the cassette with warm 1 M sodium hydroxide for 30 min at a crossflow rate of 8 L/(min m^2) with both the retentate and filtrate valves fully open to sanitize it.
4. Measure the clean water flux of the sanitized cassette as a reference for the performance of a clean cassette. At a transmembrane pressure (TMP) of 0.8 bar, the filtrate flux is 4 L/(min m^2).
5. Flush the cassette with 1× PBS to equilibrate the pH of the cassette. Equilibration is complete when the filtrate measures pH 7.4.
6. Perform ultrafiltration of the exosome-containing conditioned medium at TMP = 0.8–1.0 bar, with a filtrate flux of 2–1.5 L/(min m^2). Reduce sample volume from 5000 mL to around 100 mL including dead volume.
7. If desired, change the retentate to 1× PBS with 5–10× volume.
8. Pour out the retentate in the reservoir and drain it from the tubing and cassette into a clean bottle.

9. Flush the cassette with 1× PBS to remove the majority of the proteins in the tubing and cassette.
10. Next, flush the cassette with warm (50 °C) 1 M sodium hydroxide for 1 h, according to the manufacturer's recommendations. Fully open both retentate and filtrate valves.
11. Measure the clean water flux again at TMP = 0.8 bar. The cassette is considered clean if the measured clean water flux is between 70 and 110 % of that measured in **step 4**. The cassette may still be dirty and require further cleaning if the clean water flux is less than that before use, or its integrity may be compromised if the clean water flux exceeds that before use.
12. Disassemble the filter assembly and store the cassette in 0.1 M sodium hydroxide. Wash the holding device with water. Wash the tubing and reservoir with soapy water.

3.5.2 Size Exclusion Chromatography and Concentration

1. Connect the custom Size Exclusion Column to the AKTA Explorer.
2. Prime the AKTA Explorer using SEC buffer and then equilibrate the column for 30 min at a flow rate of 4 mL/min.
3. Connect one outlet to a GE Fraction Collector Frac-950 for collecting the purified sample at 4 °C.
4. Using a 3 mL syringe, inject 1.5 mL of sample into the sample loop.
5. The program used for size exclusion chromatography can be found in Appendix. Table 1 summarizes the ATKA run conditions.
6. The first peak, P1, at the detection wavelength of UV 280 nm will correspond to the purified exosome (*see* Fig. 1).
7. Using the fraction collector, collect P1 in 0.5 mL fractions.
8. Pool these fractions into one tube. Mix well.
9. Use a Vivaspin 20 to concentrate the pool by 20×.

Table 1
AKTA run conditions

Column	TSKgel G3000SW (13 μm), 600 mm × 7.5 mm
Column temperature	25 °C
Fraction collector temperature	4 °C
Flow rate	1 mL/min
Injection volume	1.5 mL
Fraction volume	0.5 mL
Run time	50 min

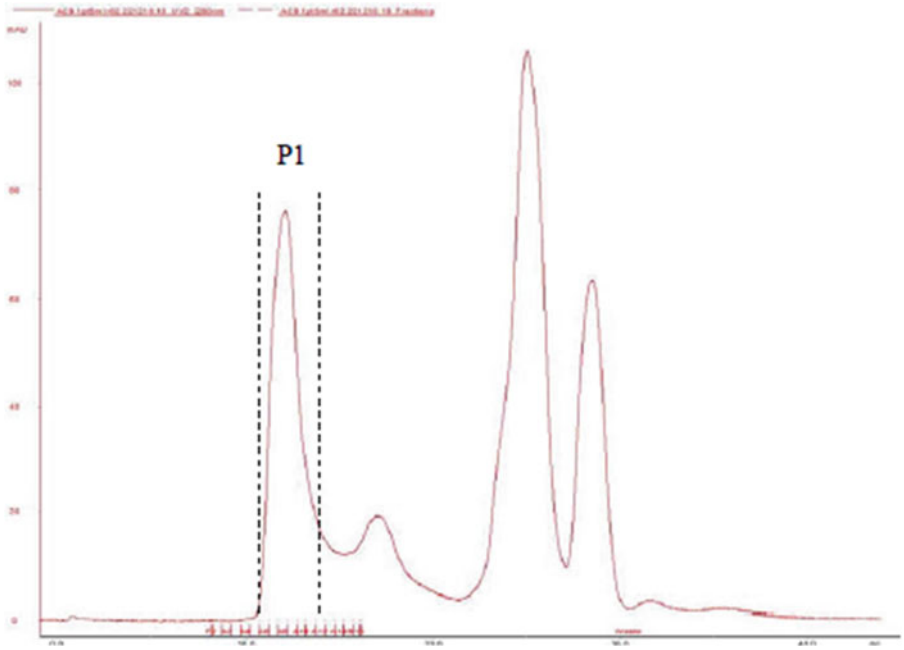

Fig. 1 Typical AKTA chromatogram of exosome purification

3.5.3 Analysis of Purified Exosomes

1. Connect the HPLC Size Exclusion Column to the Shimadzu HPLC.
2. Prime the HPLC using SEC buffer and then equilibrate the column for 30 min at a flow rate of 0.5 mL/min.
3. Inject 20 μL of 20× concentrated and purified exosomes into the column.
4. Follow Table 2 for HPLC run conditions: The elution time for exosomes is ~12 min at the detection wavelength of UV 220 nm (*see* Fig. 2).
5. Upon completion of the run, ASTRA V software is used to determine the hydrodynamic radius, Rh, of the purified exosomes. The Rh of exosomes normally ranges between 50 and 60 nm.

3.6 Exosome Protein Quantification

3.6.1 BSA Standard Preparation

1. Prepare 32 μg/mL BSA standard by adding 8 μL 2 mg/mL BSA to 492 μL distilled water in a 1.5 mL microcentrifuge tube.
2. Serially dilute 220 μL of 32 μg/mL BSA with 220 μL distilled water to generate 16, 8, 4, and 2 μg/mL BSA standards in 1.5 mL microcentrifuge tubes.
3. Aliquot 220 μL distilled water in a 1.5 mL microcentrifuge tube as the blank control.

Table 2
HPLC run conditions

Column	TSKgel G3000SWxl 300 mm × 7.8 mm
Column temperature	25 °C
Flow rate	0.5 mL/min
Injection volume	20 µL
Run time	40 min
No. of replicate injections	2
No. of blank runs as negative control between samples	1

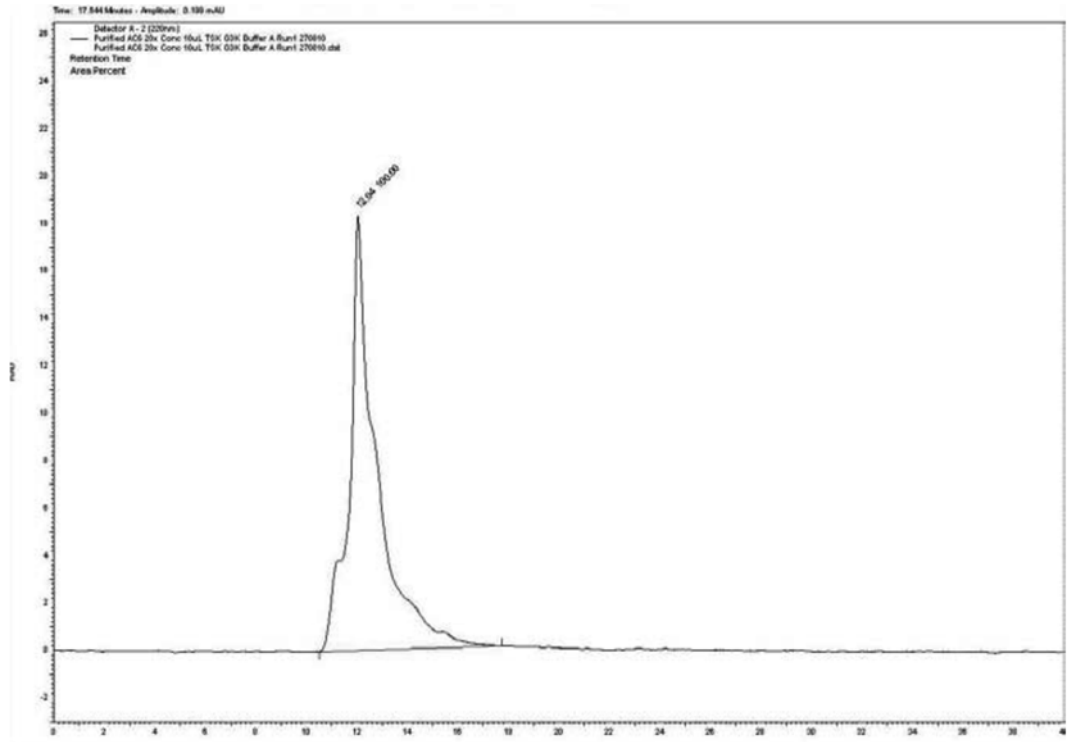

Fig. 2 Typical HPLC chromatogram of purified exosomes

4. Transfer 200 µL of standards and blank control to a 96 well flat bottom transparent microplate.

3.6.2 Sample Preparation

1. Dilute exosome sample 10× by adding 20 µL exosome to 180 µL distilled water.

2. Add 10, 20, 30 μL of the diluted exosome sample to 96 well flat bottom transparent microplate. Add distilled water to a final volume of 200 μL for each well. Prepare each well of the diluted exosome sample in triplicate.

3.6.3 Absorbance Measurement

1. Add 50 μL 1× dye reagent to each well. Mix well.
2. Measure absorbance of each well at 595 nm using a microplate reader.

3.6.4 Protein Concentration Calculation

1. Subtract the absorbance value of the blank from that of each standard and sample. The net absorbance of each standard is plotted against its known protein concentration to generate a standard curve. Set the X- and Y-axis intercept to 0 and plot the best-fit linear graph. The linear regression coefficient, r^2, must be >0.95. Otherwise, the assay should be repeated.
2. Calculate the sample protein concentration (x) by the following equation:

$$x = \frac{v \times d}{m}$$

where v is the net absorbance value, d is the dilution factor (if 10 μL of diluted exosomes was used, the dilution factor is $200/1 = 200$), and m is the slope of the standard curve.

3.7 Sucrose Density Gradient Assay

1. Prepare 50 mL of 20 mM Tris–HCl, pH 7.4 by diluting 1 mL of 1 M Tris–HCl, pH 7.4–50 mL with distilled water.
2. Weigh 2 g of sucrose and put in a 15 mL tube labeled as Tube A and 6 g of sucrose into a 15 mL tube labeled as Tube B.
3. Dissolve the sucrose in Tube A and Tube B with 7 mL of 20 mM Tris–HCl, pH 7.4 before slowly topping to 10 mL with the same buffer (*see* **Note 4**).
4. Filter the sucrose solutions using a 0.22 μm syringe filter.
5. Aliquot sucrose solution from Tube A and Tube B into each of 14 labeled 1.5 mL microcentrifuge tubes as shown in Table 3.
6. Vortex each of the 14 labeled 1.5 mL microcentrifuge tubes to mix the two sucrose solutions.
7. Prepare the sucrose gradient in a 4 mL ultracentrifuge tube by first loading 0.45 mL sucrose solution from tube 1 before layering 0.25 mL sequentially from tubes 2–14. Repeat to prepare a second sucrose density gradient (*see* **Note 5**).
8. Load 0.5 mL exosome sample on top of the gradient. Sample can be diluted with 20 mM Tris–HCl, pH 7.4 if the volume is less than 0.5 mL.
9. Carefully load the ultracentrifuge tube in a SW60 Ti rotor.

Table 3
Preparation of sucrose solution

Label	Tube A (mL)	Tube B (mL)
1	1	0.00
2	0.93	0.07
3	0.86	0.14
4	0.79	0.21
5	0.72	0.28
6	0.65	0.35
7	0.57	0.43
8	0.5	0.5
9	0.43	0.57
10	0.36	0.64
11	0.29	0.71
12	0.22	0.78
13	0.15	0.85
14	0.07	0.93

10. Spin overnight (≥16.5 h) 200,000 × *g* at 4 °C using slow acceleration. Deceleration should be slow or preferably without the brake.
11. After ultracentrifugation, carefully take out the ultracentrifuge tube from the rotor.
12. Carefully pipette 320 μL from the top of the ultracentrifuge tube into preweighed 1.5 mL Eppendorf tubes to collect 13 fractions (*see* **Note 6**).
13. Weigh each tube. After subtracting the weight of the tube, the density of each fraction is calculated using the following equation:

$$\text{density} = \frac{\text{mass}}{\text{volume}}$$

14. Take an identical aliquot volume from each of the 13 tubes containing the sucrose gradient. Detect the presence of exosomes markers (e.g., CD9) in each fraction by western blot hybridization. The density ranges of the sucrose where

exosome markers are detected represent the density range of the exosomes.

15. Figure 3 gives an overview of the whole assay.

3.8 Nanosight

1. Calibrate the NanoSight LM10 using 100 nm polystyrene beads as per the manufacturer's instruction before measuring the size distribution of the exosome sample.
2. Dilute the purified exosome to a particle number concentration of between 10^8 and 10^9 particles per mL (*see* **Note 7**).
3. Open the NTA 2.3 Analytical Software, navigate to the capture screen, select basic mode, and set the camera level to 10.
4. Aspirate the diluted exosome with a 1 mL syringe and slowly inject the sample into the viewing unit device (*see* **Note 8**).
5. Hold the syringe in place by loading it into the syringe holder of the NanoSight Syringe Pump.
6. Set the infusion rate to 1000, click "load," after 10 s change the infusion rate to 20.
7. Using the script control function, set 3× 60 s recording time and click "run."

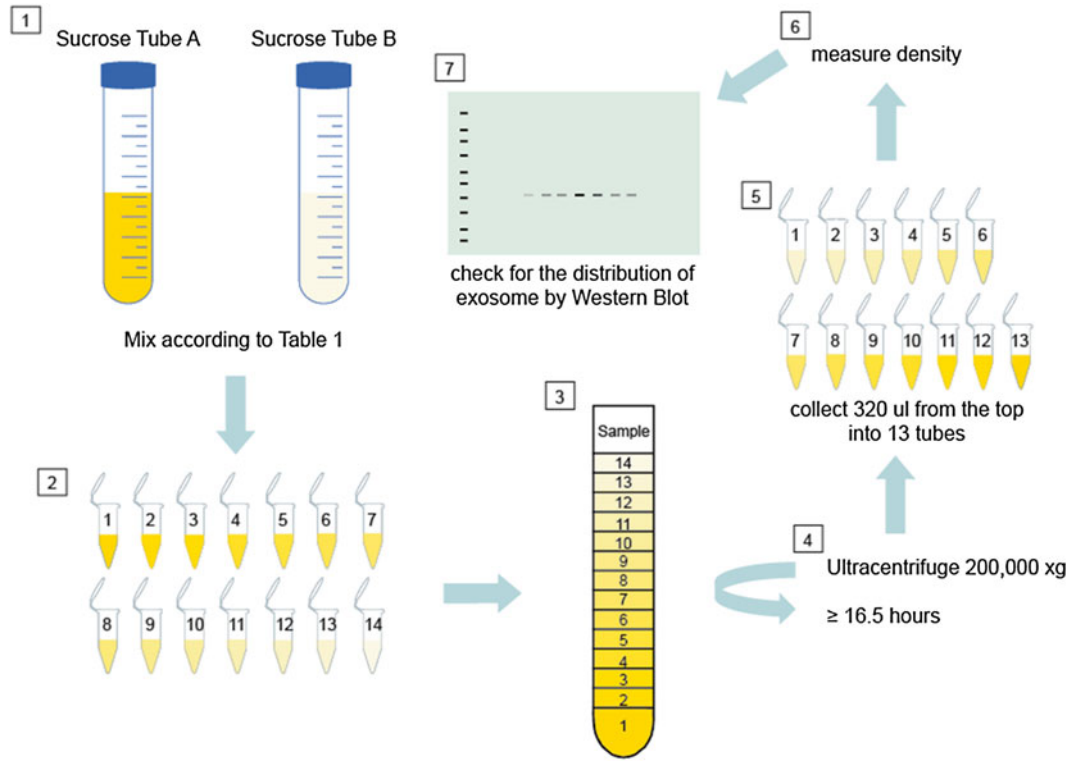

Fig. 3 Flow chart of the sucrose density gradient assay

8. Make sure that 20–100 particles can be seen in the field of view; if not, dilute or concentrate the sample accordingly.
9. After the recording, analyze the recording according to the user's manual to estimate the size distribution.

4 Notes

1. When handling live virus, wear proper personal protective equipment and soak all used disposable culture ware, tubes, serological pipettes in bleach solution for at least one hour before disposing as per regular biological waste.
2. This virus-containing medium is enough for 1 well (in a 6 well plate) only. Prepare more according to the user's needs.
3. It is recommended to always maintain hESC-derived MSCs between 25 and 80 % confluency or ~15–50,000 cells per cm^2. Upon reaching 80 % confluence, it is recommended to passage hES-MSCs at a 1:3 or 1:4 split ratio.
4. It takes about 10 min to fully dissolve the sucrose.
5. Cut 5 mm off the pipette tip before using it. After loading each layer of sucrose gradient, the interface between the layers should be visible.
6. Cut 5 mm off the pipette tip before using it. Aspirate the sample slowly and steadily.
7. 1000–10,000× dilution with PBS will be sufficient to achieve the concentration.
8. Care should be taken to avoid the introduction of bubbles at this stage.

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5 Appendix: Purification Method

Main method:

¤ (Main)
0.00 Base CV (26.5)#Column_volume [31] Any
0.00 Flow 1 {mL/min}
0.00 Watch_Pressure Greater_Than 2.9 [32] PAUSE
0.00 BufferValveA1 A12
0.00 ColumnPosition Position3

0.00 Alarm_Pressure Enabled 2.9 [32] 0.00 [32]
0.00 Wavelength 220 {nm} 280 {nm} 260 {nm}
¤ 0.00 Block Equilibration
(Equilibration)
0.00 Base SameAsMain
0.5 AutoZeroUV
0.50 End_Block
¤ 0.00 Block Sample_injection
(Sample_injection)
0.00 Base Volume
0.00 InjectionValve Inject
1.5 InjectionValve (Load)#Load_volume
1.50 End_Block
¤ 0.00 Block Fractionation
(Fractionation)
0.00 Base SameAsMain
0.25 OutletValve F2
0.25 Fractionation 12 mm 0.5 [31] FirstTube Volume
0.55 FractionationStop
0.55 OutletValve WasteF1
1.10 End_Block
¤ 0.00 Block Reequilibration
(Reequilibration)
0.00 Base SameAsMain
0.5 End_Block
0.00 End_method

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