Tips for isolating highly pure exosome subpopulations for western blot

Introduction

Exosomes are 30–150 nm extracellular vesicles containing RNA and protein cargo. They are constantly secreted by all cells *in vitro* and *in vivo*. Exosomes are being investigated for their potential to be repurposed as therapeutics due to the important roles they play in key processes such as intercellular communication, eradication of obsolete molecules, facilitation of the immune response, antigen presentation, programmed cell death, angiogenesis, inflammation, coagulation, and dissemination of oncogenes from tumor cells.

Currently there is no gold standard for identification of exosomes [1]. Rather than relying on one method, a combination of methods is recommended that includes electron microscopy (EM), various instruments for analysis of nanosized objects (e.g., ZetaView™, qNano™, and NanoSight™ systems), and western blot for verification using markers such as CD63 and analysis of subpopulations. Care must be taken when isolating vesicles and preparing them for downstream analysis. Here we present a method for exosome isolation using Invitrogen™ Dynabeads™ magnetic beads followed by downstream western blot analysis.

Materials and methods

SW480 cells (ATCC) were cultured to confluence in RPMI 1640 medium (10% fetal calf serum, 1 mM sodium pyruvate) in bottles at 37°C, 5% CO_2. The medium was replaced with 50 mL of fresh medium. Conditioned medium was harvested and centrifuged twice (300 x *g*, 10 min, 2–8°C; 2,000 x *g*, 30 min, 2–8°C) to remove cells and debris. Pre-enrichment of exosomes was performed by adding Invitrogen™ Total Exosome Isolation Reagent to the medium, which was then incubated at 4°C overnight and centrifuged (10,000 x *g*, 1 hr, 4°C). Concentrated exosomes were resuspended in PBS. Immunomagnetic isolation of exosomes was performed using Invitrogen™ Exosome-Human CD9 Isolation Reagent (from cell culture). This reagent consists of Dynabeads magnetic beads coated with a primary antibody specific for the CD9 membrane antigen expressed on most human exosomes. Isolated exosomes were analyzed by western blot using Invitrogen™ Bolt™ gels, Mini Gel Tank, and Mini Blot Module. Exosome proteins were detected on PVDF membranes using Invitrogen™ CD9 and CD63 Monoclonal Antibodies, TrueBlot™ ULTRA anti–mouse IgG HRP (Rockland Immunochemicals, Cat. No. 18-8817-30), and Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate.

Results

Western blot analysis of pre-enriched and immuno-isolated exosomes

The amount of protein derived from exosomes can be low. To help ensure a sufficient western blot signal, a gel system with large wells was used (Bolt gels with a 40 µL capacity per well). Exosomes were lysed by adding 6 µL of 5X RIPA buffer and 1.25 µL of 25X protease inhibitor cocktail to 24 µL of pre-enriched exosomes, sonicating for 10 sec, then incubating on ice for 15 min. Lysed exosomes were prepared for electrophoresis by adding 10 µL of Invitrogen™ 4X Bolt™ LDS Sample Buffer then incubating at 70°C for 10 min. The entire sample (40 µL) was applied to the gel for electrophoresis.

For exosome isolation, using Dynabeads magnetic beads coated with primary antibodies ensures recovery of a very clean population of exosomes, starting with the pre-enriched sample (obtained, for example, with Total Exosome Isolation Reagent or ultracentrifugation). Using this method, subpopulations of exosomes can be analyzed by western blot. To maximize the western blot signal, use of a high amount of Dynabeads magnetic beads is recommended. This provides a larger total surface area for exosome binding in addition to improving the binding kinetics.

To maximize signal intensity, Bolt gels with a 40 µL capacity per well were utilized. Lysis buffer was first prepared by combining 6 µL of 5X RIPA buffer, 1.25 µL of 25X protease inhibitor cocktail, and 24 µL of water. Exosomes were then lysed by adding 30 µL of lysis buffer to the magnetic bead–exosome complexes, sonicating for 10 sec, then incubating on ice for 15 min. The tube was placed on a magnet, and the supernatant was transferred to a new tube. Lysed exosomes were prepared for electrophoresis by adding 10 µL of 4X Bolt LDS Sample Buffer to the supernatant then incubating at 70°C for 10 min. The entire sample (40 µL) was applied to the gel for electrophoresis.

After electrophoresis, the proteins were transferred to a PVDF membrane with high binding capacity and preference for hydrophobic (membrane) proteins. The Mini Gel Tank and Mini Blot Module were used for wet protein transfer. Antibodies targeting tetraspanins like CD63 often require nonreducing conditions in sample preparation for western blot, thus no 2-mercaptoethanol was included in this process. CD63 was detected using Invitrogen™ CD63 Monoclonal Antibody (clone Ts63). This protein appears as a smear between 30 and 60 kDa. Different smear patterns may occur depending on the exosome origin, indicating that the glycosylation pattern is different. CD9 was detected using Invitrogen™ CD9 Monoclonal Antibody (clone Ts9), which as it produces a single band around 24–27 kDa. Note that not all exosomes are CD9 positive, and the expression level varies among different sources. For exosomes from sources known to produce CD-low or negative exosomes, detection of CD81 using Invitrogen™ CD81 Monoclonal Antibody (clone M38) might be considered. After incubation with TrueBlot ULTRA anti–mouse IgG HRP and detection with SuperSignal West Dura Extended Duration Substrate, signal intensity and duration were suitable for imaging devices that use CCD cameras, such as the Invitrogen™ iBright™ Imaging System. The results demonstrated strong CD9 signal for pre-enriched exosomes (Figure 1, lane 1) and exosome populations recovered by immunomagnetic isolation using Exosome-Human CD9 Isolation Reagent (Figure 1, lanes 2–4).

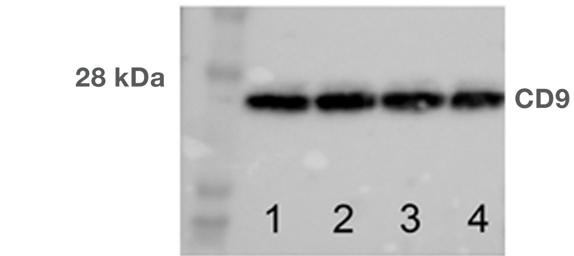

Figure 1. Western blot analysis of exosomes for CD9 marker. Exosomes were isolated from SW480 cells using the Total Exosome Isolation Reagent (lane 1) and immunomagnetically with Dynabeads CD9 (lanes 2–4).

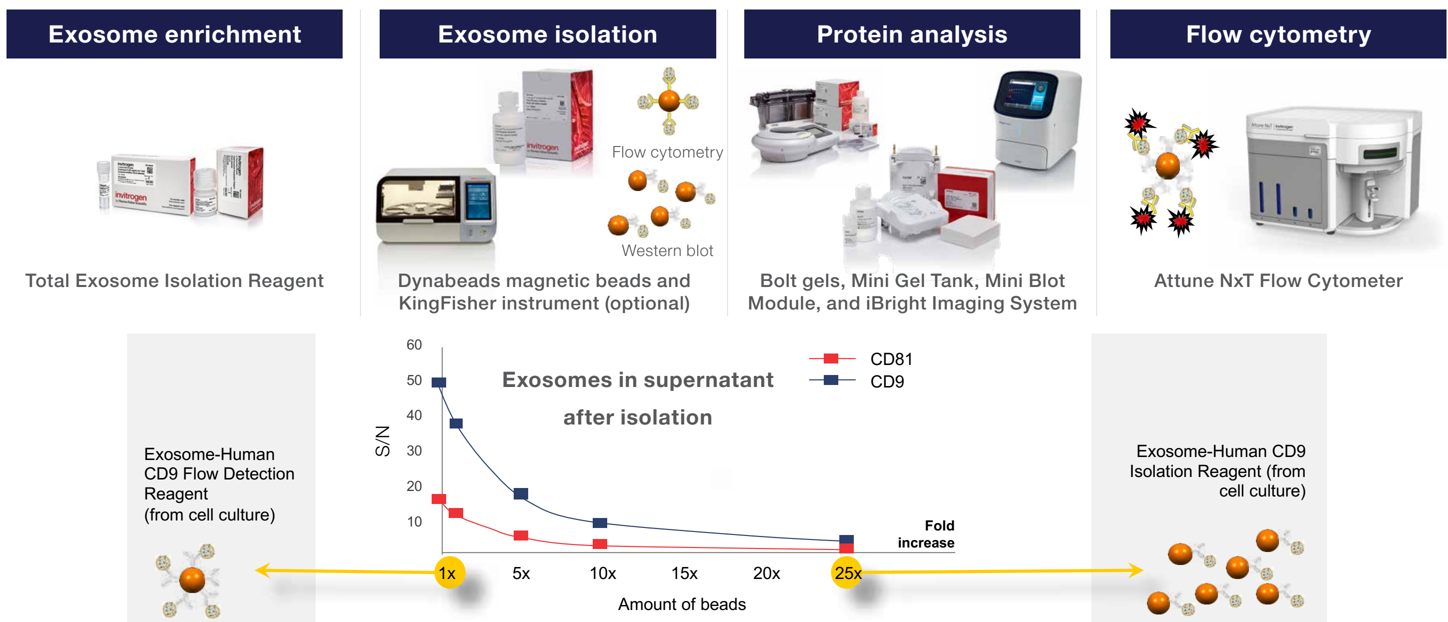

Figure 2. Exosome isolation and analysis workflow. The graph shows the effect of the amount of Dynabeads magnetic beads used for vesicle isolation. Exosomes remaining in the supernatant after isolation were analyzed by flow cytometry and are presented as signal-to-noise (S/N) ratio.

A set of western blot and flow cytometry experiments was designed to determine conditions that provide the strongest western blot signal (Figure 2). The workflow included two sequential exosome isolation steps after pre-enrichment. The first isolation step was used for western blot analysis. From the remaining supernatant a second capture step was performed using conditions suitable for flow cytometry. We used increasing amounts of Dynabeads magnetic beads in the first capture step, starting with the same amount of Dynabeads magnetic beads called for by the flow cytometry protocol (1x beads), and increasing up to 25x the number of beads used for flow cytometry. In the second round of exosome isolation, we measured the exosome depletion efficiency by isolating exosomes from the supernatant using conditions optimized for flow cytometry. The results showed that increasing the amount of isolation beads in the first isolation step lead to gradual reduction of exosomes found in the supernatant. These results were confirmed by western blot analysis of the exosomes obtained from the first isolation step (Figure 3), where increasing levels of CD9 and CD63 were detected with higher amounts of Exosome-Human CD9 Isolation Reagent.

Conclusions

The presence of $CD9^+$ and $CD63^+$ exosome subpopulations can be established by western blot analysis. Isolation of exosomes is typically performed using ultracentrifugation methods or by precipitation with the Total Exosome Isolation Reagent. Immunomagnetic isolation using Dynabeads magnetic beads enables isolation of highly pure exosome subpopulations positive for certain surface markers. The amount of magnetic beads and gel loading volume can be easily scaled for maximum detection sensitivity.

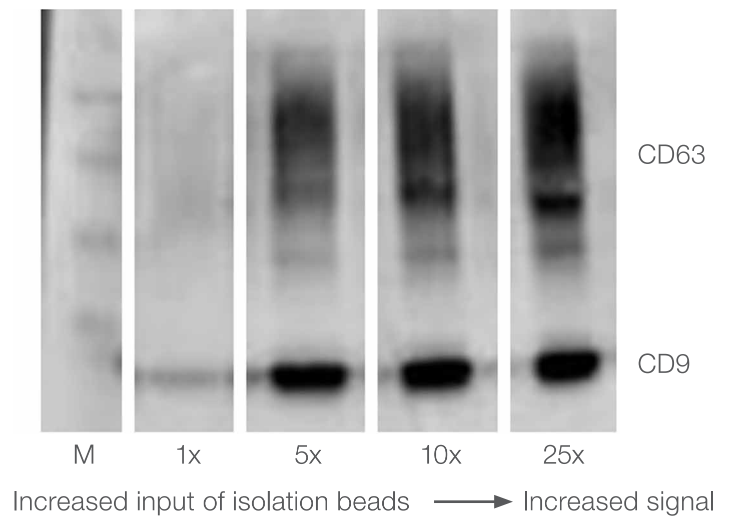

Figure 3. Western blot analysis of exosomes isolated from SW480 cells with increasing amounts of Dynabeads magnetic beads. 1x represents the amount of Dynabeads magnetic beads used for flow cytometry. Analysis was performed using CD9 and CD63 Monoclonal Antibodies.

Ordering information

Product	Cat. No.
SuperSignal West Dura Extended Duration Substrate	34075
Total Exosome Isolation Reagent (from cell culture media)	4478359
4X Bolt LDS Sample Buffer	B0007
Mini Gel Tank	A25977
Mini Blot Module	B1000
Bolt 4-12% Bis-Tris Plus Gels, 10-well	NW04120BOX
Bolt Western Pack B (PVDF)	B1000B
SeeBlue Plus2 Pre-stained Protein Standard	LC5925
SuperSignal West Dura Extended Duration Substrate	37071
Exosome-Human CD9 Isolation Reagent (from cell culture)	10614D
Exosome-Human CD9 Flow Detection Reagent (from cell culture)	10620D
DynaMag-2 Magnet	12321D
CD9 Monoclonal Antibody (Ts9)	10626D
CD81 Monoclonal Antibody (M38)	10630D
CD63 Monoclonal Antibody (Ts63)	10628D

Reference

1. Lötvall J et al. (2014) Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles* 3:26913.

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