Chapter 12

Extracellular Vesicle Isolation and Analysis by Western Blotting

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Abstract

Extracellular vesicles (EVs) are released by mammalian cells and are thought to be important mediators of intercellular communication. There are many methods for isolating EVs from cell culture media, but the most popular methods involve purification based on ultracentrifugation. Here, we provide a detailed protocol for isolating EVs by differential ultracentrifugation and analyzing EV proteins (such as the tetraspanins CD9, CD63 and CD81) by western blotting.

Key words Exosomes, Extracellular vesicles, Exosome isolation, Extracellular vesicle isolation, Ultracentrifugation, Immunoblotting, Western blot, Tetraspanins, CD63, CD81, CD9

1 Introduction

Extracellular vesicles (EVs) are involved in intercellular communication. All mammalian cell types secrete heterogeneous vesicles of a variety of sizes [1]. Here, we focus on small EVs, which are often called exosomes. Although the exact definition of exosomes varies in different papers and remains debated [2], we define an exosome as any EV between 30 and ~200 nanometers (nm). The upper limit is imposed by using a 0.22 μm filter.

There are many different protocols for isolating EVs, which yield vesicles of varying purity (all protocols, to some degree co-isolate soluble proteins) [3]. We describe an ultracentrifugation-based protocol, which remains one of the most commonly used protocols for isolating extracellular vesicles from cell culture media [4]. There are many variations on this protocol, and our protocol includes a 0.22 μm filtration step. This cutoff provides a specific size

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range for the isolated extracellular vesicles, which enhances reproducibility in EV analysis across cell types or conditions.

EVs can be characterized by a wide variety of techniques, including profiling the proteins they contain. Tetraspanins are a group of proteins that contain four transmembrane domains and certain characteristic features. The tetraspanins CD9, CD63 and CD81 are transmembrane proteins that are commonly found in extracellular vesicles across cell types [5]. In addition to our EV isolation protocol, we describe a detailed protocol for analysis of these characteristic EV markers by western blotting.

2 Materials

Store all materials at room temperature unless otherwise stated.

2.1 Cell Culture

1. Cells and cultureware.
2. Fetal bovine serum (FBS)-depleted media or defined media without FBS (*see* **Note 1**).

2.2 EV Isolation

1. PBS without Ca^{2+}/Mg^{2+}.
2. HEPES buffer (optional) (*see* **Note 2**).
3. 50 mL Falcon tubes (Fisher Scientific).
4. 0.22 μm Steriflip filter tubes (Fisher Scientific).
5. Ultracentrifuge and rotor.
6. Polyallomer ultracentrifuge tubes (Beckman Coulter).

2.3 Western Blot

1. Sample Buffer: Bolt 4× LDS Sample Buffer (Thermo Fisher Scientific). Store at 4 °C.
2. Bolt 10× Reducing Buffer (optional) (Thermo Fisher Scientific) (For proteins that require reducing conditions, not tetraspanins; *see* **Note 3**).
3. RIPA buffer (optional) (*see* **Note 4**).
4. A660 or BCA protein quantification assay (optional) (*see* **Note 5**).
5. Running buffer: 100 mL 20× MES SDS running buffer (Thermo Fisher Scientific), 1900 mL deionized water.
6. 4–12% Bis-Tris Gels (Thermo Fisher Scientific). Store at 4 °C.
7. Gel tank (XCell SureLock® Mini, Thermo Fisher Scientific) and electrophoresis equipment.
8. MagicMark XP Western protein standard (Thermo Fisher Scientific). Store at −20 °C.
9. SeeBluePlus2 protein ladder (Thermo Fisher Scientific). Store at 4 °C.

10. XCell II Blot Module and sponges (Thermo Fisher Scientific) (*see* **Note 6**).
11. Methanol.
12. Transfer buffer: 100 mL Bolt 20× transfer buffer (Thermo Fisher Scientific), 400 mL methanol, 1500 mL deionized water.
13. PVDF or nitrocellulose membranes (Thermo Fisher Scientific).
14. Milk powder.
15. Tween 20.
16. PBST: PBS with 0.1% vol/vol Tween 20. Store at 4 °C.
17. Cold room with a tilting rocker (not orbital).
18. Plastic containers to hold membranes, such as PerfectWestern™ containers.
19. Flat tweezers for handling membranes.
20. Antibodies to proteins of interest.
21. HRP-conjugated secondary antibody for visualization (Bethyl Laboratories).
22. HRP substrate, such as SpectraQuant™-HRP CL Chemiluminescent detection reagent (BridgePath Scientific).

3 Methods

3.1 EV Isolation

1. Culture cells under standard conditions to 50–70% confluency.

Day 1
For suspension cells:

1. Spin down desired total number of cells (*see* **Note 7**) in six 50 mL Falcon tubes at 300 × *g* for 5 min.
2. Aspirate media and resuspend each cell pellet in 40 mL FBS-depleted or defined media without FBS (*see* **Note 1**). Transfer contents of each Falcon tube to T75 flask and return to incubator.

For adherent cells:

1. Aspirate media from twelve 15 cm plates.
2. Add 20 mL FBS-depleted or defined media without FBS per plate (*see* **Note 1**). Return cells to incubator.

Day 2

1. After 24 h, take off all media and divide among 50 mL falcon tubes.
2. Spin at 300 × *g* for 10 min at RT (to pellet the cells).

3. Transfer supernatant to new 50 mL tubes leaving cell pellet behind. If cell protein is to be analyzed alongside EVs, one cell pellet can at this step be resuspended in the desired lysis buffer (*see* **Notes 4** and **5**).
4. Spin at 2000 × *g* for 10 min at RT (to pellet any dead cells).
5. Transfer supernatant to new 50 mL tubes leaving cell pellet behind.
6. Spin supernatant at 16,500 × *g* for 20 min at 4 °C (to pellet large EVs).
7. Transfer supernatant to new 50 mL tubes, leaving pellet behind.
8. Pass supernatant through Steriflip 0.22 μm filter.
9. Transfer supernatant to polyallomer ultracentrifuge tubes. Centrifuge at 120,000 × *g* (26,500 RPM with SW32Ti rotor) for 70 min at 4 °C.
10. Remove most of supernatant, leaving ~2 cm of media above pellet. Add 5 mL PBS to each tube. Vortex on medium speed for a few seconds. Fill to top of each tube with PBS.
11. Again, centrifuge at 120,000 × *g* for 70 min at 4 °C.
12. Aspirate all of supernatant with Pasteur pipet without touching bottom of tube where pellet is located (*see* **Note 8**).
13. Resuspend pellet either in PBS or directly in the desired lysis buffer for western blot (*see* **Notes 4** and **5**).

3.2 Western Blot

14. Add 100 μL 1× Sample Buffer to each pellet, or add 4× sample buffer to a final concentration of 1× (i.e., add 25 μL 4× sample buffer to 75 μL sample) in a pre-isolated EV sample. Vortex on high speed to mix. If in ultracentrifuge tubes pipet up and down to further disrupt pellet, then transfer to Eppendorf tubes.
15. Incubate at 70 °C for 10 min.
16. Make 2 L of 1× MES SDS running buffer (100 mL buffer into 1900 mL deionized water).
17. Make 5% milk buffer: 2.5 g dried milk into 50 mL PBST (PBS + 0.1% v/v Tween 20). Tumble at 4° for an hour (*see* **Note 9**).
18. Prepare 4–12% bis-tris gel in gel tank. Add 1× MES running buffer to top of gel. Do not forget to rinse wells.
19. Load wells. For ladder use 3 μL MagicMark + 6 μL SeeBlue-Plus2, in separate lanes if possible (*see* **Note 10**).
20. Run 40 min at 150 V, 22 min at 200 V, or until blue dye reaches gel foot.

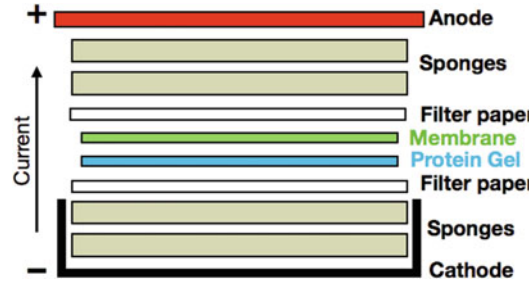

Fig. 1 Schematic of membrane transfer sandwich. This should be constructed from the bottom (cathode) up, using as many sponges as necessary to put even pressure across the surface of the membrane with no air bubbles, and all internal components except gel pre-soaked in transfer buffer

21. While gel is running, prepare for transfer (see XCell surelock manufacturer instructions [6] for more detail).
22. Make 2 L transfer buffer (100 mL Bolt 20× transfer buffer, 400 mL methanol, 1500 mL water).
23. If using PVDF membranes, place one membrane in empty tip box lid with a few milliliters methanol to activate it. Rinse several times with transfer buffer, dumping excess into large sandwich making tray, then rock gently in hand for several minutes. For nitrocellulose membranes, simply soak in transfer buffer for a minute.
24. Soak sponges in transfer buffer, squeezing out bubbles as much as possible (*see* **Note 11**). Briefly immerse filter papers in transfer buffer as well.
25. Build sandwich up from the bottom in the following order: anode, sponges, filter paper, gel, transfer membrane, filter paper and sponges (*see* Fig. 1, **Note 12**).
26. When sandwich is ready for gel, take gel out of tank and rinse it off. Crack open plastic casing. Cut off wells and foot so that remainder is completely flat and lay carefully on filter paper.
27. Squeeze sandwich together in holder and insert into gel tank. If reusing the same tank make sure to pour out gel running buffer and rinse with deionized water.
28. Use fresh transfer buffer to fill in sandwich from the top. Open and close clamp several times to let the buffer soak down through.
29. Fill the rest of the gel box with deionized water, which will serve as a heat sink.
30. Put on lid and run 1.5–2 h at 30 V, tapping firmly on occasion to remove bubbles (*see* **Note 11**).

31. When done, turn off current, pull out sandwich in holder, put it back in large tray (minus transfer buffer) then unpack it carefully. Peel away filter paper very slowly to check for protein transfer (*see* **Note 13**).
32. As soon as you peel off membrane, take a blade and cut off upper right hand corner to mark "top" face (face which was touching gel).
33. Place membrane in PerfectWestern box containing 5–10 mL milk buffer (as much as necessary to cover membrane completely). If using PVDF membranes, ensure that the membrane does not dry out at any step.
34. To block, place membrane in milk buffer on rocker in the cold room and let rock at least half an hour. Conduct all further steps in the cold room if possible.
35. After at least half an hour, pour off block and add 10 mL primary antibody diluted 1:1000 in milk buffer (*see* **Note 14**). Leave overnight rocking in the cold room.

Day 3:

1. Pour off primary, take PBST, pour in, swish, pour off, 2×, then do three washes in PBST of ~10 min each, rocking in the cold room.
2. Add 10 mL secondary antibody diluted 1:2000 in milk buffer (*see* **Note 14**). Leave rocking in the cold room for at least 1 h.
3. Pour off secondary, take PBST, pour in, swish, pour off, 2×, then do three washes in PBST of ~10 min each, rocking in the cold room.
4. Bring the membrane (in fresh PBST), equal volumes of each component of HRP substrate (reagent A and B; *see* Subheading 2.3, **item 21**) and an empty falcon tube to the imaging stage.
5. Mix reagent A and B together immediately before use. Pour PBST off membrane and pour A/B mix on. Let sit for a minute then image, using tweezers to handle membrane (*see* **Note 15**).

4 Notes

1. Since fetal bovine serum contains bovine EVs, it is important for downstream analysis that media from which EVs will be isolated is either FBS-free or has been depleted of vesicles by overnight ultracentrifugation at 120,000 × *g*. A convenient formulation is to make media with 2× FBS and ultracentrifuge it overnight, then remove and keep the supernatant, diluting it 1:1 in the base media to bring it to 1×. Some cells will still not like this media and so we advise collecting EVs for 24 h.

2. For storage of EVs at −80 °C we recommend the addition of HEPES buffer to a final concentration of 20 mM to stabilize pH over freeze–thaw cycles (to PBS or other buffers).
3. Protein gel electrophoresis can be either denaturing or nondenaturing ("native," i.e., retaining the original folded structure) and either reducing (where Cys-Cys disulfide bonds are specifically broken) or nonreducing. Though reducing can help to solubilize a concentrated or complex sample, tetraspanins such as CD63, CD81 and CD9 require nonreducing electrophoresis for western blotting, as the epitope recognized by antibodies to these proteins usually relies on several disulfide bonds to fold properly and be recognized.
4. Transmembrane proteins, particularly those with four or more membrane-spanning regions, can be difficult to extract from lysates. We have had success extracting tetraspanins with LDS sample buffer alone (Subheading 2.3, **item 1**) but other proteins may require some optimization of lysis buffer for efficient extraction. RIPA buffer is one of the harsher common buffers and is well suited for this purpose. When extracting membrane proteins from cells, it is often helpful to centrifuge the lysate at high speed (>12,000 × *g*) for 10 min and take the supernatant, leaving behind the membrane and insoluble material which can interfere with electrophoresis.
5. Many common protein quantification assays (such as A660 and BCA) rely on a colorimetric readout, and are thus incompatible with the bromophenol blue-containing LDS sample buffer. This protocol does not explicitly describe how to quantify protein in a lysate, but note that if you do wish to quantify the protein in your samples, you should lyse the cells or vesicles in RIPA (Subheading 2.3, **item 2**) or another clear buffer, quantify, and then add 4× LDS sample buffer to 1× concentration prior to immunoblotting.
6. The reagents listed as subheading 2.3, **items 10–13** are required for a traditional wet transfer of proteins to a membrane. These can be substituted with other materials of your choice for dry or semidry transfer. For example, we have found the iBlot dry blotting system from Thermo Fisher is convenient and effective, though not all labs may have the required equipment.
7. The total number of cells per isolation should be determined by the total volume of media from which you are able to isolate EVs. The limiting factor will likely be the volume capacity of your ultracentrifuge tubes (e.g., the SW32Ti rotor can hold six tubes with a volume of ~38 mL each, so the max volume per isolation is 228 mL). Start with a few extra milliliters of media per flask to account for some loss throughout the

centrifugation steps and culture the number of cells necessary to achieve 50–70% confluence in this volume.

8. The pellet at this stage will most likely not be visible. It is possible to remove all but 20–30 μL of the supernatant by tilting the tube to pool the liquid on one side and carefully avoiding touching the center of the tube bottom. We have also found that it is helpful to remove all but ~2 cm of supernatant and wait 30 s before aspirating the final few milliliters, as otherwise some liquid clings to the sides of the tube and makes the final residual volume >50 μL.
9. The proteins in the milk buffer associate with proteins in the membrane and block nonspecific antibody interactions. There are many formulations of blocking solution available but we have found milk to be cheap and effective. It is important to make this buffer fresh (it should be a few days old at most and stored at 4 °C with rotation).
10. MagicMark XP is a protein standard ladder containing IgG binding sites (you will see it on the final western blot, not in the gel), while SeeBlue is a prestained protein standard ladder which you should see in the gel and membrane but not in the final blot. These can be mixed if necessary but will run better in separate wells. SeeBlue is useful for evaluating how far the gel has run and if the transfer was successful (*see* **Note 13**) as well as for horizontally cutting the membrane in order to blot for proteins of different molecular weights, e.g., CD63 and CD81 (*see* Fig. 2).
11. Air bubbles anywhere in the sandwich can prevent successful transfer of proteins to the membrane in that spot, so it's important to squeeze the sandwich tightly and firmly tap the XCell mini tank periodically (as many times as is convenient) while transfer occurs.
12. Use as many sponges as necessary to form a tight sandwich. Generally at least three sponges on either side of the gel and membrane (six total) will suffice, but the tighter the better. *See* Fig. 1 for schematic.
13. Carefully peel away the top corner of the membrane closest to where the SeeBlue ladder was run and check for the location of the colored bands. If the transfer worked, some or all of them should now be on the membrane instead of the gel. Specifically, check that the SeeBlue bands in the molecular weight range of your protein of interest (for example, the 28 kDa band is close to the size of CD81) are on the membrane. If they are still on the gel, you can carefully reconstruct the sandwich (ensure that the gel and membrane do not shift relative to one another) and run it slightly longer. Keep in mind that running the transfer for too long will cause the lower molecular weight bands to

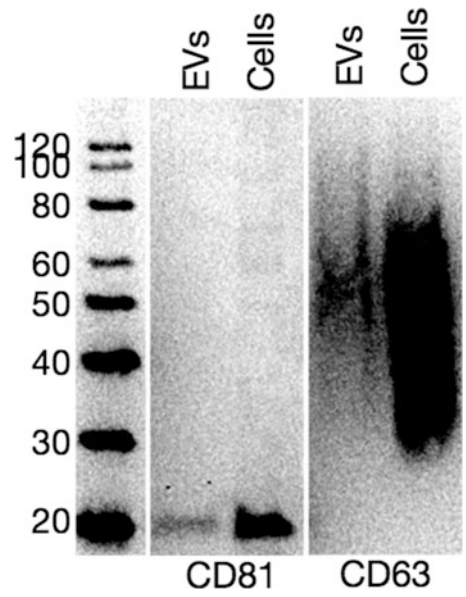

Fig. 2 Western blot showing CD81 and CD63 blotting on K562 EV lysate (7 μg) and cell lysate (86 μg), resuspended in RIPA and quantified before addition of LDS Sample Buffer. Note that CD63 appears as a smear in the range of 30–60 kDa; this is due to heavy glycosylation and is expected [7]. Each membrane was blocked for 30 min at 4 °C in milk buffer, then incubated with primary antibody diluted in 10 mL milk buffer for 12 h (1:1000 dilution mouse anti-human CD81, clone M38, or 1:1000 dilution mouse anti-human CD63, clone TS63), washed three times for 10 min each in PBST, then incubated for 2 h with 10 mL secondary antibody (Rabbit anti-mouse HRP, 1:2000 dilution in milk buffer), washed three times for 10 min each in PBST, then imaged on a Bio-Rad ChemiDoc MP system with SpectraQuant'™-HRP CL Chemiluminescent detection reagent

pass through the membrane onto the filter paper, at which point they cannot be recovered.

14. As different antibodies have different affinities for their targets, it is often necessary to experimentally determine the optimal antibody dilutions for immunoblotting. Generally these fall within 1:100 and 1:5000 and are lower (i.e., more dilute) for the secondary antibody. We recommend starting with a higher dilution (more concentrated) to ensure a strong signal and diluting further as necessary to eliminate background or conserve reagents.
15. If using Image Lab software to visualize the blot, it can be set to "signal accumulation mode" to determine optimal exposure time by monitoring blot over the course of imaging.

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