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Isolation and Characterization of Exosomes from Skeletal Muscle Fibroblasts

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Corresponding Author:	Alessandra d'Azzo St. Jude Children's Research Hospital Memphis, TN UNITED STATES
Corresponding Author's Institution:	St. Jude Children's Research Hospital
Corresponding Author E-Mail:	sandra.dazzo@stjude.org
Order of Authors:	Diantha van de Vlekkert
	Xiaohui Qiu
	Ida Annunziata
	Alessandra d'Azzo
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TITLE:

Isolation and Characterization of Exosomes from Skeletal Muscle Fibroblasts

AUTHORS & AFFILIATIONS:

Diantha van de Vlekkert, Xiaohui Qiu, Ida Annunziata, Alessandra d'Azzo

Department of Genetics, St. Jude Children's Research Hospital, Memphis, TN, USA

Corresponding Author:

Alessandra d'Azzo (sandra.dazzo@stjude.org)

Email Addresses of Co-authors:

Diantha van de Vlekkert (diantha.vandevlekkert@stjude.org)

Xiaohui Qiu (xiaohui.qiu@stjude.org)

Ida Annunziata (ida.annunziata@stjude.org)

KEYWORDS:

skeletal muscle, primary fibroblasts, exosomes, ultracentrifugation, sucrose density gradient, western blot

SUMMARY:

This protocol illustrates the 1) the isolation and culture of primary fibroblasts from the adult mouse gastrocnemius muscle as well as 2) purification and characterization of exosomes using a differential ultracentrifugation method combined with sucrose density gradients followed by western blot analyses.

ABSTRACT:

Exosomes are small extracellular vesicles released by virtually all cells and secreted in all biological fluids. Many methods have been developed for the isolation of these vesicles, including ultracentrifugation, ultrafiltration, and size exclusion chromatography. However, not all are suitable for large scale exosome purification and characterization. Outlined here is a protocol for establishing cultures of primary fibroblasts isolated from adult mouse skeletal muscles, followed by purification and characterization of exosomes from the culture media of these cells. The method is based on the use of sequential centrifugation steps followed by sucrose density gradients. Purity of the exosomal preparations is then validated by western blot analyses using a battery of canonical markers (i.e., Alix, CD9, and CD81). The protocol describes how to isolate and concentrate bioactive exosomes for electron microscopy, mass spectrometry, and uptake experiments for functional studies. It can easily be scaled up or down and adapted for exosome isolation from different cell types, tissues, and biological fluids.

INTRODUCTION:

Exosomes are heterogeneous extracellular vesicles ranging in size from 30–150 nm. They are established key players in physiological and pathological processes, given their ubiquitous distribution in tissues and organs[1,2]. Exosomes carry a complex cargo of proteins, lipids, DNA

types, and RNA types, which vary according to the type of cells from which they are derived[1-3]. Exosomes are enriched in proteins that have different functions (i.e., tetraspanins, including CD9 and CD63) are responsible for fusion events. For example, heat shock proteins HSP70 and HSP90 are involved in antigen binding and presentation. Additionally, Alix, Tsg101, and flotillin participate in exosome biogenesis and release and are widely used as markers of these nanovesicles[2-4].

Exosomes also contain a variety of RNAs (i.e., microRNAs, long noncoding RNAs, ribosomal RNAs) that can be transferred to recipient cells, where they influence downstream signaling[3]. Being enclosed by a single unit membrane, exosome bioactivity depends not only on the cargo of proteins and nucleic acids, but also on lipid components of the limiting membrane[1]. Exosomal membranes are enriched in phosphatidylserine, phosphatidic acid, cholesterol, sphingomyelin, arachidonic acid, and other fatty acids, all of which can influence exosome stability and membrane topology[2,3]. As a result of the cargo and lipid arrangement, exosomes initiate signaling pathways in receiving cells and participate in the maintenance of normal tissue physiology[1,2,4,5]. Under certain pathological conditions (i.e., neurodegeneration, fibrosis, and cancer), they have been shown to trigger and propagate pathological stimuli[4,6-11].

Owing to their ability to propagate signals to neighboring or distant sites, exosomes have become valuable biomarkers for the diagnosis or prognosis of disease conditions. In addition, exosomes have been used experimentally as vehicles of therapeutic compounds[2,12]. The potential application of these nanovesicles in the clinic makes the isolation method increasingly important in order to achieve maximum yield, purity, and reproducibility. Different techniques for the isolation of exosomes have been developed and implemented. Generally, exosomes can be isolated from conditioned cell culture media or body fluids by differential centrifugation, size exclusion chromatography, and immune capture (using commercially available kits). Each approach has unique advantages and disadvantages that have been discussed previously[1,2,13,14].

The outlined protocol focuses on the 1) isolation and culture of primary fibroblasts from adult mouse gastrocnemius muscle and 2) purification and characterization of exosomes released into the culture medium by these cells. A well-established protocol for the isolation of exosomes from primary fibroblasts for functional studies is currently lacking. Primary fibroblasts do not secrete large amounts of exosomes, making the isolation and purification process challenging. This protocol describes the purification of large amounts of pure exosomes from large culture volumes while maintaining their morphological integrity and functional activity. Purified exosomes obtained from conditioned medium have been used successfully in in vitro uptake experiments to induce specific signaling pathways in recipient cells. They have also been used for comparative proteomic analyses of exosomal cargos from multiple biological samples[4].

PROTOCOL:

All procedures in mice were performed according to animal protocols approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

1. Preparation of solutions and media

1.1. Prepare digestion solution by mixing 15.4 mL of PBS with 2.5 mL of 20 mg/mL collagenase P (5 mg/mL final concentration), 2 mL of 11 U/mL dispase II (1.2 U/mL final concentration), and 100 µL of 1.0 M $CaCl_2$ (5 mM final concentration).

1.2. Prepare 500 mL of primary fibroblasts medium (DMEM complete) by mixing 440 mL of DMEM with 50 mL of FBS (10%), 5.0 mL of pen/strep (100 U/mL and 100 µg/mL, respectively) and 5.0 mL of glutamine supplement (2 mM). Filter-sterilize the medium with a 0.22 µm pore size vacuum filter.

1.3. Prepare exosome-free serum by overnight ultracentrifugation of FBS in 38.5 mL polypropylene tubes at 100,000 x *g* at 4 °C and transfer the supernatant to a new tube.

1.4. Prepare 500 mL of exosome-free medium by mixing 440 mL of DMEM with 50 mL of exosome-free serum (10%), 5 mL of pen/strep (100 U/mL and 100 µg/mL), and 5 mL of glutamine supplement (2 mM). Filter-sterilize the medium with a 0.22 µm pore size vacuum filter.

1.5. Prepare 0.5 M Tris-HCl (pH 7.4) by dissolving 6.057 g of Tris base in 90 mL of dH_2O and adjusting the pH to 7.4 with HCl. Adjust the volume to 100 mL with dH_2O.

1.6. Prepare 10 mM Tris-HCl (pH 7.4)/1 mM $Mg(Ac)_2$ solution by mixing 97.9 mL of dH_2O with 2 mL of 0.5 M Tris-HCl (pH = 7.4) and 100 µL of 1 M $Mg(Ac)_2$. Add protease inhibitors to the solution just before use and keep the solution on ice.

1.7. Prepare sucrose density gradient solutions on ice according to **Table 1**. These solutions are sufficient to prepare two sucrose gradient tubes.

1.8. Prepare 100% TCA by adding 40 mL of dH_2O to 100 g of TCA powder and mix until fully dissolved. Adjust the final volume with dH_2O to 100 mL.

1.9. Prepare 80% ethanol by mixing 20 mL of dH_2O with 80 mL of 100% ethanol.

1.10. Prepare western blot running buffer by mixing 1.8 L of dH_2O with 200 mL of 10x running buffer.

1.11. Prepare western blot transfer buffer by mixing 1.4 L of dH_2O with 200 mL of 10x transfer buffer and 400 mL of methanol. Precool the transfer buffer to 4 °C.

1.12. Prepare 20x Tris-buffered saline (TBS) by dissolving 122 g of Tris base and 180 g of sodium chloride in 850 mL of dH_2O (pH 8.0). Adjust the volume to 1 L with dH_2O.

1.13. Prepare blocking buffer by dissolving 5 g of nonfat dry milk with 1 mL of 10% Tween-20, 5

mL of 20x TBS, and 94 mL of dH_2O.

1.14. Prepare antibody buffer by dissolving 3 g of BSA with 1 mL of 10% Tween-20, 5 mL of 20x TBS, and 94 mL of dH_2O.

1.15. Prepare washing buffer by mixing 100 mL of 20x TBS and 20 mL of 10% Tween-20 (10x) with 1.88 L of dH_2O.

1.16. Prepare developing solution by mixing 1 volume of luminol/enhance with 1 volume of stable peroxide buffer.

2. Dissection of gastrocnemius (GA) mouse muscle[15,16]

2.1. Prepare 50 mL tubes containing PBS on ice.

2.2. Euthanize the mice in a CO_2 chamber followed by cervical dislocation.

2.3. Remove the gastrocnemius muscle from both legs and transfer them to the tube with PBS on ice.

NOTE: Remove the soleus muscle from the GA muscle before transferring the GA muscle to PBS.

3. Mouse primary fibroblast isolation and culture

3.1. Weigh the muscles and transfer them to a 10 cm dish under a biosafety hood. Mince the muscles with scalpels until it becomes a fine paste.

3.2. Transfer the finely minced muscle paste to a 50 mL tube and add 3.5x volume/mg tissue of digestion solution to the tube and incubate for 45 min at 37 °C. Mix the suspension thoroughly with a 5 mL pipet every 10 min (this will aid in complete dissociation).

NOTE: Alternatively, a tissue dissociator can be used for this step.

3.3. Add 20 mL of DMEM complete to the cell suspension to inactivate the digestion solution. Transfer to a 70 µm nylon cell strainer placed on a 50 mL tube. Collect the flow-through and wash the cell strainer with an additional 5 mL of DMEM complete.

3.4. Centrifuge the cell suspension at 300 x *g* at room temperature (RT) for 10 min and carefully remove the supernatant.

3.5. Resuspend the pellet in 10 mL of DMEM complete and seed the cells into a 10 cm dish.

3.6. Culture the cells at 37 °C, 5% CO_2, 3% O_2 (passage 0 or P0).

NOTE: 1) These cultures need to be maintained at a low oxygen level to ensure physiological-like conditions (O_2 level in skeletal muscle is around 2.5%)[17]. 2) The passage number (e.g., P0) of a cell culture is a record of the number of times the culture has been subcultured. 3) Primary fibroblasts at P0 are routinely cultured until 100% confluency plus 1 day (and only for P0). This is to purge other types of cells and assure that the cells present in the culture are only fibroblasts, depleted of myogenic cells based on microscope examination. The skeletal muscle from an adult animal at 2 months of age contains about 2% myogenic progenitors[4]. In addition, myogenic progenitors usually do not attach to the uncoated dishes; therefore, they are lost during subculturing[18-20]. Myogenic cells require a different medium (F10) supplemented with 20% FBS and basic fibroblast growth factor (bFGF)[18]. In DMEM complete, medium fibroblasts have a higher proliferation rate than the myogenic cells, which results in the elimination of these contaminating cells before the first passage.

3.7. Rinse the P0 cells with PBS when 100% confluent plus 1 day. Add 1 mL of trypsinization solution and incubate at 37 °C, 5% CO_2, 3% O_2 to detach the cells. Stop the enzymatic activity by using 10 mL of DMEM complete.

3.8. Centrifuge the cells at 300 x *g* for 10 min at RT and resuspend the pellet in 20 mL of DMEM complete and seed into a 15 cm dish (passage 1 or P1). The cells are grown until 80%–90% confluency and can be expanded until passage 3.

NOTE: Depending on the downstream experiments passage 1, passage 2 (P2) or passage 3 (P3) can be used.

4. Seeding of cells and collection of conditioned medium

4.1. Wash the fibroblasts (P1, P2, or P3) with 10 mL of PBS, add 2.5 mL of trypsinization solution to the cells and incubate at 37 °C, 5% CO_2, 3% O_2. Stop enzymatic activity by using 10 mL of DMEM complete.

NOTE: After passage 4, primary cells are discarded and should not be used for further experiments, as they change characteristics.

4.2. Collect the cells and centrifuge at 300 x *g* at RT for 10 min.

4.3. Resuspended the cell pellet in 10 mL of exosome-free medium and count the cells.

4.4. Seed the cells at 1.5–2.0 x 10^6 cells per dish (15 cm) and incubate at 37 °C, 5% CO_2, 3% O_2.

4.5. Collect the conditioned medium between 16–24 h in 50 mL tubes on ice.

NOTE: If cells are not 80% confluent, fresh exosomal free medium can be added again and collected after an additional 16–24 h period. The two collections can be combined for further processing.

5. Purification of exosomes using differential and ultra-centrifugation

NOTE: All steps are performed at 4 °C or on ice.

5.1. Centrifuge the conditioned medium at 300 x *g* for 10 min for removal of live cells and transfer the supernatant to a new 50 mL tube.

5.2. Remove dead cells by centrifugation at 2,000 x *g* for 10 min and transfer the supernatant to a 38.5 mL polypropylene tube.

5.3. Centrifuge the supernatant at 10,000 x *g* for 30 min for the removal of organelles, apoptotic bodies, and membrane fragments. Transfer the supernatant to a 38.5 mL ultra-clear tube.

5.4. Ultracentrifuge the supernatant at 100,000 x *g* for 1.5 h to pellet the exosomes.

5.5. Carefully discard the supernatant, leaving approximately 1 mL of conditioned medium, and wash the exosome pellet in a total volume of 30 mL of ice-cold PBS.

5.6. Centrifuge at 100,000 x *g* for 1.5 h and carefully discard the supernatant by pipetting, being careful not to disturb the exosomal pellet.

5.7. Resuspend the pellet in ice-cold PBS (~25 µL per 15 cm dish) and measure the protein concentration using the BCA protein assay kit and a microplate reader at 562 nm.

NOTE: The protein concentration is measured by a 1:2 dilution with 0.1% Triton-X100 in dH_2O. The yield of exosomes from a 15 cm dish (20 mL of conditioned medium) of primary fibroblasts at 80% confluency is ~3–4 µg.

6. Characterization of exosomes by sucrose density gradient

6.1. Load the sucrose gradient in a 13 mL ultra-clear tube by pipetting (from the bottom up) the following: 1.5 mL of 2.0 M, 2.5 mL of 1.3 M, 2.5 mL of 1.16 M, 2.0 mL of 0.8 M, and 2.0 mL of 0.5 M sucrose solutions.

6.2. Mix 30–100 µg of exosomes with 0.25 M sucrose solution and adjust the volume to 1 mL.

6.3. Load carefully the exosomes on top of the gradients and ultra-centrifuge the samples for 2.5 h at 100,000 x *g* and 4 °C.

6.4. Remove the tubes from the ultra-centrifuge and place them on ice and collect 1.0 mL fractions starting from the top of the gradient. Transfer them to 1.7 mL tubes on ice.

6.5. Add 110 µL of 100% TCA to each tube, mix well, and incubate for 10 min at RT.

6.6. Centrifuge for 10 min at 10,000 x *g* and RT and carefully discard the supernatant.

6.7. Resuspend the pellet in 500 µL of pre-cooled 80% ethanol and leave the samples to wash for 10 min at -20 °C.

6.8. Centrifuge for 10 min at 10,000 x *g* and 4 °C and discard the supernatant.

6.9. Dry the pellet for 10–15 min at RT and resuspend the pellet in PBS for in vitro experiments. Measure the protein concentration using BCA protein assay kit or resuspend the pellet directly in 14.5 µL of dH_2O, 5 µL of 4x Laemmli buffer, and 0.5 µL of 1 M DTT for western blot analyses.

7. Exosome detection by western blot analysis

7.1. Mix 5–10 µg of exosomes from step 5.7 with 5 µL of 4x Laemmli buffer, and 0.5 µL of 1 M DTT, then adjust the final volume to 20 µL with dH_2O. Heat samples from step 5.7 and step 6.9 for 5 min at 98 °C.

7.2. Load the samples into a 10% TGX stain-free gel and load the protein ladders according to the manufacturer's recommendations.

NOTE: Choose the percentage of the gel according to the molecular weight of the protein of interest.

7.3. Run the gel at 100 V for ~1 h in running buffer until the loading dye is at the bottom of the gel.

NOTE: The run time may vary according to the equipment used or type and percentage of gel.

7.4. Image the gel. Images of the stain-free gels are used as the loading control for immunoblots.

7.5. Transfer the gel using a PVDF membrane for 1.5 h at 80 V or overnight at 30 V at 4 °C.

7.6. Block the membranes in blocking buffer for 1 h at RT.

7.7. Incubate the membranes for specific antibodies (**Table 2**) in antibody buffer overnight at 4 °C with agitation.

7.8. Wash the membranes in washing buffer and incubate the membranes with the appropriate secondary antibodies (**Table 2**) for 1 h at RT with agitation.

7.9. Wash the membranes in washing buffer and image the membranes using develop solution and a camera-based imager. Alternatively, use an X-ray film to develop the membranes.

REPRESENTATIVE RESULTS:

This protocol is suitable for the purification of exosomes from large volumes of conditioned medium in a cost-effective manner. The procedure is highly reproducible and consistent. **Figure 1** shows transmission electron microscopy (TEM) image of exosomes purified from the culture medium of mouse primary fibroblasts. **Figure 2** shows the protein expression pattern of canonical exosomal markers, and the absence of cytosolic (LDH) and ER (calnexin) protein contaminants. **Figure 3** shows the distribution of canonical exosomal markers (Alix, CD9, and CD81) after sucrose density gradients.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative TEM image of exosomes isolated from culture medium of mouse primary fibroblasts. Shown are the relatively uniform sizes of these nanovesicles. Across black lines denote the diameter of the individual vesicles. Scale bar = 100 nm.

Figure 2: Immunoblot of exosome lysates probed with antibodies against exosome markers. Alix, CD81, CD9, flotillin1, syndecan1, syntenin1, cytosolic (LDH), and ER (calnexin) markers.

Figure 3: Exosomes separated by a sucrose density gradient. Individual fractions were probed on a western blot with antibodies against Alix, CD9, and CD81. Based on the fractionation pattern of the markers, exosomes consistently sediment in fractions 3–6, which correspond to densities of 1.096–1.140 g/mL.

Table 1: Sucrose density gradient solutions.

Table 2: Primary and secondary antibodies.

DISCUSSION:

A critical step for the successful isolation of exosomes from the culture media, as outlined in this protocol, is the proper establishment and maintenance of primary mouse fibroblast cultures from adult skeletal muscle. These cultures need to be maintained at a low oxygen level to ensure physiological-like conditions (O_2 level in skeletal muscle is ~2.5%)[15]. Primary fibroblasts will change characteristics when passed in culture too many times. Hence, a low passage number is imperative for ideal exosome yield. Purified exosomes should either be used immediately for experimental purposes or kept frozen in aliquots at -80 °C until needed. Another important step in the protocol is the use of sucrose solutions prepared fresh every time. A limitation of the method is that it is time-consuming, because primary fibroblasts do not generally secrete high amounts of exosomes, like other secretory cells. Therefore, large cultures should be employed, which result in large volumes of conditioned media to be processed.

The advantage of differential centrifugation used here is that it represents a scalable approach (up to liters) and is the method of choice to isolate exosomes from primary fibroblasts. An alternative method for larger volumes (up to 100 mL per column) is size exclusion chromatography (SEC). This method is fast and can separate proteins from exosomes. The

column does not pellet exosomes, so further concentration or centrifugation steps are required. One of the disadvantages of the SEC method is that the column can be clogged over time and overloaded. Other current methods are based on the use of small volumes of biological fluids (i.e., urine, CSF, serum, plasma) and commercially available kits. Although these kits ensure reproducibility, they are costly and do not always produce a high yield of pure exosomes. The outlined protocol for exosome purification is straightforward and can be applied to different types of cells, whole tissues and organs, or other biological fluids depending on the experimental need.

DISCLOSURES:

None of the authors have any conflicts of interest to declare.

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Figure 1

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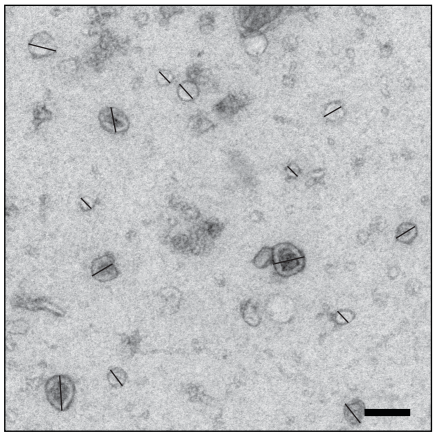

Figure 2

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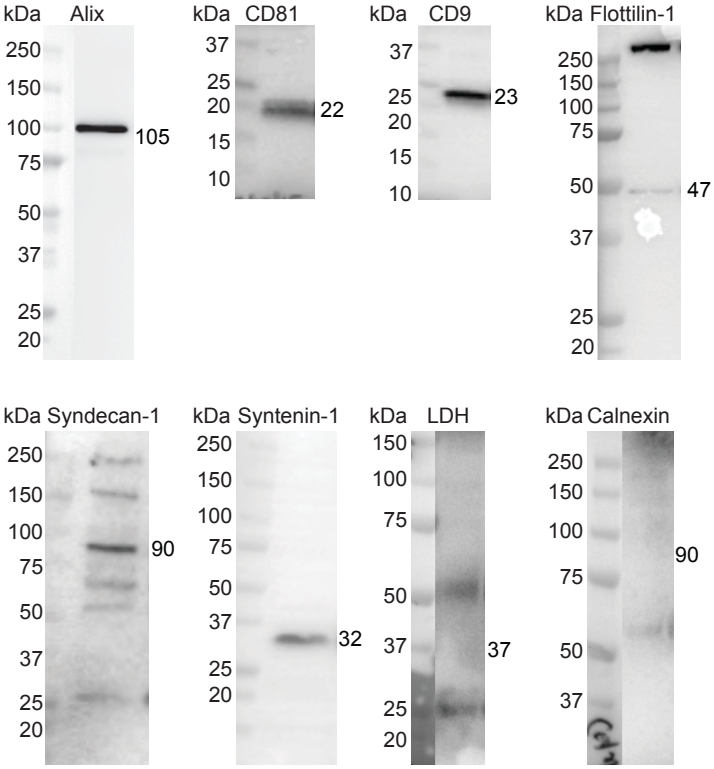

Figure 3

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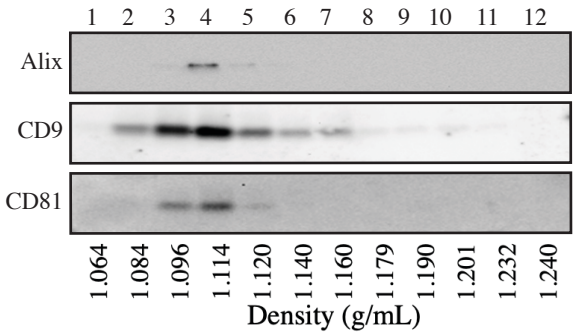

Table 1

	Sucrose (g)	Sucrose working solution (mL)
2.0 M	2.74	4.0
1.3 M	2.67	6.0
1.16 M	2.38	6.0
0.8 M	1.37	5.0
0.5 M	0.86	5.0
0.25 M	0.26	3.0

Table 2

Click here to access/download;Table;dAzzo Table 2 Antibodies1.xlsx

Antibody	Host	Company	Catalog number
CD9	Rat	BD Biosciences	553758
CD81	Mouse	Santa Cruz Biotechnologies	sc-166029
Alix	Rabbit	d'Azzo lab	Alix
Flotilin1	Mouse	BD Biosciences	610820
Syndecan1	Rabbit	Life Technologies	36-2900
Syntenin1	Rabbit	Millipore/Sigma	AB15272
LDH	Goat	Chemicon	AB1222
Calnexin	Goat	Santa Cruz Biotechnologies	sc-6465
rat-HRP	Donkey	Jackson Imm. Res Lab	112-035-003
Mouse-HRP	Goat	Jackson Imm. Res Lab	115-035-044
Rabbit-HRP	Goat	Jackson Imm. Res Lab	111-035-144

Name of Material/ Equipment	Company	Catalog Number
10 cm dishes	Midwest Scientific, TPP	TP93100
15 cm dishes	Midwest Scientific	TP93150
BCA protein assay kit	Thermo Fisher Scientific, Pierce	23225
Bovine serum albumin Fraction V	Roche	10735094001
CaCl2	Sigma	C1016-100G
Centrifuge 5430R with rotors FA-35-6-30/ FA-45-48-11	Eppendorf	022620659/5427754008
Chemidoc MP imaging system	BioRad	12003154
Collagenase P	Sigma, Roche	11 213 857 001
cOmplete protease inhibitor cocktail	Millipore/Sigma, Roche	11697498001
Criterion Blotter with plate electrodes	BioRad	1704070
Criterion TGX stain-free protein gel	BioRad	5678034
Criterion vertical electrophoresis cell (midi)	BioRad	NC0165100
Dispase II	Sigma, Roche	04 942 078 001
Dithiothreitol	Sigma/Millipore, Roche	10708984001
Dulbecco's Modification Eagles Medium	Corning	15-013-CV
Dulbecco's Phosphate Buffered Saline	Corning	21-031-CV
Ethanol 200 proof	Pharmco by Greenfield Global	111000200
Falcon 50 mL conical centrifuge tubes	Corning	352070
Fetal Bovine Serum	Gibco	10437-028
Fluostar Omega multi-mode microplate reader	BMG Labtech	
GlutaMAX supplement	Thermo Fisher Scientific, Gibco	35050-061
Hydrochloric acid	Fisher Scientific	A144S-500
Immobilon-P Transfer membranes	Millipore	IPVH00010
Laemmli sample buffer (4x)	BioRad	1610747
Magnesium acetate solution	Sigma	63052-100ml
Non-fat dry milk	LabScientific	M-0842
O_2/CO_2 incubator	Sanyo	MC0-18M
Penicillin-Streptomycin	Thermo Fisher Scientific, Gibco	15140-122
Premium microcentrifuge tubes	Fisher Scientific, Midwest Scientific	AVSC1510
Protected disposable scalpels	Fisher Scientific, Aspen Surgical Bard-Parke	372610
Running buffer	BioRad	1610732
Sodium Chloride	Fisher Scientific, Fisher Chemical	S271-3

Stericup Quick release-GP sterile vacuum filtration system	Millipore	S2GPU05RE
Sterile cell strainer (70 mm)	Fisher Scientific, Fisher brand	22-363-548
Sucrose	Fisher Scientific, Fisher Chemical	S5-500
SuperSignal west Femto	Thermo Fisher Scientific	34096
Thin wall Polypropylene tubes	Beckman Coulter	326823
Transfer buffer	BioRad	16110734
Trichloroacetic Acid	Sigma	91228-100G
Tris base	BioRad	1610719
Triton-X100 solution	Sigma	93443-100mL
TrypLE Express Enzyme	Thermo Fisher Scientific, Gibco	12604-013
Tween-20	BioRad	#1610781
Ultra-centrifuge Optima XPM	Beckman Coulter	A99842
Ultra-clear tube (14x89 mm)	Beckman Coulter	344059
Ultra-clear tubes (25x89 mm)	Beckman Coulter	344058
Water bath Isotemp 220	Fisher Scientific	FS220

Comments/Description

100 mg

10% 18-well, midi-gel

neutral protease, grade II

10,000 U/ml
1.7 mL

500 mL

No phenol red

Editorial comments:

1. Your response to Reviewer 3's first point is unclear. How exactly do you 'assure that the only cell type present in the culture is fibroblasts'? Do you, e.g., check for any fibroblast/myoblast markers?

We agree with the Review Editor and have now added a detailed note in 4.6 c to address this point.

2. There are no longer any black lines in Figure 1, although the legend says that there are; please clarify.

We thank the Review Editor for bringing this to our attention. We have now added black lines back in Figure 1.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript and corrected any spelling/grammar error if present.

2. Please provide email addresses for all authors in the manuscript itself.

The email addresses of all authors were added to the manuscript.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Glutamax, TryplE

We have limited the use of commercial language in this manuscript.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the essential steps of the protocol for the video.

We have about 1.5 pages for film content including:

1. Mouse primary fibroblast isolation and culture;
2. Purification of exosomes using differential and ultracentrifugation.

2. For each protocol step/substep, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. 3: Please include more information about this dissection, possibly including references.

We have now added two references to describe the dissection of the GA muscle in more detail.

Figures:

1. Please remove ‘Figure 1’ etc. from the figures themselves.

We have now removed the “Figure” from the figures themselves.

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

We have ensured that all references are listed correctly.

2. Please do not abbreviate journal titles.

We have replaced abbreviations with full Journal titles.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have verified the information on all materials and equipment used in the protocol.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a well written and time manuscript from Vlekkert et al. Overall it is in excellent condition and should be accepted after considering only a few minor suggestions. This reviewer believes it will be very helpful to many investigators working to purify EVs from muscle fibroblasts.

Major Concerns:

none.

Minor Concerns:

The EVs are challenging to see in Figure 1 and this image needs brightness / contrast enhancement.

We thank the Reviewer for bringing this to our attention. We have now improved Figure 1 to better visualize the exosomes.

It would be nice to see a full WB for figure 2 including input lanes rather than the single band crops to inspect enrichment of these EV markers. if possible.

Following the Reviewer' suggestion, we have now replaced the cropped images with full Western blot images in Figure 2.

SEC (IZON) has become a leading approach for EV isolation but does have limitations as well (volume constraints, over loading etc), a few more sentences discussing this point should be added to the discussion.

Following the Reviewer' comment we have now added additional discussion points.

2.8 - the description could be improved by clarifying that the TCA should be dissolved in a volume of water to obtain a final solution of 100 mL.

We have changed the description for the TCA solution in point 2.8 of the "Preparation of solutions and media" section.

4.7 (note) - it is unclear what is meant by P1, P2, or P3. is this referring to the number of days of culturing in vitro ? this should be clarified throughout the manuscript.

We thank the Reviewer for this suggestion and have clarified this point in note 4.6.

7.5 "Add 110 mL of 100% TCA", is this a typo? is it micro liters?

The Reviewer is correct. The volume should be 110 μl (micro liters). We have corrected this in the text.

Reviewer #2:

Manuscript Summary:

In this manuscript van de Vlekkert et al. outline protocols for the establishment of cultures of primary fibroblasts isolated from adult mouse skeletal muscles, and for the purification and characterization of exosomes from the culture media of these cells. Their method is based on the use of sequential centrifugation steps followed by sucrose density gradients. The main advantage of their work is that they bring forward that these cultures need to be maintained at a low oxygen level to ensure physiological-like conditions and that low passage number is imperative for ideal exosome yield since primary fibroblasts will change characteristics when passed in culture too many times. The protocol is well written and all the steps are well described.

Major Concerns:

Authors should emphasise the importance of their protocol in the Abstract. Moreover, in the introduction, authors should list the problems faced for the purification of exosomes from primary fibroblasts isolated from adult mouse skeletal muscles and bring forward the objectives of the suggested protocol.

Following the Reviewer' suggestions, we have now adjusted the text accordingly.

Minor Concerns:

I think it is better to use exosome-free serum and exosome-free medium rather than exosome serum and exosome medium in 2.3 and 2.4.

We agree with the Reviewer and have now changed the text accordingly.

Reviewer #3:

Major Concerns:

1- the authors did not characterize the mixed cellular population they obtain from skeletal muscle, they only mention the fibroblasts, but I'm confident that they obtain also myogenic cells. They have to demonstrate If they obtain only fibrostast cells.

We agree with the Reviewer and have now added a detailed note to 4.6 to address this point.

2-it is important to indicate the volume of fibroblast conditioned-medium or the amount of muscle they minced in relation to the yield in exosomes

Following the Reviewer's suggestion, we have now added a note to 6.7 to address this point.

3-in the discussion the authors claimed that their procedure uses large amount of volumes while other procedures rely on small volumes, in my opinion this is a limitation because several times researchers manage small amount of samples.

The authors have to explain what they claim thinking at the advantage of big volumes

We have explained this point in more detail in the Introduction and Discussion.

4- In literature, exists a publication in which authors used growth medium conditioned by human myoblasts to obtain exosomes, not mentioned, that used both CD81 and CD63 to identify the exosomes, I was wandering why the authors did not have used CD63. It could be that exosomes from mouse muscle fibroblasts have less amount of this marker or did not have it with respect to human samples?

There is no specific reason for not using the anti-CD63 antibody. We have used multiple canonical markers for exosomes, including CD9 and CD81, in this manuscript to show the purity of the samples. CD63, just like CD9 and CD81, is a tetraspanin decorating the exosome membrane and can also be used as a marker for exosomes.

5-an attempt to compare data obtained with those present in literature deserves merit

Following the Reviewer's comment we have now modified the Introduction and the Discussion.

Minor Concerns:

1-it was intended to see a video, but I do not have it

No video is planned until the manuscript is finalized, according to JOVE online instruction on publishing process.

2- update literature

We have updated the literature.