

## Video Article

# Protein Digestion, Ultrafiltration, and Size Exclusion Chromatography to Optimize the Isolation of Exosomes from Human Blood Plasma and Serum

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## Abstract

Exosomes, a type of nanovesicle released from all cell types, can be isolated from any bodily fluid. The contents of exosomes, including proteins and RNAs, are unique to the cells from which they are derived and can be used as indicators of disease. Several common enrichment protocols, including ultracentrifugation, yield exosomes laden with soluble protein contaminants. Specifically, we have found that the most abundant proteins within blood often co-purify with exosomes and can confound downstream proteomic studies, thwarting the identification of low abundance biomarker candidates. Of additional concern is irreproducibility of exosome protein quantification due to inconsistent representation of non-exosomal protein levels. The protocol detailed here was developed to remove non-exosomal proteins that co-purify along with exosomes, adding rigor to the exosome purification process. Five methods were compared using paired blood plasma and serum from five donors. Analysis using nanoparticle tracking analysis and micro bicinchoninic acid protein assay revealed that a combined protocol utilizing ultrafiltration and size exclusion chromatography yielded the optimal vesicle enrichment and soluble protein removal. Western blotting was used to verify that the expected abundant blood proteins, including albumin and apolipoproteins, were depleted.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/57467/>

## Introduction

Exosomes are nanovesicles (ranging in size from 30 nm to 150 nm) released by almost all the cells in the human body to facilitate cell-to-cell communication processes<sup>1,2</sup>. Interestingly, the composition of exosomes changes depending on the cells of origin as well as the health status of the individual<sup>3,4,5</sup>. Additionally, exosomes can be retrieved from several biological fluids such as: saliva, urine, and blood<sup>2</sup>. Because of these features, exosomes are considered to be a good source of disease biomarkers. Unfortunately, there is no standard method for the isolation of exosomes. Some laboratories consider multistep centrifugation, with a final high-speed step at 100,000 x g using density gradients as the gold standard method for exosome isolation. However, recent studies have shown that ultracentrifugation induces aggregation of exosomes with soluble proteins and other exosomes, in addition to affecting exosome integrity, both of which may hamper downstream applications<sup>6,7</sup>. Other common methods of exosome isolation include, but are not limited to: precipitation by commercial polyethylene glycol (PEG) based reagents, centrifugal ultrafiltration, and size-exclusion chromatography (SEC). The commercial reagents using polyethylene glycol (PEG) polymers enrich exosomes by causing them to precipitate and form a pellet. Limitations using this polymer are contamination with residual PEG polymer and an abundance of soluble non-exosomal proteins in the final product. Ultrafiltration utilizes centrifugation to purify and concentrate vesicles using a cellulose membrane; exosomes are retained above the filter, while smaller impurities and other proteins pass through the membrane<sup>7,8</sup>. Just like other methods, centrifugal ultrafiltration has a limited capacity to purify exosomes due to the retention of high levels of non-exosomal proteins, including protein complexes and aggregates. Finally, SEC purification uses porous resin to separate molecules by size. SEC has shown promising results, overcoming most of the problems experienced with other methods by capturing the majority of contaminant proteins and preserving exosomal integrity, since isolation is based on gravity or low-pressure systems<sup>7,9</sup>. However, the co-isolation of larger protein aggregates and lipoproteins<sup>10</sup> during SEC affects the purity of the final exosome preparation. While some methods have been tested for exosome purification from cell culture supernatants and plasma<sup>7</sup>, or only plasma<sup>9,11</sup>, there is no information about the performance of methods directly comparing blood plasma and serum from the same individual.

Here, we focus on the purification of blood exosomes by comparing a variety of workflows to determine if vesicle enrichment techniques are translatable between plasma and serum. Nanoparticle tracking analysis and western blot were used to quantify the differences in exosome concentration, purity, and protein composition in the end products. The final method, detailed in this protocol, increases vesicle numbers and reduces non-exosomal protein levels. Importantly, a drastic reduction of common co-precipitating proteins including albumin and apolipoproteins is demonstrated. The high abundance of these two proteins in blood and the frequency at which they co-purify with exosomes causes inconsistencies between "purified" samples, skewing the downstream analyses. This protocol includes the use of a commercially available SEC

resin; the resin is composed of porous beads in which proteins smaller than 700 kDa can enter the beads. Once inside, the proteins are retained by an octylamine ligand. The eluate is composed of exosomes and molecules larger than 700 kDa<sup>12</sup>. Additionally, in order to reduce the protein aggregates and apolipoproteins which evade bead trapping, we include a protease digestion step in the workflow. Currently, there is no single technique capable of maximizing exosome yield while reducing co-purifying non-exosomal proteins. This study shows that a purification protocol that combines a protease digestion pretreatment with multiple recovery and purification methods can be used to increase exosome yield and purity from blood serum and plasma.

## Protocol

This work was determined not to be human subject research upon initial review from Colorado State University's Institutional Review Board (IRB), as all human samples were obtained as de-identified samples from the Bioreclamation IVT biorepository and were collected under IRB approved protocols.

### 1. Preparation of Crude Sample (either Plasma or Serum) by Pelleting Larger Vesicles and Cellular Debris

**Note:** Safety consideration: plasma, serum, and other biological fluids are biohazardous materials and must be handled with special care, while wearing basic personal protective equipment, including gloves and lab coat. It is highly recommended that biological samples are processed in a biosafety cabinet; if not available, the use of eye-protection is recommended.

1. Equilibrate a serum or a plasma sample by placing the tube under non-shaking conditions in a 4 °C refrigerator overnight (from either -20 or -80 °C storage).
2. Aliquot 250 µL of the sample into a microcentrifuge tube using a 1,000 µL pipette.
3. Centrifuge the sample at 18,000 x g for 30 min at 4 °C.
4. Using a 200 µL pipette, remove 200 µL of the cleared supernatant and add it to a new microcentrifuge tube. Avoid any pelleted material while transferring the supernatant. Discard the pelleted material.
5. Quantify the protein content of the sample by bicinchoninic acid (BCA) assay, using the manufacturer's protocol. To perform the assay, dilute the sample 1:50 in 1x phosphate-buffered saline (PBS). Test each sample in duplicate.
 

**Note:** Use 1x PBS throughout the protocol unless stated otherwise. On average, 200 µL of clarified serum or plasma (after 18,000 x g) will yield 15 mg of total protein. Other sample types, with lower or higher protein content, may require additional BCA dilutions.
6. Aliquot 10 mg of the sample into a new microcentrifuge tube. Use the concentration obtained in step 1.5 to calculate the corresponding volume of sample. Aliquot the sample using a 200 µL pipette.
 

**Note:** It is recommended to store the remaining sample at -80 °C. Aliquot the sample in different tubes at 10 mg per tube to avoid multiple freeze-thaw cycles in future use.

### 2. Digestion of Non-exosomal Blood Proteins

**Note:** The digestion step is designed to reduce the non-exosomal proteins which can co-purify with exosomes. If preservation of exosome surface proteins is required for downstream analyses, it should be taken into consideration that this step has the potential to interfere with this analysis. The detection of the exosomal CD63, a surface-exposed tetraspanin protein, was not significantly negatively impacted by this process.

1. Prepare proteinase K solution at a concentration of 500 µg/mL in PBS. Add the corresponding amount of proteinase K in a conical tube. Then add the buffer and vortex for 30 s, 3x at room temperature.
2. Add 50 µL of proteinase K solution to the 10-mg sample aliquot and mix gently by pipetting up and down using a 200 µL pipette.
3. Incubate the sample at 37 °C in a water bath for 30 min. Place the tube in a float tube rack and avoid complete immersion of tube in the water bath to avoid potential leakage.
4. Transfer the sample and floating tube rack to a 60 °C water bath for 10 min, to inactivate the proteinase K.

### 3. Removal of Small Proteins and Peptides by Centrifuge Ultrafiltration

1. Rinse an ultrafiltration device containing 100 kDa molecular weight cut-off (MWCO) filter with PBS prior to use. Do this by adding 500 µL of PBS to the filter, using a 1,000 µL pipette. Spin the device at 3,700 x g for 5 min. Discard any remaining retentate as well as the eluate.
 

**Note:** The sample volume capacity of the ultrafiltration device must be 0.5 mL - 4 mL to ensure that the final reduced sample volume of 50 µL is achievable.
2. Take the sample from step 2.4 and increase the volume to 500 µL by adding PBS. Pipette the sample into the pre-rinsed ultrafiltration device.
 

**Note:** On average, the sample volume from step 2.4 is 185 µL (120 to 150 µL of sample and 50 µL of proteinase K solution).
3. Place the ultrafiltration device in a fixed-angle benchtop centrifuge at 3,700 x g at 4 °C until the sample has reduce to a volume of 50 µL.
 

**CAUTION:** When using ultrafiltration devices with dead stop volume, care must be taken to avoid complete dryness of the filter membrane during centrifugation steps.
4. Add 500 µL of PBS directly into the filter membrane and pipette up and down 5 times. Centrifuge as in step 3.3. Perform this wash step a total of 3 times.
5. Transfer the final 50 µL retentate in a new microcentrifuge tube.
6. Rinse the filter membrane with 200 µL PBS, pipetting up and down 10 times, and transfer the wash to the tube from step 3.5.
7. Place the filter holder in the inverse position in a new tube. Run a reverse spin recovery for 5 min at 2,000 x g to retrieve remaining sample from the membrane. Pool the sample with the sample from step 3.5.

## 4. Purification of Vesicles by Size Exclusion Chromatography (SEC)

1. Add 850  $\mu$ L of SEC slurry, with beads having a MWCO of 700 kDa, into an empty, capped gravity flow column using a 1,000  $\mu$ L pipette. Place the columns in a size appropriate rack fitted with a drip tray.
2. Uncap the bottom of the column and let the liquid drain for 5 min. Once drained, add 10 mL of PBS to wash the resin. Allow 20 min for the wash to drain from the column.
3. Place the concentrated, proteinase K treated sample from step 3.5 into a 15-mL conical tube, and increase the sample volume to 5 mL adding PBS with a serological pipette. Mix the tube gently by rocking for 1 min and then slowly apply the sample to the column prepared in step 4.2 with a serological pipette.  
**Note:** Once the cap of the column is removed, the sample will flow through the resin by gravity; the 5-mL sample will completely flow through the column in 10 min.
4. Collect the flow through in a new 15 mL conical tube.
5. Using a serological pipette, apply the collected material to the resin again to remove any remaining material below 700 kDa.
6. Collect the flow through in a new 15 mL conical tube. Wash the resin twice adding 1 mL of PBS. Collect the flow through in the tube from step 4.5; the total final volume will be roughly 7 mL.  
**Note:** After step 4.6, the sample will be diluted about 35 times from the original sample volume; the following steps will reduce the volume and concentrate the purified exosome sample.

## 5. Concentration of Purified Exosomes and Total Protein Quantification

1. Rinse an ultrafiltration device with a 3 kDa MWCO filter with PBS prior to use, as described in step 3.1.
2. Add the sample from step 4.6 to the ultrafiltration device and centrifuge in a swinging-bucket rotor at 3,700  $\times$  g at 4  $^{\circ}$ C. Buffer will pass through the filter and can be discarded. Concentrated exosomes will be retained above the filter. Centrifuge the sample until the volume above the filter (retentate) is reduced to 200  $\mu$ L.
3. Transfer the retentate to a new microcentrifuge tube.
4. Rinse the filter membrane with 200  $\mu$ L PBS by pipetting over the membrane 10 times and transfer the wash to the tube from step 5.3. This will allow for the collection of any residual exosome sample.
5. Bring sample volume up to 500  $\mu$ L by adding PBS. Mix by slowly pipetting up and down 10 times.  
**Note:** The protocol can be paused here. Samples should be stored at 4  $^{\circ}$ C overnight or at -20/-80  $^{\circ}$ C for long term storage.
6. Quantify the protein content of the sample by BCA assay, using the manufacturer's protocol. Dilute the sample 1:10 and 1:50 in PBS. Run each sample in duplicate.  
**Note:** Starting with 10 mg of total protein of serum or plasma, the total protein yield in purified exosomes from step 5.6 is, on average, 150  $\mu$ g. Additional dilutions may be necessary if samples other than serum or plasma are used; yield may vary with sample type.

## 6. Quantification and Sizing of Exosomes by Nanoparticle Tracking Analysis (NTA)

1. Add 5  $\mu$ g of purified exosomes (as quantified in 5.6) to 1 mL of PBS and vortex for 15 s on low-medium speed.
  1. Place the sample in a 1 mL disposable syringe. If available, set the syringe in an automatic syringe pump set to inject the diluted exosome sample at a rate of 30  $\mu$ L/min.
  2. Perform NTA measurements using video capture settings: screen gain of 3 - 4 and camera level of 12 - 13.
  3. Define the script for analysis to run three technical replicates for a minimum of 30 s using a constant flow for every replicate. For the analysis, set the capture threshold at 5.  
**Note:** Details regarding the use of the automatic syringe, and settings for video capture are available in the reference<sup>13</sup>. Capture and analysis settings for NTA must be consistent across different samples to assure comparability.

## 7. Determination of Soluble Protein Reduction

1. Add 1 - 10  $\mu$ g of purified exosomes to SDS sample buffer and perform polyacrylamide gel electrophoresis (PAGE) using a 4 - 12% Bis-Tris Gel in 1x MES SDS running buffer for 35 min at 200 V. Transfer resolved proteins to a nitrocellulose 0.2  $\mu$ m membrane by applying a voltage of 50 V for 1 - 1.5 h. Perform western blot analysis to evaluate the presence of albumin, apolipoproteins A and B, and the exosome marker CD 63 following standard methodologies.  
**Note:** Full protocols for the methods in 7.1 can be found in reference<sup>14</sup>; specifically, SP007: Running of polyacrylamide gels and SP011: Western blot protocol.
2. Separate 10  $\mu$ g of purified exosomes using PAGE as in step 7.1 and stain the protein bands using coomassie dye, following standard recommendations, to visualize protein variation and reduction between samples.  
**Note:** Further details regarding the protocol for western blots specific to CD63 are described by Diaz *et al.*<sup>15</sup>

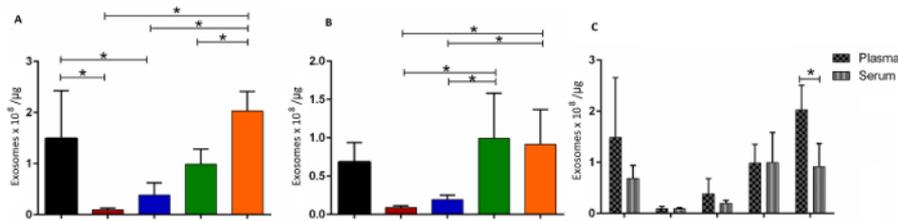
## Representative Results

The data presented below were obtained using paired plasma and serum samples from five healthy blood donors. To determine the effects of each processing step, five variations of the presented protocol were performed, and exosome recovery and non-exosome protein removal were compared. The methods trialed included: 1) SEC 700 kDa + Ultrafiltration 3 kDa; 2) Ultrafiltration 100 kDa; 3) Proteinase K + Ultrafiltration 100 kDa; 4) SEC 700 kDa + Ultrafiltration 100 kDa, and 5) Proteinase K + Ultrafiltration 100 kDa + SEC 700 kDa + Ultrafiltration 3 kDa.

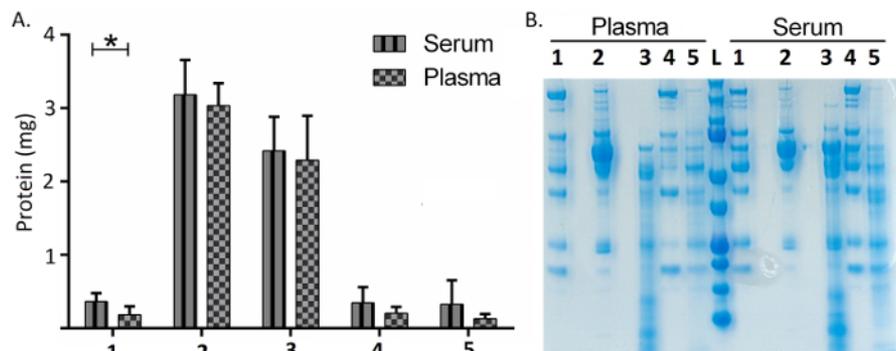
Collectively, all methods involving SEC 700 kDa (1, 4, and 5) produced a significantly higher number of vesicles per microgram of protein in both types of sample, serum, and plasma (Figure 1A-B). However, method 5 generated a significantly higher number of vesicles per microgram of plasma compared to serum (Figure 1C). Conversely, the protein concentration in purified exosome samples was significantly lower while using the SEC 700 kDa-based methods (1, 4, and 5). One-way ANOVA and Tukey's multiple comparisons tests showed that the final protein concentration after methods 2 and 3 were significantly higher than the final protein concentration after methods 1, 4, and 5 ( $p < 0.001$ ). The differences were consistent using plasma or serum, however, with method 1 the final protein concentration of purified exosomes was significantly higher (t-test,  $p < 0.05$ ) when using serum (Figure 2A). To evaluate the protein distribution of purified exosomes by the different five methods, 10  $\mu\text{g}$  of sample from each method were resolved in a PAGE and stained with coomassie dye. The results suggest that the majority of the protein present in the "purified exosomes" from methods 2 and 3 was albumin signified by the strong band  $\sim 65$  kDa (Figure 2B).

The next step was to confirm the increase in purity of isolated exosomes based on the presence of the albumin, the most abundant protein in the blood. The ultrafiltration process showed a marked concentration of albumin in the exosomal fraction that was partially reduced by pre-treating the sample with proteinase K (Figure 3A, lanes S and P). The inclusion of SEC 700 kDa in the process decreased the amount of albumin (Figure 3A, lanes P1/S1 and P4/S4) but the combination of SEC 700 kDa, proteinase K, and ultrafiltration showed the most efficient removal of albumin from purified exosomes (Figure 3A, lane P5/S5). Recent studies have shown that apolipoproteins are also commonly co-isolated during exosome purification<sup>10,16</sup>. Specifically, ApoB, which is present in low density lipoproteins, was found to be highly concentrated in purified exosomes from human blood<sup>10</sup>. Therefore, we evaluated the co-isolation of the lipoproteins ApoB and ApoA-1. Ultracentrifugation methods and the combination of Ultracentrifugation and SEC 700 kDa showed a similar amount of ApoB compared to the amount detected in serum and plasma by western blot (Figure 3B, lanes P1/S1, P2/S2, and P4/S4; Supplementary figures 1/2). Interestingly, the addition of proteinase K resulted in the complete degradation of ApoB from the purified sample (Figure 3B, lanes P3/S3 and P5/S5). Similarly, ApoA-1, the major protein component of high density lipoproteins in human plasma, was reduced by all methods involving either SEC 700 kDa or proteinase K (Figure 3C). Although, much like albumin, the 100 MWCO ultrafiltration on its own was unable to significantly reduce the amount of co-purifying ApoA-1.

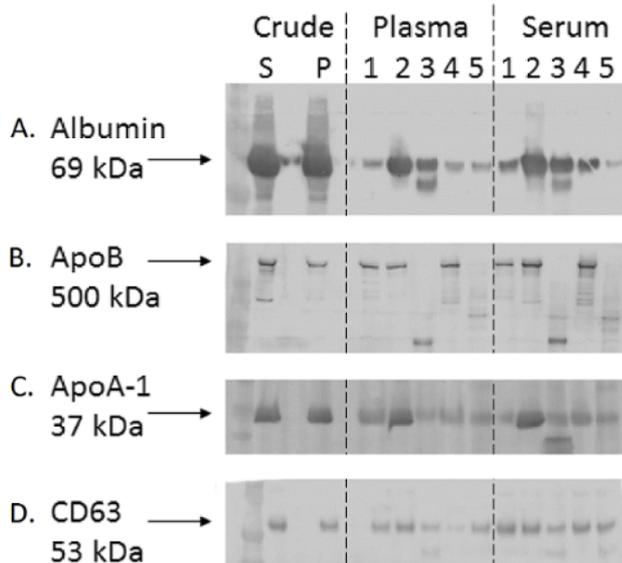
Finally, to determine the effect of proteinase K on proteins exposed to the external membrane of exosomes, we evaluated the presence of the CD63 tetraspanin, a hallmark protein of exosomes. An analysis of Western blots showed that the protein was not only detectable in methods using proteinase K (Figure 3D, lanes P3/S3 and P5/S5), but the bands for CD63 using method 5 had a more intense signal (Figure 3D, lanes P5/S5). This finding suggests method 5 yields either a higher concentration of exosomes or the increase of CD63 antibody binding due to the decrease of protein aggregates associated with the exosome surface.



**Figure 1: Exosome yield from plasma and serum after five different purification methods.** (A) Concentration of exosomes obtained from plasma (n = 5). (B) Concentration of exosomes obtained from serum (n = 5). (C) Comparison between the numbers of exosomes per microgram of protein from 5 paired sets of plasma and serum. Numbers on the x-axis represent each method used, 1 = SEC 700 kDa + Ultrafiltration 3 kDa; 2 = Ultrafiltration 100 kDa; 3 = Proteinase K + Ultrafiltration 100 kDa; 4 = SEC 700 kDa + Ultrafiltration 100 kDa, and 5 = Proteinase K + Ultrafiltration 100 kDa + SEC 700 kDa + Ultrafiltration 3 kDa. In A and B, the differences were calculated by one-way ANOVA and Tukey's multiple comparisons tests. In C, differences between serum and plasma per method were calculated by t-test. \* $p < 0.05$ ; Error bars: 95% of Confidence Interval. [Please click here to view a larger version of this figure.](#)



**Figure 2: Protein yield in purified exosomes from plasma and serum using five different methods.** (A) Comparison of the total protein yield in the purified exosomes obtained from plasma and serum (n = 5). (B) Representative picture of a coomassie staining of 10  $\mu\text{g}$  of purified exosomes resolved by SDS-PAGE; n = 1, paired plasma and serum. Numbers in the figure represent each method, 1 = SEC 700 kDa + Ultrafiltration 3 kDa; 2 = Ultrafiltration 100 kDa; 3 = Proteinase K + Ultrafiltration 100 kDa; 4 = SEC 700 kDa + Ultrafiltration 100 kDa, and 5 = Proteinase K + Ultrafiltration 100 kDa + SEC 700 kDa + Ultrafiltration 3 kDa. In A, differences among plasma and serum-derived samples were calculated by t-test. \* $p < 0.05$ . [Please click here to view a larger version of this figure.](#)



**Figure 3: Reduction of the normal contaminants of exosomes isolated from human blood and stability of the hallmark protein CD63. Western blot evaluation of: (A) Albumin, 1 µg per lane; (B) Apolipoprotein B, 1 µg per lane; and (C) Apolipoprotein A1, 10 µg per lane; (D) CD63, 1 µg per lane. L = Ladder; S = crude serum; P = crude plasma. Purified exosome: purification using 5 different methods. Numbers in the figure represent each method, 1 = SEC 700 kDa + Ultrafiltration 3 kDa; 2 = Ultrafiltration 100 kDa; 3 = Proteinase K + Ultrafiltration 100 kDa; 4 = SEC 700 kDa + Ultrafiltration 100 kDa, and 5 = Proteinase K + Ultrafiltration 100 kDa + SEC 700 kDa + Ultrafiltration 3 kDa. Representative results from the paired serum and plasma of one patient; trends were consistent between donor samples. Protein loading varied by blot; normalization was based on BCA determination of total sample concentration. Total protein load per lane was specified for each blot. [Please click here to view a larger version of this figure.](#)**

**Supplementary Figure 1: Original uncropped western blots from Figure 3. [Please click here to view a larger version of this figure.](#)**

**Supplementary Figure 2: Density quantification of Albumin, ApoB, ApoA-1, and CD63 bands from Figure 3; ImageJ software was used. [Please click here to view a larger version of this figure.](#)**

## Discussion

An optimized method to increase the purity and yield of exosomes from blood will increase the ability to accurately mine extracellular vesicles as a source of biomarkers for several diseases. The standard methods currently used to isolate exosomes, specifically, ultracentrifugation and precipitation methods, have several disadvantages including exosome aggregation and pelleting of soluble proteins<sup>7,17,18</sup>. Alternatively, the use of SEC has shown a significant improvement in exosome isolation, protecting exosomes from aggregation, and improving the removal of common contaminant non-exosomal proteins<sup>9,11,18,19</sup>. However, apolipoproteins<sup>10</sup>, larger vesicles, and protein aggregates, including albumin, are often co-isolated with exosomes, if SEC alone is used. Two critical steps in the presented protocol aid in overcoming the last two mentioned limitations. First, the centrifugation of the plasma or serum sample at 18,000 x g precipitates most of the larger vesicles present in the sample. Second, the pre-treatment of serum or plasma samples with proteinase K, a serine protease with broad specificity<sup>20</sup>, is critical to reduce the amount of albumin and the apolipoproteins A-1 and B associated with the exosomes during purification. We combined the use of the proteinase K with an ultrafiltration step using a membrane with a MWCO of 100 kDa to partially elute small proteins and peptides, and then we adapted the use of a SEC resin with a MWCO of 700 kDa to further clear smaller peptides/proteins from the exosomes. This resin, originally optimized for virus isolation, captures remaining large proteins and/or complexes under 700 kDa. The principle of isolation of this SEC resin allows a gentle recovery of the exosomes, since the flow of the sample through the resin is driven by gravity. This allows for less aggregation and the retained integrity of the exosomes, overcoming two of the major limitations of ultracentrifugation-based methods. Lastly, it was demonstrated by Baranyai *et al.* that the dilution of the plasma before SEC processing improves exosome recovery<sup>21</sup>. In this protocol, we have included a dilution step before SEC to increase the contact time of the sample with the resin.

An important limitation of the presented protocol is the potential digestion of proteins in the exosomal membrane by proteinase K treatment. This can impair downstream experiments if surface-exposed membrane proteins are required for downstream analyses including western blot, ELISA, or flow cytometry. To partially test the effect of proteinase K on membrane associated proteins, we analyzed the protease-treated samples for the presence of CD63, a tetraspanin protein, which is normally present in exosomes. These results showed that use of proteinase K with the described conditions of digestion time and concentration did not lead to significant CD63 degradation. However, further evaluation of membrane-associated protein stability based on specific research interests will be required.

In conclusion, the presented protocol offers several advantages over current existing methods. Each step included in the protocol has shown in independent studies to be beneficial for the purity of the exosomes: pelleting of larger vesicles, serum or plasma dilution, and SEC. Additionally, as mentioned above, a proteinase digestion step was included to remove apolipoproteins and protein aggregates as well as a concentration step at the end of the purification process that benefits exosome stability and yield. Finally, this protocol can be easily adapted to isolate exosomes from larger volume samples like urine or cell culture supernatants.

## Disclosures

The authors have nothing to disclose.

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