# Automated pull-down of extracellular vesicles (EVs) on the KingFisher system using Dynabeads magnetic beads—standardizing EV capture and analysis

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## Goal

Exploring the biology of extracellular vesicles (EVs) and their potential use in clinical applications has gained considerable interest over the last several years by researchers. In biomarker and liquid biopsy assays, EVs are regarded as complementary or even superior to both cell-free DNA (cfDNA) and circulating tumor cells (CTCs). So far, the main focus has been on the nucleic acid cargo of EVs, namely the microRNA (miRNA) and mRNA. However, the protein cargo of EVs is highly complex, involved in numerous biological functions, and ripe for further in-depth analysis. In addition, researchers believe EVs show potential for therapeutic applications such as drug delivery, immune modulation, and cell-based regenerative medicine. Here we present an automated system for the isolation of EVs for further downstream protein analysis using Invitrogen™ Dynabeads™ magnetic beads and the Thermo Scientific™ KingFisher™ Flex instrument.

## Introduction

Exosomes (50–150 nm) are extracellular vesicular structures secreted by all cells in culture and found in body fluids [1]. Exosomes are involved in antigen presentation [2], apoptosis, angiogenesis, inflammation, and coagulation [3]. They can activate signaling pathways or deliver nucleic acids to distant cells [4-6]. Tumor-derived exosomes can enhance cancer progression, suppressing the immune response and transferring oncogenes from tumor host cells [7]. Targeting exosomes could increase the efficacy of therapeutic antibodies.

Exosomes are formed by invagination of the endosomal membrane, forming multivesicular bodies containing miRNA and mRNA [6,8-11]. The protein composition mirrors their endocytic origin and includes multivesicular body (MVB) machinery, tetraspanins (e.g., CD9, CD81), heat shock proteins, as well as lipid-related proteins and phospholipases [12-13] and their possible role in intercellular communication [14]. Currently, exosomes are isolated by differential ultracentrifugation, density gradients or cushions [15], size-exclusion chromatography [16], or precipitation [17-19]. To obtain ultrapure exosomes or isolate potential subpopulations of exosomes, a manual or automated immunomagnetic isolation strategy can be applied by targeting exosomal markers [20,21].

## Materials and methods

### Cell culture and exosome pre-enrichment

SW480 cells (ATCC) were cultured to confluence in RPMI 1640 medium (10% fetal calf serum, 1 mM sodium pyruvate) in bottles (37°C, 5% $CO_2$). The medium was replaced with 50 mL fresh medium. Control Jurkat cells (ATCC) were seeded at 0.4 x $10^6$ cells/mL in RPMI 1640 medium and grown for 3 days (37°C, 5% $CO_2$). Two centrifugation steps (300 x *g*, 10 min, 2–8°C; 2,000 x *g*, 30 min, 2–8°C) were performed on conditioned medium before exosome pre-enrichment by ultracentrifugation or precipitation. Ultracentrifugation was performed using an Optima™ XPN-100 Ultracentrifuge (Beckman Coulter; 100,000 x *g*, 70 min, 4°C) with a Type 45 Ti rotor. The Invitrogen™ Total Exosome Isolation Kit was used for precipitation of exosomes. Enriched exosomes were resuspended in PBS.

### Magnetic capture of exosomes and analysis

Exosomes were captured using Invitrogen™ Exosome-Human CD9 Isolation Reagent (Cat. No. 10614D) or Invitrogen™ Dynabeads™ Protein G (Cat. No. 10003D) coupled to Invitrogen™ CD9 Monoclonal Antibody (Ts9) (Cat. No. 10626D). For magnetic capture using multiple exosomal markers, prototype magnetic beads targeting CD9, CD63, or CD81 were mixed prior to capture. Prototype magnetic beads coated with antibodies targeting CD9, CD63, and CD81 on each bead were used for comparison. For generic capture of exosomes, prototype magnetic beads targeting exosome membranes were used for isolation.

20 µL of magnetic beads (flow cytometry: 1 x $10^7$ beads/mL; western blotting: 1.3 x $10^8$ beads/mL) were washed in 200 µL PBS (0.1% BSA, 0.2 µm filtered). 100 µL cell medium containing exosomes were added to the magnetic beads and incubated for 16–20 hr at 4°C. The magnet was applied, and exosome-coated beads were washed in 0.3 mL of PBS (0.1% BSA) for flow cytometry. 100 µL was used for staining with Mouse Anti-Human CD9 PE (BD Biosciences, Cat. No. 555372) and incubated for 45–60 min on a shaker (1,050 rpm) at room temperature (RT) in the dark. Flow cytometry analysis using the LSRFortessa™ system (BD Biosciences) or western blotting was performed as described [23].

### Electron microscopy

Immunolabeling and negative-staining transmission electron microscopy were performed using Invitrogen™ CD81 Monoclonal Antibody (Cat. No. 10630D), glow-discharged carbon-coated copper grids, uranyl acetate, rabbit anti-mouse antibody, and protein A gold (CMC Utrecht, Netherlands) [22].

### Chemiluminescence assay

Labeled antibodies were prepared by mixing 50 µg IgG in sodium phosphate with acridinium ester for 30 min at RT. The reaction was quenched with 20 mM sodium phosphate containing 10% lysine for 15 min at RT. The antibody was purified using a NAP-5 column (GE, Cat. No. 17-0853-02) and TBST. Light emission was triggered with 1.32% $H_2O_2$ and 0.35 M NaOH. Chemiluminescence detection was performed with the Centro LB 960 Luminometer (Berthold Technologies).

### Automated immunoprecipitation and analysis

The Thermo Scientific™ KingFisher™ Flex Purification System With 96 Deep-Well Head (Cat. No. 5400640) and BindIt™ 3.3.1 software was used for automated immunoprecipitation and downstream analysis. Electrophoresis and western blotting were performed using the Invitrogen™ Bolt™ system.

## Results

### Workflow

A typical workflow, exemplified here by flow cytometry, is divided into 3 major steps (Figure 1). The first step is the pre-enrichment step. In this step, the cell culture medium is collected. Pre-enrichment is done either by ultracentrifugation or precipitation. Alternative methods such as filtration or size-exclusion chromatography are also used. Specific capture using exosome surface markers as targets is performed in the second step. The third step is labeling of the captured exosomes.

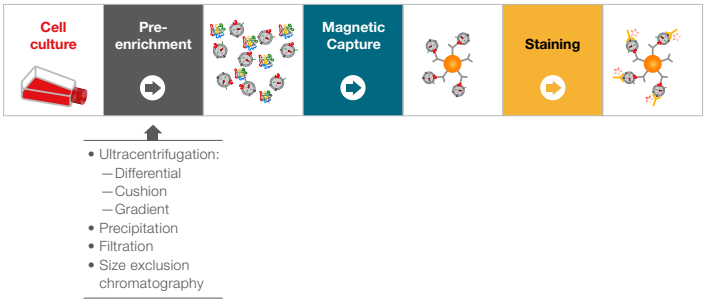


**Figure 1. Exosome enrichment, specific capture, and analysis workflow.**

### Magnetic bead optimization

The downstream application determines the number of magnetic beads used for exosome capture (Figure 2). For flow cytometry, a strong signal is obtained by keeping the number of beads very low. This strategy helps ensure a high density of exosomes per magnetic bead and a good signal. If the remaining supernatant is analyzed to quantify the number of exosomes left, the data is expected to demonstrate that very few exosomes have been pulled out. In contrast, western blotting requires a large total surface area in order to give a strong signal but leaves very few exosomes in the supernatant.

### Flow cytometry of exosomes isolated with magnetic beads

A typical flow cytometry signal after isolation of CD9-positive exosomes from SW480 cells is demonstrated in Figure 3B. As a control, CD9-negative exosomes from Jurkat cells were included (Figure 3C). The exosomes were isolated by targeting CD9 followed by staining. The scatter plot and gating profile is demonstrated in Figure 3A.

### Flow cytometry and western blot analysis of exosomes

CD9-labeled exosomes from SW480 cells were isolated directly without pre-enrichment and analyzed by flow cytometry and western blot. Figure 4A shows a correlation between increased signal-to-noise ratio (S/N) and increased bead input following flow cytometry analysis. Figure 4B compares direct isolation with exosomes pre-enriched by ultracentrifugation (UC) or precipitation. The results were compared with equal amounts of exosomes.

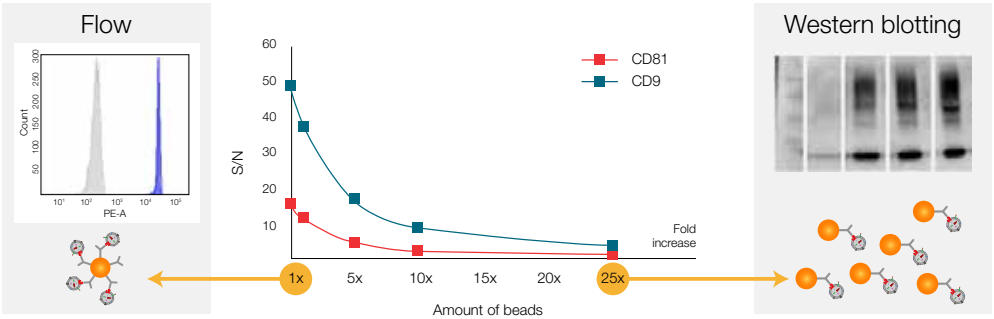


**Figure 2. Dynabeads magnetic bead optimization.**

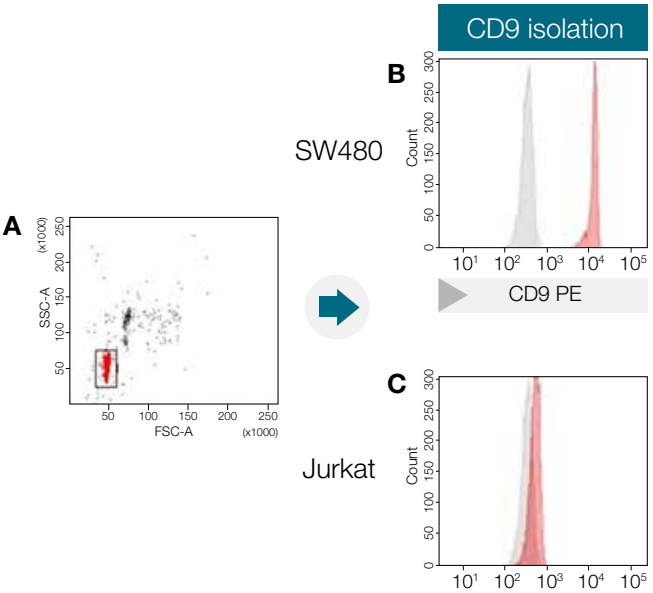


**Figure 3. Flow cytometry of exosomes isolated with Dynabeads magnetic beads.**

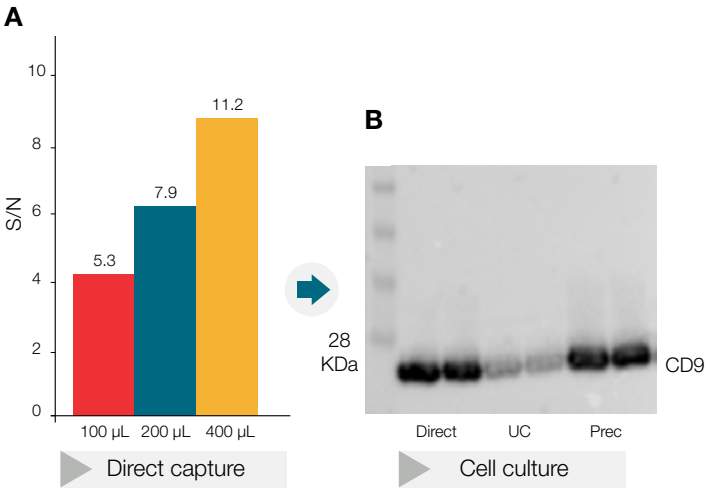


**Figure 4. Flow cytometry and western blot analysis of exosomes isolated with Dynabeads magnetic beads.**

### Capture using multiple exosomal markers

Flow cytometry and western blot analysis were performed after exosome isolation by targeting CD9, CD63, and CD81. In Figure 5A, magnetic beads targeting CD9, CD63, or CD81 were mixed prior to capture followed by CD9 staining and flow cytometry. In Figure 5B, magnetic beads coated with antibodies targeting CD9, CD63, and CD81 on each bead were used for isolation followed by CD9 staining and flow cytometry. Figure 5C shows exosomes isolated using each method and analyzed by western blotting.

### Generic isolation, release, and recapture

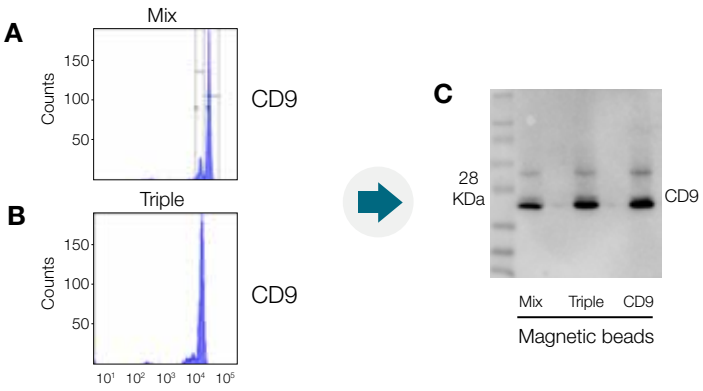


**Figure 5. Capture using Dynabeads magnetic beads with multiple exosomal markers.**

Magnetic beads targeting exosome membranes were used for generic isolation of exosomes. The exosomes were then analyzed by transmission electron microscopy (TEM), western blotting, and flow cytometry. Figure 6A shows ultrastructural analysis of the isolated exosomes by TEM. After isolation and release, captured exosomes were analyzed by western blotting (Figure 6B) and by immunolabeling and negative-staining TEM (Figure 6C). The released exosomes were recaptured and processed for flow cytometry (Figure 6D).

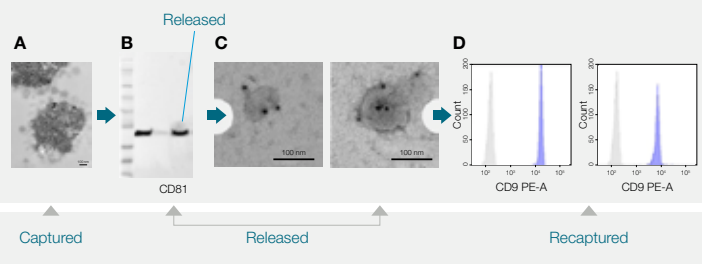


**Figure 6. Isolation, release, and recapture of exosomes using magnetic beads targeting exosome membranes.**

### Automation

A chemiluminescence assay for comparing performance using manual or automated isolation is shown in Figure 7. In this method, antibodies are labeled with acridinium prior to binding to Dynabeads Protein G. The amount of antibodies bound is estimated by measuring chemiluminescence.

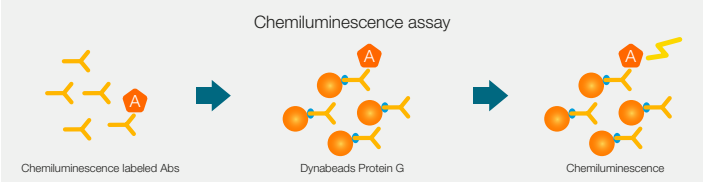


**Figure 7. Assay for comparing performance of manual vs. automated isolation.**

### Automated vs. manual pull-down, recovery, and reproducibility

Manual and automated pull-down of target is compared in Figure 8A, indicating slightly better recovery of target when using the manual protocol. The variation is very similar for manual and automated methods. Day-to-day and operator variation was addressed, and showed an overall coefficient of variation (CV) of 5.0% (Figure 8B).

**A**

| Sample | KingFisher system | Manual |
|---|---|---|
| 1 | 185,300 | 199,863 |
| 2 | 187,178 | 204,333 |
| 3 | 182,220 | 202,336 |
| 4 | 182,645 | 207,603 |
| 5 | 178,543 | 202,148 |
| 6 | 181,268 | 198,240 |
| 7 | 178,185 | 205,363 |
| 8 | 180,840 | 201,458 |
| Mean | 182,023 | 202,668 |
| STD | 3,082 | 3,017 |
| CV | 1.7 % | 1.5 % |

**B**

| Day 1, operator A | Day 2, operator A | Day 1, operator B |
|---|---|---|
| 231,884 | 257,407 | 256,915 |
| 234,854 | 257,253 | 263,366 |
| 233,403 | 253,504 | 260,675 |
| 230,895 | 259,065 | 264,146 |
| 234,249 | 258,486 | 256,563 |
| 238,061 | 257,595 | 260,865 |
| 230,513 | 261,522 | 289,933 |
| 233,182 | 258,035 | 257,354 |

| | | |
|---|---|---|
| Overall | Mean | 250,405 |
| | STD | 12,564 |
| | CV | 5.0 % |

**Figure 8. Recovery and reproducibility using automated and manual pull-down.**

### Background staining

Background analysis using silver staining after automated or manual immunoprecipitation (IP) is shown in Figure 9. Similar background staining was observed using both methods. For comparison, lysate prior to IP and antibody were included.

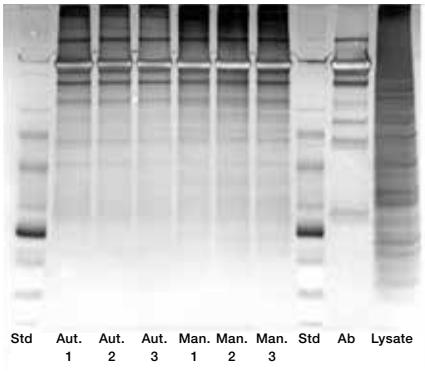


**Figure 9. Background staining after manual or automated IP.**

### Manual vs. automated IP

A comparison of manual and automated IP was performed using the KingFisher Flex Purification System and Dynabeads Protein G coupled to CD9 antibody (Figure 10). Similar results were observed after western blotting and CD9 staining using both methods.

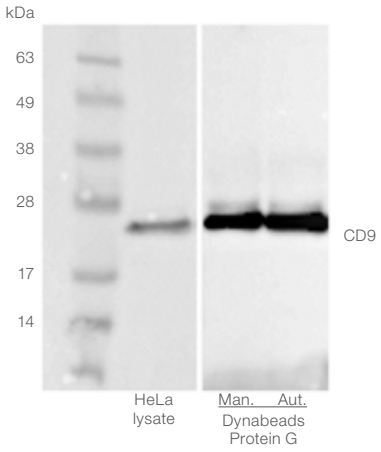


**Figure 10. CD9 staining after manual or automated IP.**

## Conclusions

Standardization of EV isolation and characterization has been a highly requested need of the International Society of Extracellular Vesicles (ISEV) for several years. A highly reproducible method for pull-down and analysis was developed using the KingFisher Flex Purification System in combination with Dynabeads magnetic beads. Critical parameters identified affecting automated pull-down include:

- Mixing conditions during washing (loss of binding)
- Number of washing steps (loss of binding)
- Elution volume (increased yield with increased volume)

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