

## Direct Isolation of Exosomes from Cell Culture: Simplifying Methods for Exosome Enrichment and Analysis

### Abstract

Exosomes, (50-150 nm sized vesicles), are secreted by all cells and found in all body fluids investigated. Isolation and characterization of exosomes from cell culture systems and body fluids provide valuable information about the biological system. The level of exosomes in human serum will vary depending on many factors such as age, sex, time of sample collection (circadian rhythm/nutrition status), and of particular interest during diseased conditions. Such information may possibly be used for early detection of disease, monitoring of disease and/or effect of treatment.

The established standard for exosome isolation is differential ultracentrifugation a method which cannot discriminate between exosome subpopulations or other particles with similar size and density.

Here we have established a direct method for specific isolation of exosomes from cell culture supernatant suitable for a range of different downstream applications. Magnetic beads targeting human CD9 or CD81, common exosomal markers, were used to first isolate and characterize pre-enriched exosomes addressing critical factors (volume, time and exosome concentrations) to establish optimal and comparable isolation conditions. Finally, exosomes were isolated and characterized directly from cell culture media.

In conclusion, the data demonstrates the ability to capture exosomes directly providing the possibility to characterize and compare exosomes from different sources and increasing the compatibility in terms of applications.

**Keywords:** Exosomes; Direct isolation; Flow cytometry; Western blotting; Immunoassay; Magnetic beads; CD9; CD81

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### Introduction

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

Exosomes, formed by invagination of the endosomal membrane forming multivesicular bodies contain miRNA and mRNA [6,8-11]. The protein composition mirrors their endocytic origin, MVB formation machinery, tetraspanins (e.g. CD9, CD81), heat shock proteins in addition to 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 [16] or precipitation [17-19]. To obtain ultrapure exosomes or isolation of potential subpopulation of exosomes, an immunomagnetic isolation strategy can be applied by targeting exosomal markers [20,21].

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The aim of this study was to address critical factors for direct exosome isolation and to implement this knowledge to establish an easy, reliable and direct method for exosome characterization ensuring compatibility with downstream analysis.

## Materials and Methods

### Reagents

For isolation of CD9<sup>+</sup> and CD81<sup>+</sup> exosomes Dynabeads® coated with monoclonal anti-human CD81 (Thermo Fisher Scientific, Oslo, Norway) were applied. Primary antibodies for Western blotting analysis: mouse anti-CD81 (10630D) and CD9 (10626D, Thermo Fisher Scientific, Oslo, Norway). Primary antibodies for flow cytometry: mouse anti-human CD9 PE (555372) and -CD81 PE (555676, BD Biosciences USA). HRP-coupled anti-mouse IgG Trueblot was purchased from Rockland (US). AFP-substrate: DynaLight Substrate with Rapid Glow (4475406), Life Technologies. Chemiluminescence reader: BioTek Synergy (BioRad). AFP-conjugated antibodies (CD9 and CD81) for immunoassay (in house conjugation). Protein-A gold was obtained from CMC (Utrecht, Netherlands).

### Cell culture

The SW480 cell line (ATCC) was cultured to confluence in RPMI 1640 (10% FCS, 1 mM Sodium-pyruvate) in cell culture bottles (37°C and 5% CO<sub>2</sub>) and medium was replaced with 50 mL fresh medium. Jurkat cells (ATCC) were seeded at a density of 0.4 x 10<sup>6</sup> cells per mL in fresh RPMI medium and grown for 3 days before harvest.

### Exosome harvest and pre-enrichment

The cell culture medium was replaced with fresh complete medium with FBS 3 days before exosome harvesting. Jurkat and SW480 cells (5 x 10<sup>7</sup>) were cultured for each exosome sample preparation. After 24 h, cells and debris were removed by centrifugation at 350x g for 10 minutes and 2,000 x g for 30 min. For ultracentrifugation, the samples were pre-enriched at 100,000 x g for 70 min at 4°C using a Ti45 rotor (Beckman) and Beckman ultracentrifugation tubes (355622) on a Beckman Optima XPN-100. For precipitation, Total Exosome Isolation kit (Thermo Fisher Scientific, USA) was added to the cell- and debris-free cell medium (1:2 with exosome isolation reagent and cell medium, respectively). Cell medium and the exosome isolation reagent were mixed by brief vortexing and incubated at 4°C overnight before centrifugation at 4 °C at 10,000 x g for 1 h. The pellet containing pre-enriched exosomes was resuspended in PBS (e.g. 400 µl for 10 ml cell media input).

### Size measurement of exosomes

Analysis of pre-enriched exosomes was performed as previously described [22] using the NanoSight LM10 instrument.

### Real time quantitative PCR

RNA isolation, reverse transcription and real time quantitative PCR analysis were performed as described earlier [23] using the following primers: Hs9999905\_m1 (GAPDH), Hs03023880\_g1 (ACTB), Hs9999901\_s1 (18S), Let7a: has-let-7a, 000377, and has-miR-16, 000391 (miR16).

### Immunolabeling and negative stain of exosomes for electron microscopy

Immunolabeling followed by negative stain and electron microscopy was carried out targeting CD81 as previously described [24] and analyzed at the University of Oslo, Norway on Philips CD100. Images were recorded at 80 kV. Further image processing was done with Adobe Photoshop software.

### Preparation of pre-enriched exosomes for Western immunoblotting analysis

The total pre-enriched exosome samples were prepared for Western blotting as described by Oksvold et al [22].

### Direct capture of exosomes from cell culture supernatant for flow cytometry, Western blotting analysis and immunoassay

20 µL of magnetic beads (1 x 10<sup>7</sup> beads/mL 1.3 x 10<sup>8</sup> beads/mL, and 0.5 x 10<sup>7</sup> beads/mL were used for flow cytometry, western blotting and immunoassay, respectively) and washed in 200 µL PBS (0.1% BSA, 0.2 µm filtered). 0-100 µL of cell-and debris-free cell medium containing exosomes were added to the magnetic beads and incubated for 16-20 h at 4°C while rotating/mixing unless other incubation times are indicated. After incubation, the tubes containing magnetic beads coated with exosomes were subjected to a short centrifugation followed by applying a magnetic field. Washing the exosome coated beads twice were done by removing the supernatant while being on the magnet and replaced the supernatant with 0.3 mL of PBS (0.1% BSA). Samples were then processed for flow cytometry or Western blotting. For immunoassay washing was performed two to three times in microtitre plates in 100 µL TBS (0.1% Tween-20) buffer.

### Preparation of immunoisolated exosomes for Western immunoblotting analysis

Magnetic beads coated with anti-human CD9 or CD81 were mixed with exosome samples and incubated over night at 4°C while mixing. The beads were washed in PBS (0.1% BSA) and lysed in 25 µL RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with proteinase inhibitors followed by 15 min incubation on ice. The lysate was mixed 1:1 with 2x Tris lysis buffer under non-reducing conditions. Samples were further processed for SDS-PAGE.

### Flow cytometry

CD9 or CD81 positive exosomes captured directly from cell culture media or after pre-enrichment were resuspended in 300 µL PBS with 0.1% BSA. 100 µL was used per flow staining and incubated for 45-60 minutes on a shaker (1050 rpm) at room temperature. The labelled exosomes were washed and analysed using BD LSRII and BD FACS Diva.

### Immunoassay

CD9 (also positive for CD81) positive exosomes captured directly from cell culture media with magnetic beads were stained with AP conjugated CD81 or CD9 antibodies (1:1000 in TBS-T) for 60 minutes on a shaker (1050 rpm) at room temperature. The labelled exosomes were washed three times, transferred to new wells, added substrate and analysed using BioTek Synergy (BioRad) chemiluminescence reader.

## Results

### Analysis of exosome markers in pre-enriched exosome samples

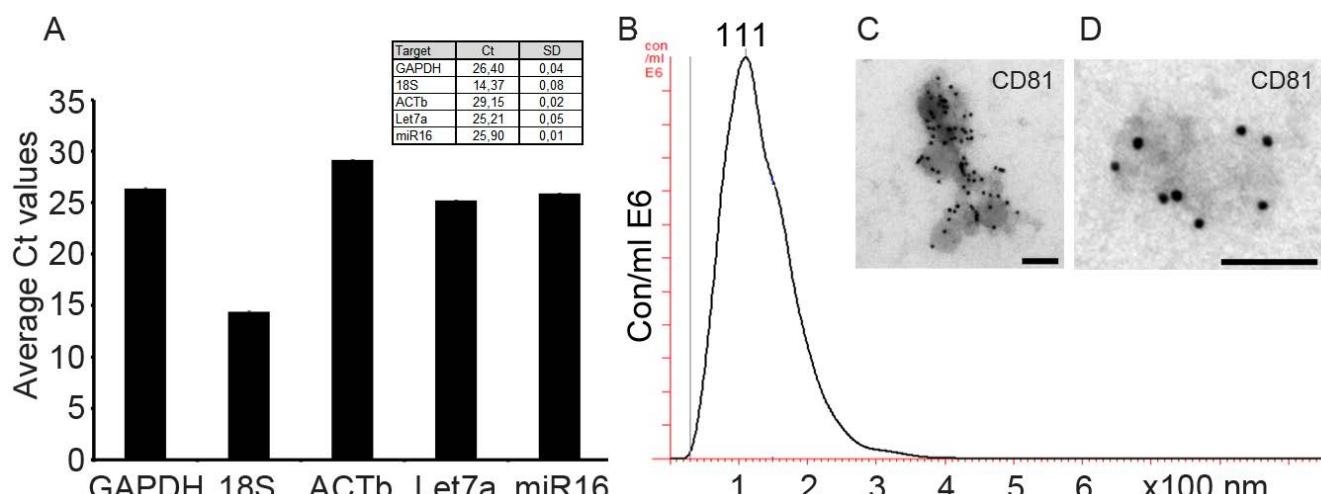
Prior to analysis, the RNA content of the exosomes was verified. SW480 cell culture media was harvested after 3 days followed by precipitation and analysis of mRNA/rRNA and microRNA content. The exosomes contained full length mRNA/rRNA (GAPDH and 18S) and microRNAs (ACTb, Let7a and miR16) typical for exosomes (**Figure 1A**). Then, the size distribution was estimated. The precipitated exosomes from SW480 cell culture media was analysed by the Nanosight LM10 instrument. The size distribution showed a size range (top at 111 nm) (**Figure 1B**) comparable to standard exosome preparations by ultracentrifugation. Finally, the pre-enriched vesicles were identified by electron microscopy. Pre-enriched exosomes were prepared from SW480 cells. Here antigens located on the surface of the exosomes can bind detection antibodies such as the tetraspanin protein CD81, commonly identified in exosomal preparations [25]. Immunoelectron microscopy confirmed the identity of the vesicles both in terms of size and expression of CD81 (**Figure 1C and 1D**).

Pre-enriched exosomes were then subjected to Western blotting analysis to target the exosomal markers CD9 and CD81. Currently, the most common method for exosome pre-enrichment is differential ultracentrifugation. Therefore, the presence of the exosomal marker CD9 and CD81 in exosomes prepared by ultracentrifugation were compared with CD9 and CD81 expression in exosomes prepared by precipitation (**Figure 2A and 2B**). Exosomes were pre-enriched from the same cell culture medium using the same amount of input volume. In addition, the same volume of exosomes applied to each well was the same. Therefore, the results from these two pre-enrichment methods

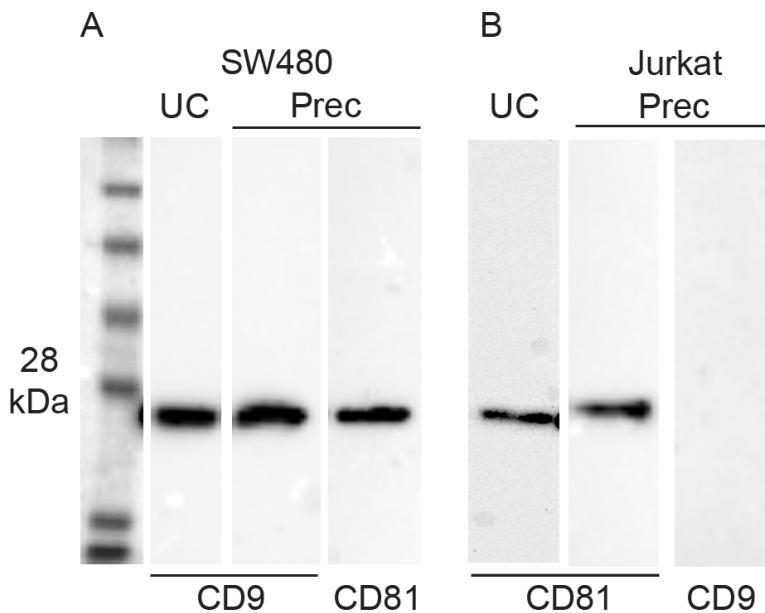
should be comparable. The data showed equal amounts of CD9 from SW480 derived exosomes using the two methods for exosome pre-enrichment (**Figure 2A**). Furthermore, CD81 was also detected in SW480 derived exosomes after pre-enrichment using precipitation (**Figure 2A**). CD81 was detected in exosomes derived from Jurkat pre-enriched by ultracentrifugation and precipitation. CD9 was absent in equal to the findings by Oksvold et al. [22]. The presence of contaminating non-exosomal vesicles were examined by staining for gp96, an endoplasmic reticulum marker. Gp96 was only observed in cell lysate and absent in pre-enriched exosomes (data not shown).

### Analysis of pre-enriched exosome sub-population

Common methods for exosome sample preparation are ultracentrifugation and precipitation. Both methods are challenging in terms of lack of vesicle specificity obtaining vesicle populations with heterogeneous size distributions indicating that other vesicles are co-enriched [26]. Size of exosomes will provide little information on FACS given the limited resolution. Magnetic beads can provide a solid support at a size detectable by flow cytometry. Increased sensitivity and removal of potential protein aggregates and possibly isolate subpopulations of exosomes were obtained by immunomagnetic separation strategy followed by downstream flow analysis. The forward/side scatter plot demonstrated how the magnetic beads were displayed in flow cytometry (**Figure 3A**). Immunomagnetic isolation of exosomes and staining with isotypic antibody demonstrating the background (**Figure 3B-3E**, grey histograms) compared to specific signal obtained by relevant CD9 and CD81 staining of bead isolated exosomes (**Figure 3B-3E**, red histograms). With the purpose of maximize the flow signal and be able to compare data the amount of magnetic beads and the isolation volume were kept constant, ensuring equal binding kinetics. Pre-enriched



**Figure 1** Analysis of precipitated exosomes. A) Precipitated exosomes released from SW480 cells were analysed for the content of characteristic exosomal mRNA/rRNA (GAPDH, 18S) and miRNAs (ActB, Let7a, miR16) by qRT-PCR. Average Ct values for three independent experiments are shown (n=3, SD). B) Precipitated exosomes from SW480 culture media were analysed by Nanosight LM10. The size distribution of total exosomes resuspended in PBS is shown. C-D) Immunolabelling of exosomes derived from SW480 cells. Bar. 100 nm.



**Figure 2** Expression of exosome surface proteins in pre-enriched exosomes. A) Exosomes from SW480 cells pre-enriched by ultracentrifugation (UC) or precipitation (Prec.) and B) exosomes from Jurkat cells pre-enriched by ultracentrifugation (UC) or precipitation (Prec.) were analysed by Western immunoblotting with antibodies against CD9 and CD81. Shown is a one representative result out of three independent experiments. The western blots are all from one single gel.

CD9<sup>+</sup> SW480 derived exosomes were isolated using magnetic beads coated with anti-human CD9 antibodies followed by CD9 staining (**Figure 3B**). The data demonstrated significant staining of CD9. Isolation of CD81<sup>+</sup> exosomes from SW480 cells showed the presence of CD81 but at lower signal (**Figure 3C**). Likewise, pre-enriched Jurkat derived exosomes confirmed the presence of CD81 (**Figure 3E**). Finally, isolation of CD9<sup>+</sup> exosomes from Jurkat cells resulted in signal equivalent to background (**Figure 3D**).

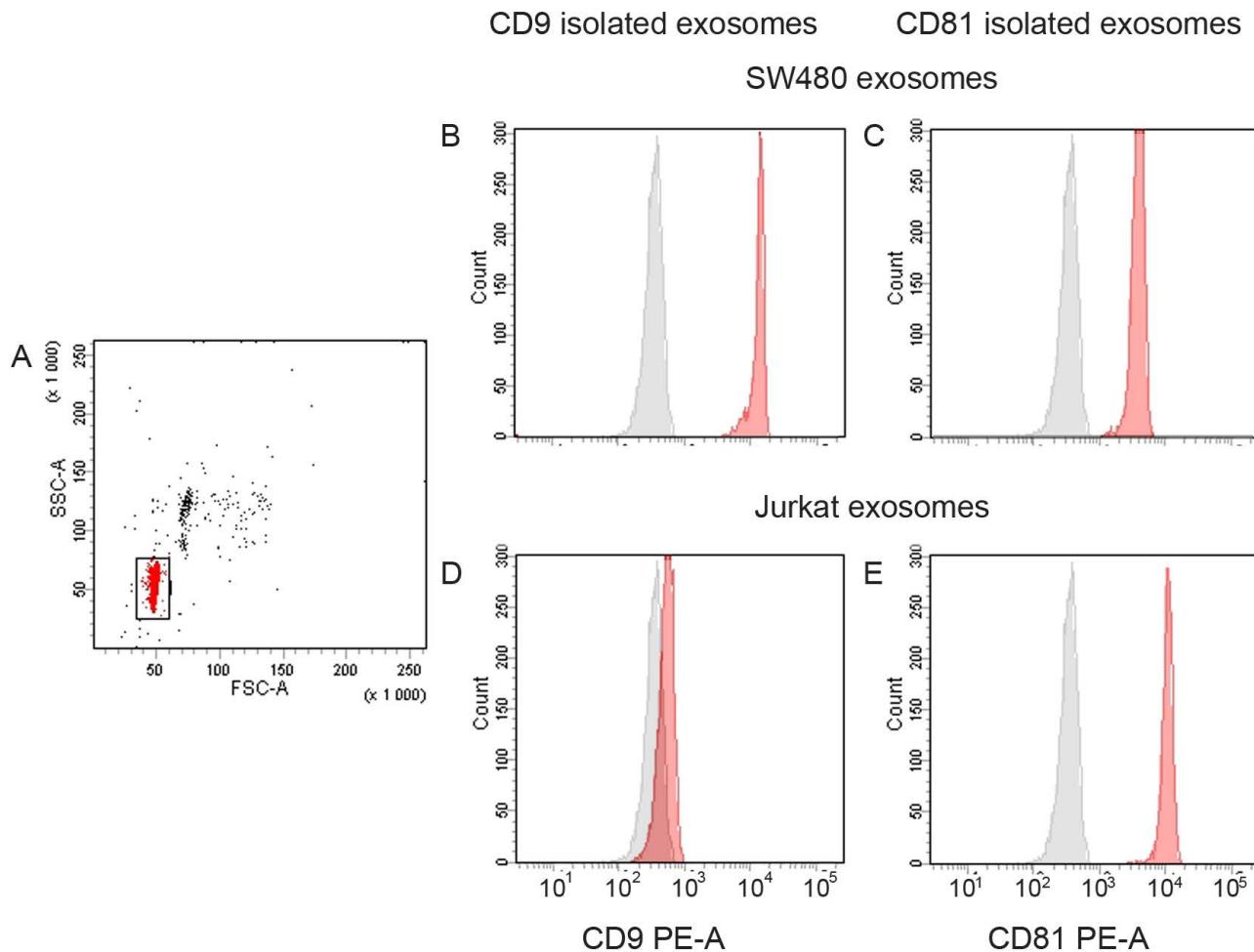
Exosomes derived from Jurkat cells were isolated using magnetic beads and prepared for downstream Western blotting analysis. Western blotting requires a larger total surface area for optimal signal. A higher number of magnetic beads were therefore used. In order to illustrate the isolation volume were kept constant in combination with increased amounts of beads. CD81<sup>+</sup> exosomes derived from Jurkat cells (**Figure 4A**) were isolated using magnetic beads coated with anti-human CD81 antibodies at a concentration equal to the concentration used for detection by flow cytometry (1x) in addition to 5x, 10x and 25x the amount of beads used for flow detection. The exosomes were processed for SDS-PAGE and Western blotting and labeled for CD81. The data showed that exosomes isolated with a bead concentration equal to what is used for flow cytometry were not detectable by western blotting. However, increasing amount of CD81 was observed when the amount of beads was increased. Finally, isolation efficiency was confirmed by analysis of the remaining exosomes in the supernatant post isolation measured by flow cytometry (**Figure 4B**). Increasing amounts of magnetic beads have been used for isolation starting with amounts equivalent to what is used for flow cytometry (1x) up to amount of beads typically used for Western blotting (25x).

### Critical factors for direct isolation of exosomes from cell culture media

In order to provide a fast and scalable protocol compatible with many downstream applications a direct approach would be beneficial. This requires attention to several factors. In order to illustrate this, exosomes derived from SW480 cells were isolated with magnetic beads coated with anti-human CD9 antibodies and subjected to different experimental conditions. First, the impact of volume of exosome containing cell culture media during isolation was addressed. Increased amount of exosome containing cell culture media (175 µl, 350 µl, 700 µl) keeping the amount of magnetic beads constant (20 µl) provided a non-linear (+) dose response curve (**Figure 5A**). At very high volumes, the reduced concentration of magnetic beads resulted in lower binding kinetics. Secondly, we looked at the effect of increased incubation time. Increased incubation time in combination with fixed amount of exosome containing cell culture media and magnetic beads (20 µl) also gave a non-linear (+) dose response curve (**Figure 5B**). Finally, we kept the isolation volume and the amount of magnetic bead constant and increased the amount of exosomes in order to ensure equal binding kinetics. The data demonstrated a linear (+) dose response curve (**Figure 5C**). For sample comparison such experimental settings are critical as it ensures equivalent binding kinetics.

### Direct isolation of exosomes from cell culture media

The optimized conditions were used for direct isolation from SW480 cell culture media for flow analysis. SW480 derived exosomes were isolated targeting CD9 followed by CD9 staining. For comparison we related direct (Direct) isolation with exosomes



**Figure 3** Flow cytometric analysis of exosomes isolated with magnetic beads. SW480 derived exosomes (B and C) and Jurkat derived exosomes (D and E) were immunoisolated from pre-enriched exosome samples using magnetic beads coated with anti-human CD9 or CD81. Exosomes were stained for exosomal antigens CD9 (B and D) and CD81 (C and E) followed flow cytometric analysis and presented as Median Fluorescence Intensity (MFI). A) Scatter plot of exosomes isolated with anti-CD81 and labelled for CD9 to demonstrate gating. Shown is one representative result out of three independent experiments.

pre-enriched by ultracentrifugation (UC) and precipitation (Prec.). To enable direct comparison of the flow analysis the exosome input was normalized for the three ways of processing conditioned cell culture medium based on the concentration factor achieved for UC and prec., respectively.

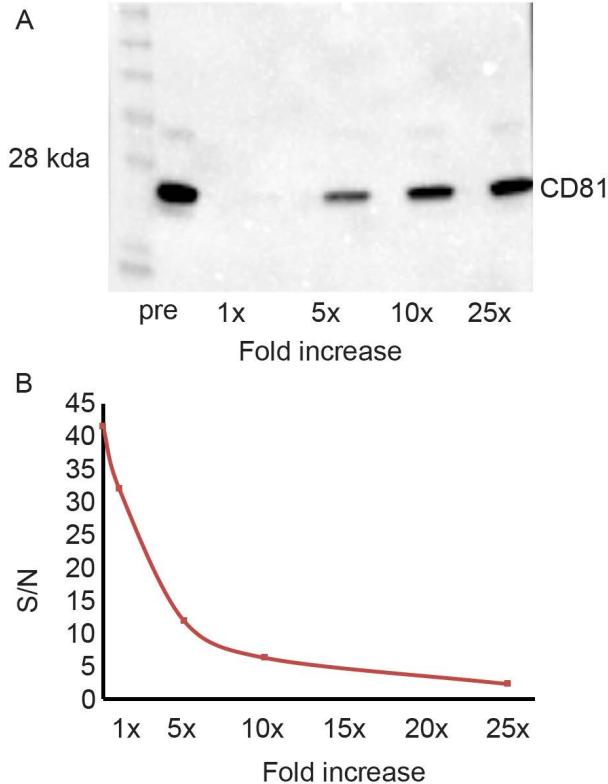
The results obtained by flow cytometry (**Figure 6A**) were supported by Western blotting (**Figure 6B**) demonstrating CD9 staining after direct isolation of CD9<sup>+</sup> exosomes (lane 1). Furthermore, CD9 staining was somewhat reduced for UC pre-enriched samples compared to direct isolation indicating loss of exosomes during UC pre-enrichment (lane 2). For isolation from exosomes pre-enriched by precipitation (lane 3), CD9 staining was comparable to direct isolation (lane 1). The isolation efficiency was confirmed by flow cytometry analysis. The supernatant after isolation for Western blotting in **Figure 6B** was subjected to a second round of isolation for downstream flow cytometry (**Figure 6C**).

Finally, CD9<sup>+</sup> exosomes isolated with magnetic beads were subjected to immunoassay (**Figure 7**). First, the correlation between results obtained by flow cytometry and immunoassay

were established. CD9<sup>+</sup> exosomes stained for CD81 (**Figure 7A**) and CD9 (**Figure 7B**) showed high degree of correlation. Secondly, the dose response was investigated. Increasing amount of cell culture media containing exosomes (two separate exosome batches (Batch A and Batch B) were isolated using magnetic beads targeting CD9 followed by CD81 (**Figure 7C**) or CD9 (**Figure 7D**) detection. As observed for flow cytometry, a dose response was observed for immunoassay.

## Discussion

Isolation and analysis of nano-sized exosomes is challenging. Today the general approach for exosome isolation is differential centrifugation. The protocol is not standardized as many variants with potential effect on the outcome are used and published. Also, several centrifugation steps are labour intensive, require costly equipment, increase risk for loss of exosomes and does not discriminate well between exosomes and contaminating structures such as larger vesicles and protein/lipid aggregates [26]. The inclusion of magnetic beads with antibodies specific for



**Figure 4** Western blot analysis of exosome surface marker CD81. Exosomes derived from SW480 were isolated by magnetic beads coated with anti-human CD81 processed for Western immunoblot analysis (A). For comparison pre-enriched exosomes prior to bead isolation was included (pre). Shown is one representative Western immunoblot out of three independent experiments. B) Depletion efficiency was measured by flow cytometry as signal to noise (S/N) of the supernatant after isolation for Western blotting. 25x represent the amount of magnetic beads used for Western blotting.

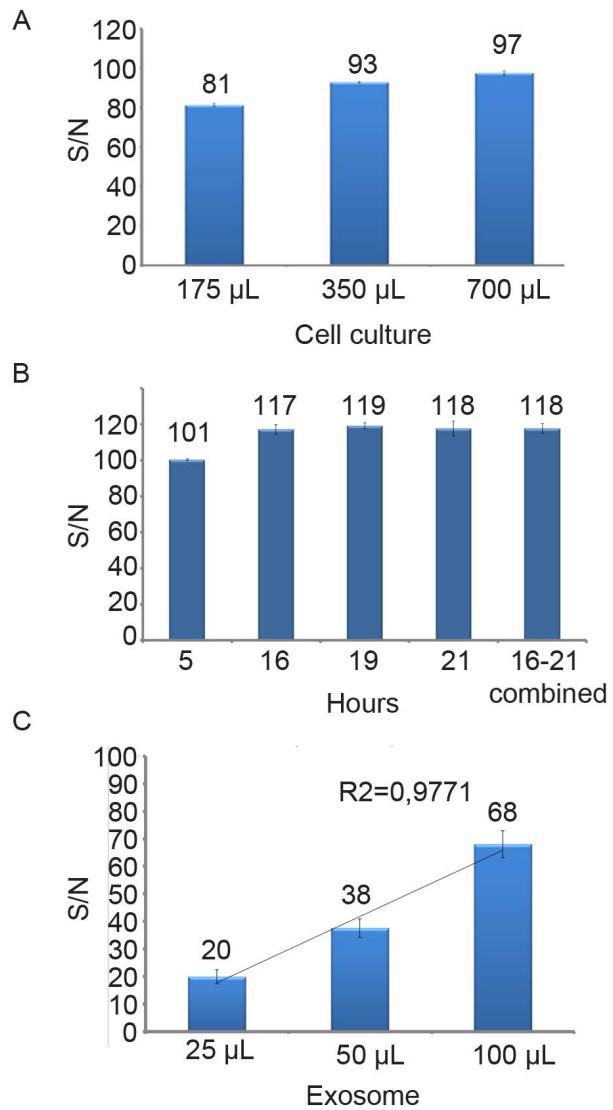
exosome surface proteins in the isolation protocol downstream of pre-enrichment has successfully overcome the issue of low purity.

Here we provide a simplified exosome isolation workflow by omitting the commonly used pre-enrichment step. We demonstrate that capturing exosomes directly from cell culture supernatant provides sufficient amounts of exosome for downstream analysis while dramatically simplify the sample preparation/handling.

Direct isolation with magnetic beads requires minimal hands-on, provides highly pure exosomes with minimal loss and enables future automation opportunities as it is compatible with the immunoassay format.

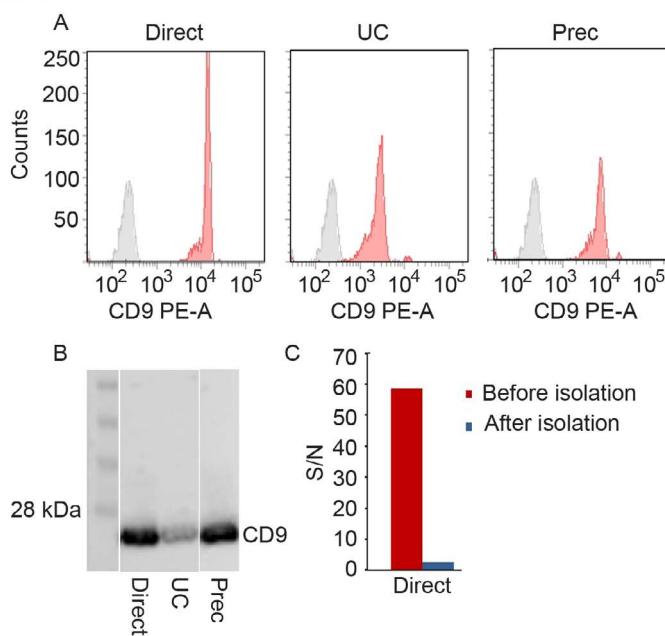
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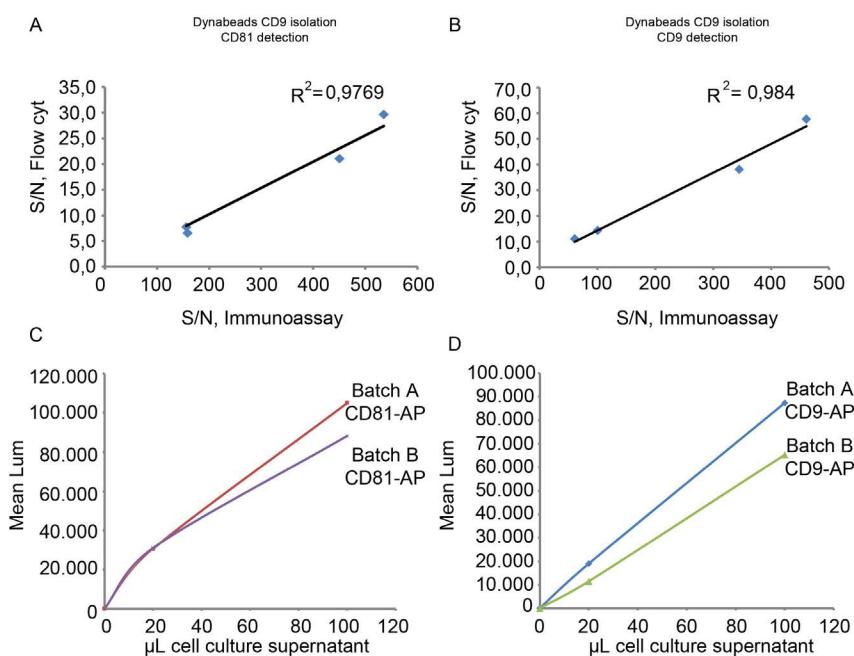


**Figure 5** Flow cytometric analysis of the effect of incubation volume (A), incubation time (B) and exosome input (C) keeping the amount of magnetic beads constant. Exosomes derived from SW480 cells were isolated using magnetic beads coated with anti-human CD9 followed by CD9 staining and analysis by flow cytometry and presented as signal to noise (S/N). A) CD9+ exosomes were isolated in 175 µL, 350 µL or 700 µL of cell culture media using 20 µL of magnetic beads. B) CD9+ exosomes were isolated from 100 µL of cell culture media by incubating 20 µL of magnetic beads in 5, 16, 19 or 21 hours at 4°C. C) CD9+ exosomes were isolated from 25 µL, 50 µL or 100 µL of added exosomes in a 100 µL total volume using 20 µL of magnetic beads.

flow assay development and analysis, flow assay validation flowcyt analysis and data interpretation. A. Kullman was responsible for the western blotting and flow cytometry analysis. M.P. Oksvold was responsible for western blotting and data interpretation. M.Li was responsible for the miRNA analysis. Norbert Roos was responsible for the electronmicroscopy. A.V. Vlassov was



**Figure 6** Flow analysis of CD9-positive exosomes isolated with magnetic beads from cell culture media, after pre-enrichment by ultracentrifugation or precipitation. SW480 cells were grown for three days before the conditioned cell culture supernatant was harvested and processed in three different ways (A) no pre-enrichment (Direct), pre-enriched by ultracentrifugation (UC) or precipitation (Prec.) followed by isolation using magnetic beads coated with anti-human CD9. The exosomes were analysed by flow cytometry after staining with CD9-PE (red histogram) or isotype-control (gray histogram). B) CD9 positive exosomes were isolated from cell culture media (Direct), pre-enriched by ultracentrifugation (UC) or precipitation (Prec), processed for Western blotting and labelled for CD9. The western blots presented were all from the same gel. C) Exosomes were isolated for Western blotting using magnetic beads specific for CD9 (25x the amount of beads used for flow analysis) and the remaining supernatant was subjected to a second round of exosome capture for flow analysis as described above and presented as signal to noise (S/N). The red and blue bars represent the flow analysis pre- and post-exosome isolation for Western blotting, respectively.



**Figure 7** CD9 positive exosomes were isolated directly from conditioned cell culture supernatant harvested from SW480 cells and processed for immunoassay detection using either CD9- or CD81-AFP (A-D). A-B) Strong correlation between data obtained by flow cytometry (median fluorescence intensity) and immunoassay (mean luminescence) are demonstrated for exosomes isolated with Dynabeads® and stained for CD81 (A) or CD9 (B). Immunoassay provided high signal to noise (S/N) compared to flow analysis (C-D) Dose response between amount of exosomes added and signal obtained by immunoassay was shown.

responsible for the experimental design and miRNA analysis. Ingrid Manger was responsible for the experimental design of immunoassay and analysis of immunoassay. A.Neurauter was responsible for the experimental design and data interpretation. K.W. Pedersen was the project lead, and responsible for the experimental design, microscopy, data interpretation and writing of the manuscript.

## Conflicts of interest

Anette Kullmann, BenteKierulf, Axl Neurauter, Mu Li, Alexander V. Vlassov, Ingrid Manger and Ketil W. Pedersen are full-time employees of Thermo Fisher Scienfic, Oslo, Norway. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

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