

## Exosome Isolation for Proteomic Analyses and RNA Profiling

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### Abstract

While the existence of exosomes has been known for over three decades, they have garnered recent interest due to their potential diagnostic and therapeutic relevance. The expression and release of specific tumor-derived proteins into the peripheral circulation has served as the centerpiece of cancer screening and diagnosis. Recently, tissue-associated microRNA (miRNA) has been shown to be characteristic of tumor type and developmental origin, as well as exhibit diagnostic potential. Tumors actively release exosomes, exhibiting proteins and RNAs derived from the originating cell, into the peripheral circulation and other biologic fluids. Recently, we have demonstrated the presence of miRNAs within the RNA fraction of circulating tumor-derived exosomes. Currently, in over 75 investigations compiled in ExoCarta, over 2,300 proteins and 270 miRNAs have been linked with exosomes derived from biologic fluids. Our previous work has indicated that these circulating exosomal proteins and miRNAs can serve as surrogates for the tumor cell-associated counterparts, extending their diagnostic potential to asymptomatic individuals. In this chapter, we compare currently utilized methods for purifying exosomes for postisolation analyses. The exosomes derived from these approaches were assessed for quantity and quality of specific RNA populations and specific marker proteins. These results suggest that, while each method purifies exosomal material, circulating exosomes isolated by ExoQuick precipitation produces exosomal RNA and protein with greater purity and quantity than chromatography, ultracentrifugation, and DynaBeads. While this precipitation approach isolates exosomes in general and does not exhibit specificity for the originating cell, the increased quantity and quality of exosomal proteins and RNA should enhance the sensitivity and accuracy of down-stream analyses, such as qRT-PCR profiling of miRNA and mass spectrometric and electrophoretic analyses of exosomal proteins.

**Key words:** Exosomes, Biofluids, Proteomics, miRNA profiling

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### 1. Introduction

The release of small vesicles by cancer and embryonic cell types was initially described by our group three decades ago (1). Since that time, the release of membranous vesicles (exosomes), ranging from 50 to 100 nm, has been demonstrated in multiple cell

types and systems (2) (see Note 1). Although we demonstrated the presence of microvesicles in the peripheral circulation of patients with cancer and pregnant women in the late 1970s (3), they have received renewed interest due to the recognition that they serve as essential intercellular communication vehicles, are key determinants of the immunosuppressive microenvironment observed in cancer and pregnancy (4, 5) and provide stability to tumor-derived proteins that can serve as diagnostic biomarkers for cancer. During the 1970s, following the development of monoclonal antibody technology, significant work was directed at the identification of new biomarkers for the development of antigen-based assays for cancer. Our original work focused on the identification of specific isoforms of placental-type alkaline phosphatase (PLAP) associated with ovarian cancer (1, 3). We defined the presence of heat-stable PLAP in the sera and ascites of patients with ovarian cancer. Using size exclusion chromatography, we attempted to purify PLAP from the blood and ascites of cancer patients. While the expected molecular weight of PLAP was approximately 70,000 Da, the enzyme activity appeared in the void volume of Sephadex G200 columns. Subsequent separations using high exclusion limit, agarose-based gels revealed a molecular weight between 100 and 150 million Daltons (1). Further analyses of a large group of female patients in a blinded study found that these high molecular weight complexes were observed in all women with cancer and in women who were pregnant; however, the 100–150 million Dalton fractions were not observed in any other groups. We now recognize that the apparent absence of exosomes in normal individuals was the consequence of the limited detection of the methods used, with pathologic conditions, such as cancer and pregnancy, linked with elevated production and release of exosomes.

Analyses of these “high molecular weight” complexes with various extraction approaches indicated that they consisted of lipids and proteins (6). Using specific markers of intracellular membranous components, these complexes expressed components associated with the plasma membrane, but failed to exhibit markers of nuclear or mitochondrial membranes. This suggested that the presence of this membranous material was not the result of cell death. The membranous complexes were shown to possess markers associated with tumors in cancer patients and with placenta in pregnant patients (6, 7). Further, analyses using metabolic inhibitors demonstrated that the release of these membranous structures was energy requiring, further indicating that the release of this material was an active cellular process and not merely the consequence of cell death (6). Our initial electron micrographs of these membranous complexes revealed that they were vesicular (6). Subsequently, Pan and Johnstone (8) described the role of these vesicles from sheep reticulocytes in the recycling of transferrin

receptors (TfR) during maturation and that these vesicles of endocytic origin were secreted extracellularly. Thus, this secreted vesicular material became identified as “exosomes.”

In addition to recycling “unwanted” proteins during maturation, we now recognize that exosomes play a significant role in intracellular communication. With current mass spectrometry-based proteomic technologies, it is clear that exosomes are comprised of distinct subpopulations of macromolecules, including proteins and RNAs, associated with cell type-linked functions. Recent data indicate that these macromolecules can be transferred to target cells and can mediate intercellular interactions, nonclassical protein secretion, signaling between neighboring cells resulting in pathologic conditions by becoming functional in their new microenvironment (9, 10). While tumor-derived exosomes can induce events associated with the pathology of cancer, the renewed interest in exosomes has been their potential diagnostic utility. Mathivanan and Simpson (11) have established a compendium of exosomal proteins and RNA (ExoCarta), since numerous studies have demonstrated the presence of tissue/cell type-specific proteins associated with exosomes. The presence of tissue/cell type-specific marker proteins associated with specific exosome populations can serve as surrogates, identifying the presence of the originating cell. Similarly, in 2008, we published the initial description of microRNA (miRNA) associated with circulating exosomes derived from ovarian cancer patients and demonstrating their diagnostic use (12), which would extend its utility to screening asymptomatic individuals.

While currently data from 75 studies have been cataloged in ExoCarta, the various groups investigating exosomal components lack consensus on the methods for isolating exosomes from biologic fluids of cancer patients, which include ultracentrifugation, chromatography, and magnetic beads. In this chapter, we compare these methods along with a new precipitation procedure, for the isolation of exosomes from biologic fluids for subsequent analyses, including RNA profiling or protein analyses using mass spectrometry and electrophoresis-based proteomic techniques.

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## 2. Materials

### 2.1. Patient Samples

To compare methods for the isolation of exosomes from biologic fluids derived from cancer patients, three ascites specimens were obtained from women diagnosed with stage III serous adenocarcinoma of the ovary.

### 2.2. Exosome Isolation Methods

1. Size exclusion chromatography. Size exclusion chromatography gel comprised of 2% agarose (Agarose Bead Technologies, Tampa, FL).

2. Magnetic beads. Anti-EpCAM antibodies coupled to magnetic microbeads (#161.02, DynaBeads, Invitrogen, Carlsbad, CA).
3. Ultracentrifugation. Beckman Optima MAX-XP centrifuge with a TLA-120.2 rotor (Beckman Coulter, Fullerton, CA).
4. ExoQuick precipitation. ExoQuick precipitation reagent #EXOQ5A-1 (System Biosciences, Mountain View, CA).

### **2.3. RNA Isolation and Analysis**

1. Trizol (#15596-028, Invitrogen).
2. Agilent small RNA chip and reagent kit (#5067-1548).

### **2.4. Exosome Protein Analysis by SDS-PAGE and Western Immunoblotting**

1. Separating buffer (4× solution): 1.5 M Tris(hydroxymethyl)aminomethane (Tris-HCl), pH 8.7, 0.4% SDS (Bio-Rad Laboratories, Hercules, CA).
2. Stacking buffer (4× solution): 0.5 M Tris-HCl, pH 6.8, 0.4% SDS (Bio-Rad Laboratories).
3. 30% Acrylamide/Bis solution (37.5:1 with 2.6% C) and N,N,N,N'-tetramethyl-ethylenediamine (TEMED, Bio-Rad Laboratories).
4. Ammonium persulfate: 10% solution in water prepared immediately prior to use.
5. Running buffer (10× solution): 250 mM Tris, 1,920 mM glycine, 1.0% (w/v) SDS, pH 8.3 (#161-0772, Bio-Rad Laboratories).
6. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad Laboratories).
7. Transfer buffer (Tris-Glycine): 25 mM Tris, 190 mM glycine, 20% (v/v) methanol plus 0.05% (w/v) SDS.
8. Nitrocellulose membrane (0.45 μm, #162-0117, Bio-Rad Laboratories) and thick filter paper (#170-5040, Bio-Rad Laboratories).
9. Tris-buffered saline, as a 10× solution (#170-6435, Bio-Rad Laboratories) with 0.1% Tween-20 (TBS-T).
10. Blocking buffer: 5% (w/v) nonfat dry milk (#170-6404, Bio-Rad Laboratories) in TBS-T.
11. Bradford protein microassay kit with BSA standard (#500-0203, Bio-Rad Laboratories).
12. Primary antibody dilution buffer: Super-Block solution (#37537, Pierce Chemical Co.).
13. Primary antibody: mouse antihuman placental alkaline phosphatase (sc-47691, Santa Cruz Biotechnology, Santa Cruz, CA).
14. Secondary antibody: Antimouse IgG conjugated to horseradish peroxidase (#170-5047, Bio-Rad Laboratories).

15. Immun-Star HRP chemiluminescent reagents (#170-5040 from Bio-Rad) and Bio-Max Light-2 film (Kodak, Rochester, NY).
16. Visualized bands were digitized and quantitated using Un-Scan-it Software (Silk Scientific Corp., Orem, UT).

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### 3. Methods

Exosomes can be isolated from blood (plasma or serum) or ascites (see Note 1). For plasma and ascites, sodium heparin (1,000 IU/L) is added prior to isolation. To isolate exosomes from biologic fluids, the ascites (2 mL) was initially centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  to remove any cellular debris.

#### **3.1. Exosome Isolation by Ultracentrifugation**

1. The cell-free ascites specimens were centrifuged at  $100,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ .
2. The pellet containing exosomes was resuspended in PBS and then recentrifuged at  $100,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ .
3. The resulting pellet was used for Trizol extraction for RNA and protein analyses.

#### **3.2. Exosome Isolation by Size Exclusion Chromatography**

1. For isolation by size exclusion chromatography, 2 mL aliquots of patient-derived cell-free ascites were applied to a 2% agarose-based gel column ( $2.5 \times 16$  cm). For optimal separation, the sample volume should be  $1/20$  of the total column volume (as defined by  $\pi r^2 h$ ).
2. The column was eluted isocratically with PBS at a flow rate of 1 mL/min, monitoring at 280 nm and fractions (2 mL) were collected.
3. The void volume fractions (based on absorbance at 280 nm) were pooled and centrifuged at  $100,000 \times g$  for 1 h at  $4^{\circ}\text{C}$ .
4. The resulting pellet was used for Trizol extraction for RNA and protein analyses.

#### **3.3. Exosome Isolation by Magnetic Beads**

1. For isolation of specific subsets of exosomes, exosomes were selectively isolated from ascites specimens by absorption to anti-EpCAM antibodies coupled to magnetic microbeads.
2. Anti-EpCAM coupled to microbeads (50  $\mu\text{L}$ ) were added to the ascites specimens (2 mL), mixed, and incubated on a shaker for 2 h at room temperature.
3. Each tube was placed in the magnetic separator and fluid removed, leaving the magnetic beads and bound exosomes attached to the side of the tube.
4. The tube was removed from the magnetic separator and the beads rinsed with 500  $\mu\text{L}$  TBS and the separation repeated.

5. After the wash step, the tube was removed from the magnetic holder and the bead/exosome complex was used for Trizol extraction for RNA and protein analyses.

### **3.4. Exosome Isolation by ExoQuick Precipitation**

1. To isolate exosomes by the precipitation approach, the ascites specimen (2 mL) was transferred to a sterile tube and 0.5 mL of ExoQuick exosome precipitation solution was added and mixed.
2. The mixture was incubated overnight (at least 12 h) at 4°C and then the mixture was centrifuged at 10,000×*g* in a microfuge for 5 min at 4°C.
3. The supernatant was aspirated and the exosome pellet was extracted using the Trizol extraction procedures for RNA and protein analyses.

### **3.5. RNA Isolation and Analysis (See Notes 2–4)**

1. Total RNA was isolated from exosomes derived from each isolation method using Trizol according to manufacturer's instructions (Invitrogen), except with the isopropanol precipitation step extended to overnight.
2. The RNA quality and yield was accessed using a GeneQuant II.
3. Small RNAs were analyzed with the Agilent 2100 Bioanalyzer Lab-on-a-Chip instrument system (Agilent Technologies, Santa Clara, CA), using the Agilent Small RNA chip and reagent kit (see Note 3).
4. Approximately 100 ng of isolated total RNA in 1 µL was applied to each run.
5. The manufacturer's recommended protocol was strictly followed to obtain Bioanalyzer profiles for the size range of 6–150 nucleotides (nt).
6. The profiles were calibrated for size (nt) using the small RNA ladder supplied with the kit, containing markers of 20, 40, 60, 80, and 150 nt in size, as reference.
7. The instrument software quantitated the peak area between 0 and 150 nt as small RNA region, the area within 10–40 nt as miRNA region, and provides percentages of miRNA detected for each sample (see Note 5).

### **3.6. Exosome Protein Analysis by Western Immunoblotting (See Note 6)**

1. Exosomal protein isolation was performed by continuing the Trizol isolation procedure, as described by the manufacturer.
2. The quantity of protein was determined by the Bradford microassay method, using BSA as a standard.
3. SDS-PAGE was performed by the method of Laemmli (13). Proteins from each exosome isolate were standardized to the original sample volume and equal volumes were applied per lane of a 12.5% SDS-PAGE gel.

4. Western immunoblotting was performed to analyze the presence of the specific marker protein, PLAP as described by Brown (14).
5. The SDS-PAGE gel was transferred to a nitrocellulose membrane, the membrane blocked for 1 h at room temperature with nonfat dried milk, and probed overnight at 4°C with primary antibody.
6. The bound immune complexes were visualized by ECL onto X-ray film and the resulting bands were quantified by digitizing the X-ray film image.

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## 4. Notes

In the late 1970s, biofluids (ascites and blood) were fractionated by size exclusion chromatography to identify and isolate circulating tumor-derived antigens as potential diagnostic biomarkers (see Notes 7–13). Based on these initial studies, we identified a “high molecular weight” form of these ovarian tumor antigens (in excess of 50 million Daltons). Due to their size, we attempted to sediment these high molecular weight antigens by ultracentrifugation prior to chromatographic separation from other blood components; however, little of this material could be pelleted (also see Notes 2 and 6). By the early 1980s, we referred to these vesicular structures as “membrane vesicles,” which were subsequently termed exosomes. A major obstacle to the study of circulating tumor proteins is their relative instability. The association of tumor associated proteins with exosomes, in contrast, creates a stable environment for these proteins. This enhanced stability has renewed interest in exosomes and their associated components as diagnostic biomarkers. The current use of mass spectrometry for proteomics on circulating biomarkers focuses on proteins in the peripheral circulation. The association of tumor-derived proteins with exosomes allows the proteins to be studied in their intact forms and with appropriate conformations:

1. Exosomes have been isolated from most biofluids (blood, ascites, milk, urine) (10, 15–19). While exosomes have been isolated from both plasma and serum with similar results, our experience is that better yields of protein and RNA are obtained with plasma.
2. For isolation of RNA for further analyses, based on the  $OD_{260}/OD_{280}$  ratios, in general, the ExoQuick precipitation (Subheading 3.4) produced an enhanced concentration of RNA and a higher ratio indicating greater purity (Fig. 1). The use of size exclusion chromatography (Subheading 3.2) to

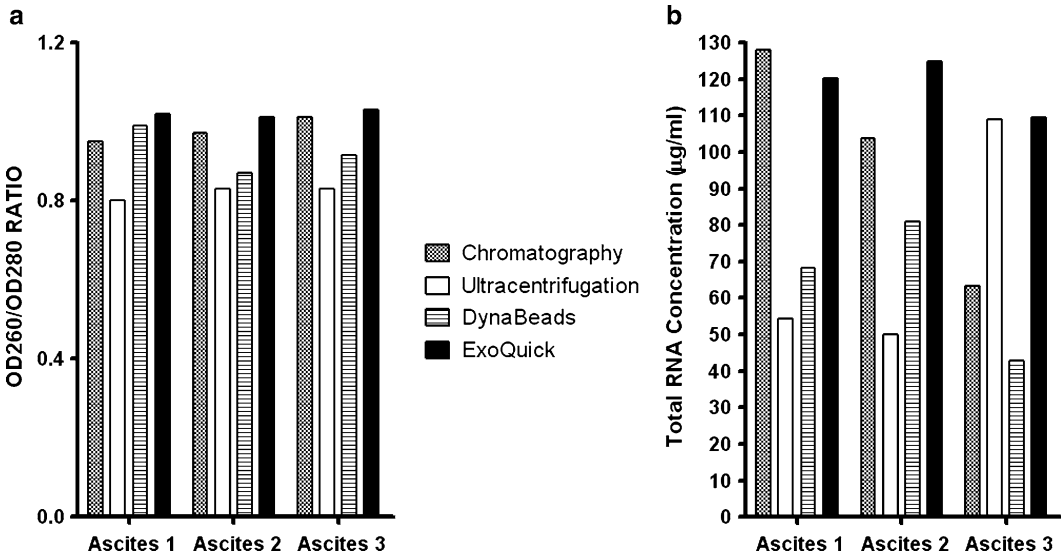


Fig. 1. Total RNA from exosomes isolated from biologic fluids (ascites) of ovarian cancer patients by size exclusion chromatography, ultracentrifugation, anti-EpCAM DynaBeads, and ExoQuick precipitation procedure. Panel (a) presents  $OD_{260}/OD_{280}$  ratio for RNA purity. Panel (b) presents total RNA isolated.

- isolate circulating exosomes also yields similar ratios of purity. Ultracentrifugation (Subheading 3.1) produced the lowest ratios for RNA integrity, potentially due to cosedimentation of protein impurities. The use of anti-EpCAM dynabeads (Subheading 3.3) isolated exosomal RNA similar in purity to chromatography, although the quantity was significantly less.
3. In our initial characterization of exosomal RNA, the analysis was limited by the use of the RNA6000 Pico LabChip kit (Agilent), which defined only the absence of 18S and 28S RNA and poorly discriminated small RNAs [12]. Thus, for this comparative characterization of small RNA populations derived from circulating exosomes, the small RNA LabChip kit was used.
  4. In terms of RNA isolation, the ExoQuick precipitation (Subheading 3.4) produced the greatest quantity of exosomal RNA in all three ascites specimens with the greatest purity. When the isolated exosomal small RNA fraction was analyzed by Bioanalyzer scanning (Fig. 2), it revealed that the exosomal RNA derived by the ExoQuick precipitation approach yielded more small RNA with a greater percent of miRNA than that observed with anti-EpCAM DynaBeads (percent of miRNA was 53% vs. 47%, respectively).
  5. Since miRNA signatures have been linked with cancer type, stage and grade, as well as predicting therapeutic responses, the enhanced isolation of miRNA is essential for accurate miRNA profiling.

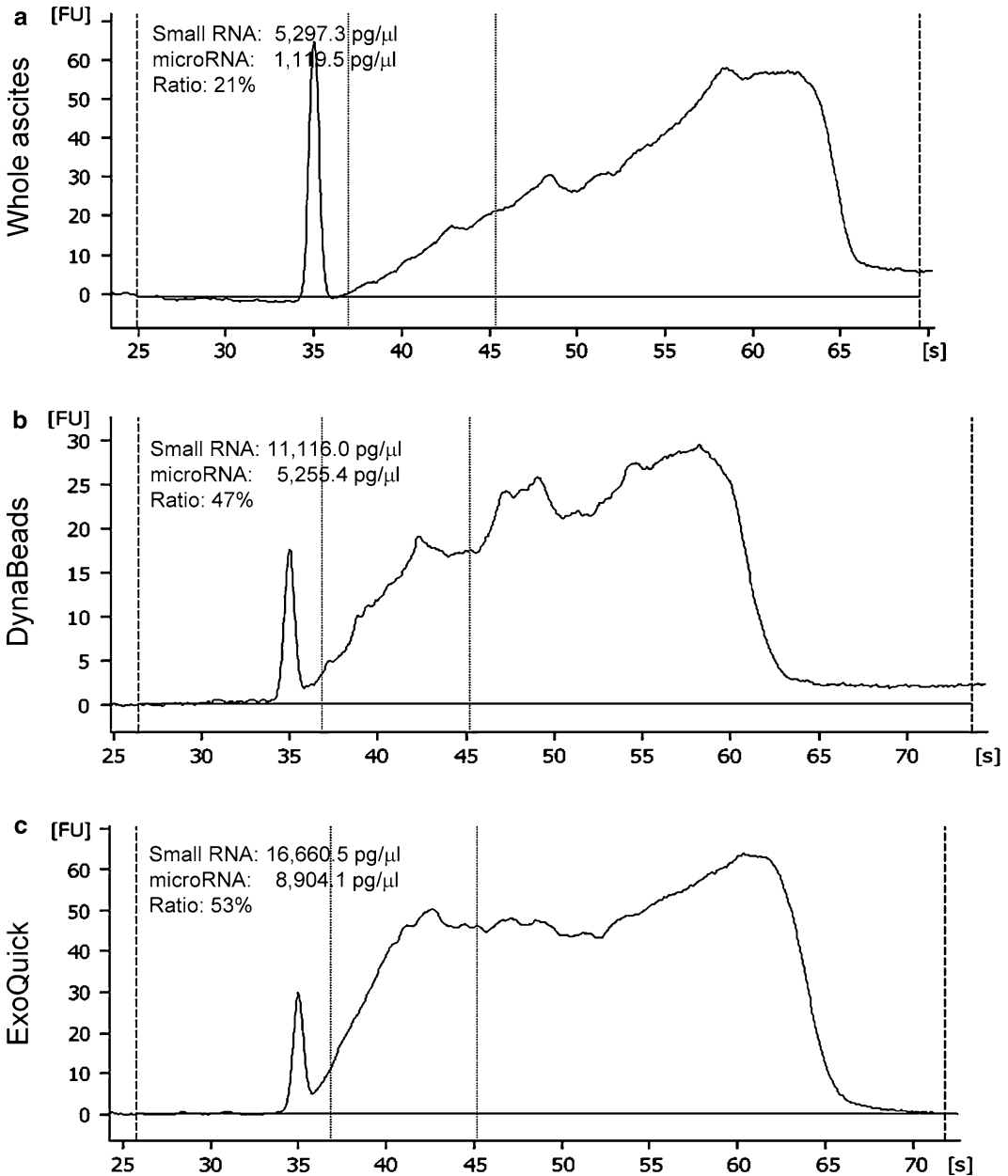


Fig. 2. Bioanalyzer electropherogram characterization of the small RNA fractions from Trizol extraction of unfractionated ovarian cancer ascites (panel a) compared with Trizol extraction of exosomes isolated from the same ascites by anti-EpCAM DynaBeads (panel b) and ExoQuick precipitation (panel c).

6. For isolation of exosomal protein for further analyses, the ExoQuick precipitation yielded an increased concentration of protein from each ascites sample (Fig. 3). The use of size exclusion chromatography (Subheading 3.2) to isolate circulating exosomes resulted in the second highest concentration of exosomal protein. Ultracentrifugation (Subheading 3.1) produced the lowest ratios of exosomal protein. The use of

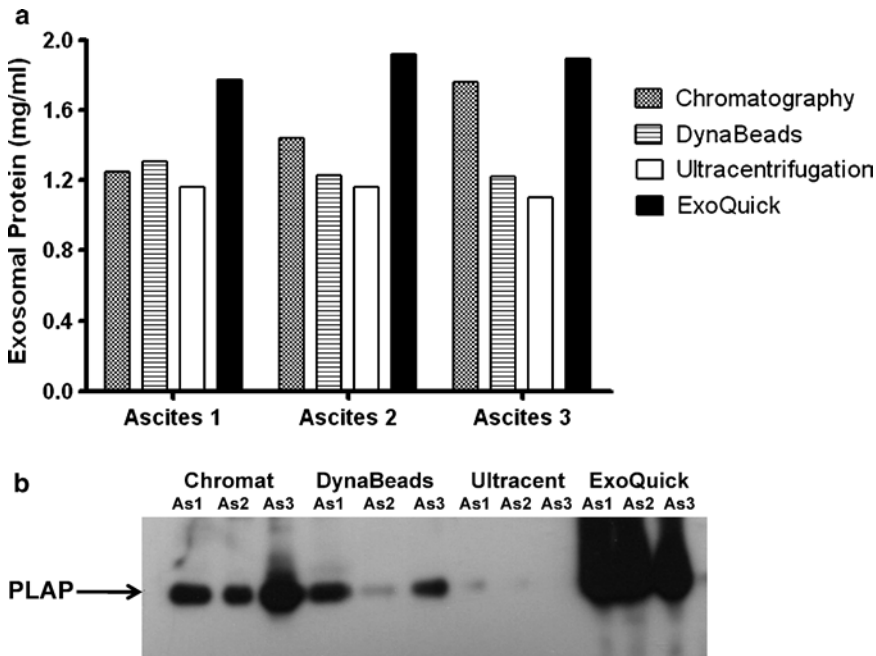


Fig. 3. Exosomal proteins isolated from biologic fluids (ascites) of ovarian cancer patients by size exclusion chromatography, ultracentrifugation, anti-EpCAM DynaBeads, and ExoQuick precipitation. Panel (a) presents total exosomal protein concentrations isolated by each technique. Panel (b) presents the western immunoblots for PLAP, visualized by ECL, associated with exosomes isolated by each approach.

anti-EpCAM Dynabeads (Subheading 3.3) isolated exosomal proteins at a level intermediate between chromatography and ultracentrifugation.

7. During the past three decades, we evaluated different approaches for exosome isolation from blood and other biofluids that would allow high-throughput isolation of intact exosomal proteins for proteomic analyses, while also retaining their biologic activities. Analysis of these isolated exosomal proteins for the level of the specific protein marker, PLAP, indicated that, when standardized to volume of the initial sample, the ExoQuick-derived exosomes exhibit the highest level of the tumor marker, followed by chromatography and DynaBeads.
8. It should be noted that chromatography, ultracentrifugation, and ExoQuick precipitation cannot preferentially isolate tumor-derived exosomes. These are general approaches that isolate all circulating exosome populations.
9. We have focused on approaches appropriate for the small sample volumes, feasible with clinical specimens. Chromatography is labor-intensive and requires specialized equipment. Chromatographic separation of exosomes exhibits an incomplete recovery of exosomes, due to their dilution

into multiple fractions and nonspecific adherence to the agarose chromatography beads. Sedimentation of exosomes requires an ultracentrifuge and we have previously demonstrated that direct ultracentrifugation of biofluids does not pellet all, or even most, of the exosomes present. This appears to be due to protein composition of the biofluid and the resulting viscosity; exosomes pellet poorly, without prior chromatographic separation (1). The ExoQuick precipitation uses only low-speed microfuge and does not dilute the biofluid-derived exosomes.

10. Of the approaches described here, only immunoaffinity, using the anti-EpCAM, can selectively derive any specific population of exosomes.
11. Since we are investigating cancers of epithelial origin, our immunoaffinity approach is based on anti-EpCAM. Based on our previous work, we are aware that antibody binding to exosomal antigens is distinct from binding their counterparts expressed on cells (1). This may relate to differences in glycosylation of exosomal proteins or other aberrant posttranslational modifications or differences in lipid/protein ratios of the membranes of exosomes versus cells, resulting in differential exposure of antigenic epitopes on integral membrane proteins or changes in protein motility due to the rigidity of exosomal membranes compared to cellular membranes. These may effectively reduce the binding of exosomal target proteins with antibodies linked to beads and reducing the levels of specific exosomes isolated.
12. Other investigators have utilized other surface markers or exosome markers to isolate exosomes from biologic fluids. Selection for A33-positive exosomes has been reported for isolating exosomes from colorectal cancer patients (11). Other investigators have also used anti-CD63 for immunoaffinity selection of circulating exosomes (9); however, as a general exosome marker, the use of CD63 isolates all circulating exosomes, cancerous and noncancerous.
13. Since the level of exosomes in biologic fluids is significantly elevated in pathologic conditions, it may not be essential to separate tumor-derived exosomes from the normal exosome background. The presence of tissue/cell type-specific marker proteins or cell-specific miRNA signatures associated with specific exosome populations can serve as surrogates, identifying the presence of the originating cell. Thus, postisolation analyses by RNA profiling or proteomics should be capable of identifying the presence of specific tissue/cell type components or signatures associated with tumor exosomes above the background of exosomes from normal cells.

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