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Original Article

Simple and Fast SEC-Based Protocol to Isolate Human Plasma-Derived Extracellular Vesicles for Transcriptional Research

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Extracellular vesicles (EVs) are membranous structures that protect RNAs from damage when circulating in complex biological fluids, such as plasma. RNAs are extremely specific to health and disease, being powerful tools for diagnosis, treatment response monitoring, and development of new therapeutic strategies for several diseases. In this context, EVs are potential sources of disease biomarkers and promising delivery vehicles. However, standardized and reproducible EV isolation protocols easy to implement in clinical practice are missing. Here, a size exclusion chromatography-based protocol for EV-isolation from human plasma was optimized. We propose a workflow to isolate EVs for transcriptional research that allows concomitant analysis of particle number and size, total protein, and quantification of a major plasma contaminant. This protocol yields $7.54 \times 10^9 \pm 1.22 \times 10^8$ particles, quantified by nanoparticle tracking analysis, with a mean size of 115.7 ± 11.12 nm and a mode size of 83.13 ± 4.72 nm, in a ratio of $1.19 \times 10^{10} \pm 7.38 \times 10^9$ particles/μg of protein, determined by Micro Bicinchoninic Acid (BCA) Protein Assay, and 3.09 ± 0.7 ng RNA, assessed by fluorescence-based RNA-quantitation, from only 900 μL of plasma. The protocol is fast and easy to implement and has potential for application in biomarkers research, therapeutic strategies development, and clinical practice.

INTRODUCTION

Extracellular vesicles (EVs) have emerged as promising shields of disease-specific or therapeutic RNAs.[1–4] EVs comprise a heterogeneous population of membranous structures that are naturally released from all cell types into the extracellular space. These structures carry a variety of molecules that reflect the biomolecular composition of the tissue and cells of origin.[2,5,6] Thus, EVs have emerged as promising disease biomarkers.[7] Moreover, EVs from healthy cells can elicit beneficial effects in disease-associated recipient cells, being increasingly identified as promising cell-free therapeutic agents.[8] Advanced technological strategies are also being developed to load specific therapeutic RNAs into EVs, as these structures are more stable, efficiently protect nucleic acids from environmental damage, and have reduced immunogenicity when compared to other nano-based drug delivery systems.[2]

Efforts performed in the field have been focused on isolating these vesicles from plasma and serum as these body fluids are abundant, easily accessible, and routinely collected through minimally invasive procedures.[9,10] However, the use of blood-derived EVs in a clinical context depends on the capacity to isolate these structures from contaminants in sufficient yields and in a reproducible, cost-effective, and simple manner.[11–15] Among the different techniques available so far, size exclusion chromatography (SEC) holds great promise for EV-based translational research as this method can be easily adapted to most research and clinical laboratories.[16,17]

SEC separates molecules, based on their size, by filtration through a resin-packed column.[16–19] This technique allows isolation of homogeneous EV populations, removes major biofluid components, avoids EV aggregation, and preserves their functional characteristics.[20–23] When compared to other methods, SEC is user-friendly, less time-consuming, has a relatively low cost, and requires a small amount of starting material.[16,17] Indeed, SEC has been successfully used for small scale analysis of EVs from clinical samples.[24] Its performance is determined by multiple variables, such as column stacking, resin pore size, or sample volume/collected volume ratio.[17,19] The use of commercial columns avoids column preparation and allows faster protocols in quality-controlled and consistent conditions, decreasing the method variability.[25] SEC is also easily automated by using accessible equipment, reducing hands-on time and user influence, and improving consistency and reproducibility.[16]

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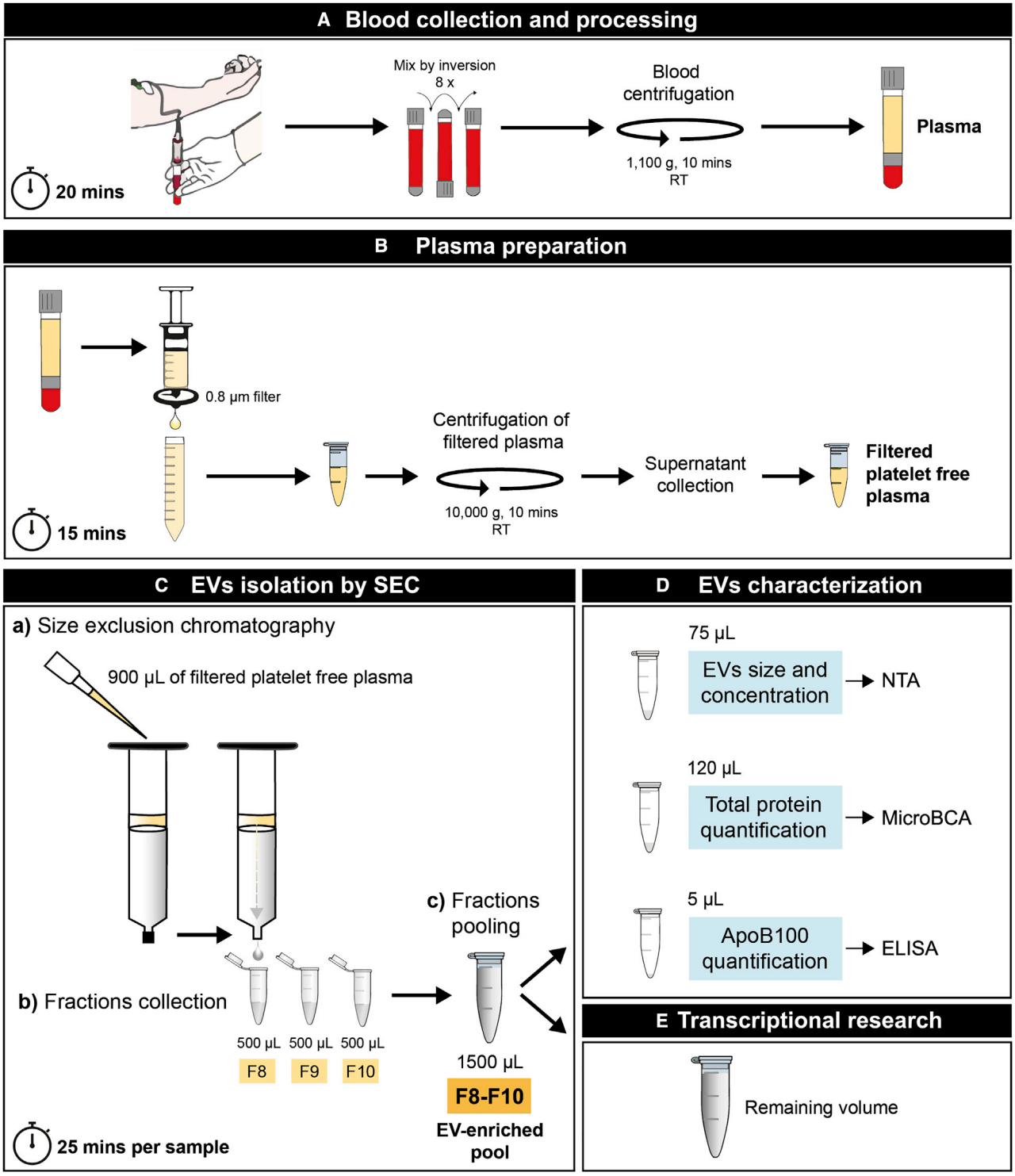


Figure 1. Workflow Summarizing the Protocol to Isolate Human Plasma-Derived EVs by SEC

(A) Blood collection and processing (≈20 min): blood is collected into plasma preparation tubes, mixed by inversion, and centrifuged 1,000 × *g* for 10 min at RT for plasma separation. (B) Plasma preparation (≈15 min): plasma samples are filtered using 0.8-µm filters and centrifuged at 10,000 × *g* for 10 min at RT. The supernatant is collected to obtain filtered platelet-free plasma. (C) EV isolation from plasma samples (≈25 min): a) 900 µL of filtered platelet-free plasma is loaded into the size exclusion chromatography column; b) $F8_{(3.5–4.0\ mL)}$, $F9_{(4.0–4.5\ mL)}$, and $F10_{(4.5–5.0\ mL)}$ are collected; c) enriched EVs $F8_{(3.5–4.0\ mL)}$, $F9_{(4.0–4.5\ mL)}$, and $F10_{(4.5–5.0\ mL)}$ are pooled in a final volume of 1,500 µL.

(legend continued on next page)

Box 1 Comparison between Current Protocol and Manufacturer's Protocol

Differences in Protocol

Input sample of 900 μL instead of 500 μL

Sample is filtered prior to SEC with an 800-nm filter

F8–$10_{(3.5–5.0\ mL)}$ are pooled instead of F7–$9_{(3.0–4.5\ mL)}$

Provides a workflow, step by step, that allows analysis of particle concentration and size, protein amount, levels of a major plasma contaminant, and downstream transcriptional applications from an input sample of only 900 μL

Differences in Results

Higher particle yield

Less cellular contamination

Less plasma contamination: higher particle/protein ratio and higher particle/APOB100 ratio

Higher RNA yield, richer in small RNAs, and low rRNA contamination

The original qEV columns (Izon Science) are among the most popular commercial SEC columns used to isolate EVs.[3,22,26–31] While the manufacturer's instructions[26,27,29,30] were strictly followed in several studies, new conditions were adopted in others (e.g., higher input volumes[22,28,31] and different collected fractions).[3,31] In the latter case, downstream analysis is often performed without prior characterization of isolated EV populations or a critical evaluation of protocol efficiency. There is an evident lack of standardization, which may lead to misleading results and interpretations.[32,33]

Here, we optimized a SEC protocol to isolate EVs from human plasma, using qEV columns, suitable for transcriptional research and clinical applications. Original qEV columns are composed of a bed volume of 10 mL Sepharose CL-2B. Sepharose CL-2B resin has a size exclusion limit of 40×10^6 Da according to its manufacturer. This is equivalent to a sphere with a diameter of 45 nm, assuming the approximate density for large proteins (1.37 g/cm^3).[26,34,35] According to our data (data not shown) and the manufacturer's instructions, up to 1,000 μL of sample can be loaded into these columns without compromising the elution profile, but higher volumes (e.g., 2,000 μL) cause a widening of the distribution profile.[26] Our protocol was developed starting with 900 μL of plasma, as biological fluids are frequently biobanked in small aliquots (500–1,000 μL) and there is always sample loss over freeze-thaw and pipetting.[36,37] A fast and simple workflow that provides sufficient yield for (1) EVs and protein quantification; (2) detection of APOB100, a major plasma contaminant; and (3) transcriptional downstream applications (Figure 1) is proposed. Major differences between this protocol and the manufacturer's protocol[26] are highlighted in Box 1.

According to *Minimal Information for Studies of Extracellular Vesicles* guidelines (MISEV2018),[33] this protocol allows EV isolation with intermediate recovery and specificity. All relevant data from our experiments were submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV190037).[38] The described protocol was optimized for transcriptional analysis of EVs isolated from human plasma samples. It is a versatile protocol that can be easily adapted for EV isolation from other biofluids (e.g., serum or cerebrospinal fluid) or scalable for other purposes (e.g., EV isolation from cell cultured conditioned media for therapeutic applications). It is also adaptable to proteomic analysis, functional assays, or other objectives besides transcriptional analysis. Higher sample volumes can be concentrated before SEC or larger commercially available columns might be used instead. If so, comparison with other protocols[8] and a similar characterization following MISEV guidelines[33] is advisable.

MATERIALS

Reagents

Blood Collection and Processing

- 21G butterfly needles (BG2134, MediPlus)
- K_2EDTA plasma preparation tubes (362799, BD Vacutainer)

Plasma Preparation

- 0.8 μm syringe filter (#SLAA033SB, Merck Millipore)
- 10 mL syringe (SS+10ES1, Terumo) or 1 mL syringe (#303172, Becton Dickinson) for non-filtered biobanked plasma samples
- 15 mL tubes (05-539-5, Fisher Scientific)
- 1.5 mL microcentrifuge tubes (509 GRD-SC-FIS, Fisher Scientific)

SEC

- qEV original columns (#SP1, Izon Science)
- Phosphate-buffered saline (PBS) solution (#BP-399, Fisher Bioreagents)
- 0.1% Triton X-100 (#BP-151, Fisher Bioreagents)
- Ethanol absolute (#E/0650DF/C17, Fisher Chemical)

(D) EV-enriched sample characterization: NTA concentration and size (75 μL), total protein content (120 μL), and APOB100 levels (5 μL) are quantified by nanoparticle tracking analysis (NTA), microBCA, and ELISA, respectively. (E) Transcriptional research: the remaining volume can be used for transcriptional analysis or other analysis that better suits the aim of the study.

- Low binding 1.5 mL microcentrifuge tubes (#509 GRD-SC-FIS, Fisher Scientific)
- Low binding 2.0 mL microcentrifuge tubes (#508 GRD-SC-FIS, Fisher Scientific)
- 10–1,000 μL filter tips (#TF102-10-FIS, #TF113-100-FIS, and #TF112-1000-FIS, Fisher Scientific)
- Recipient for disposals

EV Size and Concentration Analysis by Nanoparticle Tracking Analysis (NTA)

- 1 mL syringes (#303172, Becton Dickinson)
- PBS solution (#BP-399, Fisher Bioreagents)
- 2 mL microcentrifuge tubes (#508 GRD-SC-FIS, Fisher Scientific)
- Molecular biology grade water (BP2819, Fisher Bioreagents)

Total Protein Quantification

- Pierce MicroBCA Protein Assay Kit (#23235, Thermo Scientific)
- PBS solution (#BP-399, Fisher Bioreagents)
- 1.5 mL microcentrifuge tubes (509 GRD-SC-FIS, Fisher Scientific)

APOB100 Quantification

- ELISA kit for APOB100 levels detection (#ELH-APOB100-1, RayBiotech)

RNA Extraction

- Urine Cell-Free Circulating RNA Purification Mini Kit (#56900, Norgen Biotek)

  Note: the Exosomal RNA Isolation Kit (#58000, Norgen Biotek) was also tested and can be used by purifying RNA from 0.5 mL aliquots of sample and pooling the RNA of the 3 aliquots in the end.
- 15 mL centrifuge tubes (#430885, Fisher Scientific)
- RNA Clean-Up and Concentration Kit (#23600, Norgen Biotek)
- β-Mercaptoethanol (#125520250, Acros Organics)
- Ethanol absolute (#E/0650DF/C17, Fisher Chemical)
- Molecular biology grade water (BP2819, Fisher Bioreagents)
- Isopropanol (#P/7500/17, Fisher Chemical)

Equipment

Blood Collection and Processing

- Centrifuge (5810, Eppendorf)

Plasma Preparation

- Microcentrifuge (5415R, Eppendorf)
- Waterbath (1083, GFL)

SEC

- Column holder (RTMM-250-001 and RDS3-060-001, Labbox)

Suggestion: automatic fraction collectors can be used for automated, fast, and precise SEC fraction collection based on weight, eliminating human error and enabling high-precision large-scale standardizable EV isolation.

- Vortex

EV Size and Concentration Analysis (NTA)

- NTA device (NanoSight NS300 instrument, Malvern Instruments)

Note: this protocol was optimized with a NanoSight NS300 instrument (Malvern Instruments) with a 488 nm laser and a scientific complementary metal-oxide-semiconductor detector (sCMOS) camera module. Another device may require settings optimization.

Caution: NTA does not discriminate particle types, which may lead to an underestimation of EV concentration, especially in the sub-100 nm size range, where the sensitivity of NTA is lower.[39] For this reason, we use the term "NTA concentration".

- Vortex (F202A0173, Velp Scientifica)
- Microcentrifuge (5415R, Eppendorf)

Total Protein Quantification

- Vortex (F202A0173, Velp Scientifica)
- Thermic incubator (Raypa)
- Absorbance microplate reader (SpectraMax Plus 384 Microplate Reader, Molecular Devices)

APOB100 Quantification

- Vortex (F202A0173, Velp Scientifica)
- Plate shaker (3005, GFL)
- Thermic incubator (Raypa)
- Absorbance microplate reader (SpectraMax Plus 384 Microplate Reader, Molecular Devices)

Reagent Setup

SEC

0.2 μm sterile-filtered and degassed PBS 1×. To prepare 1 L of PBS 1×, dilute 100 mL of PBS 10× into 900 mL of water. Alternatively, prepare 1 L of PBS 1× by mixing 8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, and 0.2 g KCl in 950 mL of water. Adjust the pH to 7.3 and complete the volume to 1 L with water. Filter PBS 1× through a 0.2 μm filter membrane and degasify it by vacuum for at least 1 h. Store it at 4°C for up to 6 months.

Note: each SEC run requires 50 mL of degasified PBS.

Triton X-100 0.1% in PBS (v/v). To prepare 20 mL of 0.1% Triton X-100, add 20 μl of Triton X-100 to 19.98 mL of filtered PBS and degasify it by vacuum for at least 1 h. Store it at 4°C for up to 6 months.

Note: each SEC run requires 20 mL of 0.1% Triton X-100 in PBS.

Ethanol 20% (v/v). To prepare 15 mL ethanol 20%, add 3 mL ethanol absolute to 12 mL of water. Store it at room temperature (RT, 18–25°C) for up to 2 weeks.

Note: each column requires 12.5 mL of 20% ethanol.

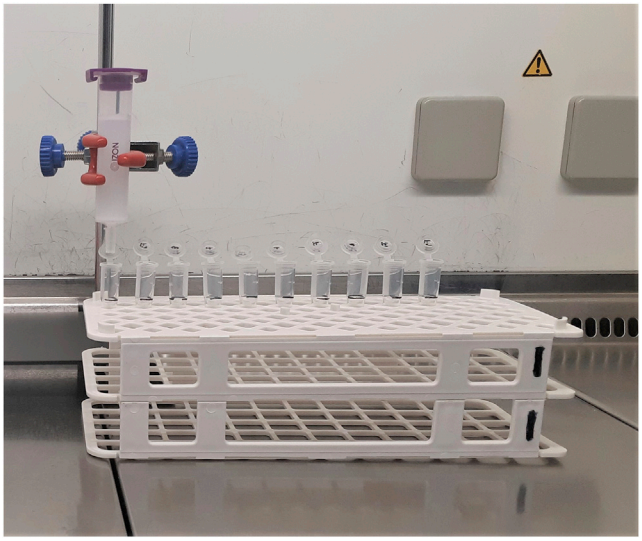


Figure 2. Size Exclusion Chromatography System Set-Up
A qEV column is placed in a holder and marked microcentrifuge tubes are displaced below. Fractions are successively collected until fraction 10, as described in the protocol.

Caution: ethanol is highly flammable and volatile. All manipulation should be carried out under a fume hood.

Total Protein Quantification

PBS 1×. Prepare the required amount as mentioned before. Filtration and degasification steps are optional. Store it at 4°C for up to 6 months.

Note: each 96 well plate requires approximately 20 mL of PBS.

APOB100 Quantification

PBS solution. Prepare the required amount of PBS solution as mentioned before. Store it at 4°C for up to 6 months.

Note: each sample is diluted in 980 µL of PBS. Triplicates require approximately 3 mL of PBS.

RNA Extraction

Ethanol 80% (vol/vol). To prepare 5 mL ethanol 80%, add 4 mL ethanol absolute to 1 mL of water. Store it at RT (18–25°C) for up to 2 weeks.

Note: each sample requires 500 µL of 80% ethanol. 10 samples require 5 mL of 80% ethanol.

Caution: ethanol is highly flammable and volatile. All manipulation should be carried out under a fume hood.

PROCEDURE

A scheme of the workflow is presented in Figure 1.

Blood Collection and Processing

1. Collect blood (8.5 mL), ideally without a tourniquet, from the peripheral veins of study subjects into K_2EDTA plasma preparation tubes.
2. Mix blood samples by completely inverting the blood tubes 8–10 times.
3. Immediately centrifuge blood samples at 1,100 × *g* for 10 min at RT (18–25°C) without a break.
4. Proceed directly to plasma preparation or divide the plasma into 1 mL aliquots and store at −80°C until further processing.

Note: any information about donors, conditions of blood collection, and technical details must be reported according to MISEV guidelines.[33,40,41]

Plasma Preparation

1. Remove the plunger from a 10 mL syringe. Attach the 0.8 µm filter to the syringe and place it on the top of a 15 mL tube.
2. Collect the cell-free plasma with a sterile Pasteur pipette and draw it into the syringe. Carefully reset the syringe plunger.
3. Slowly press the plunger to force the plasma sample rinse through the syringe and filter it into the 15 mL tube, drop by drop. Proceed with step 4 or divide plasma into 1 mL aliquots and store at −80°C until further processing.
4. Centrifuge plasma at 10,000 × *g* for 10 min at RT (18–25°C) for platelet removal.
5. Collect the supernatant into a new microcentrifuge tube.

Note: for non-filtered aliquoted plasma samples, use a 1 mL syringe instead and filter plasma into a 1.5 mL microcentrifuge tube. Proceed with step 4.

EV Isolation by SEC

Before Starting

1. Equilibrate the qEV column, PBS, and Triton X-100 0.1% in PBS to RT (18–25°C) overnight (at least).

Sample Fractioning

See Figure 2 for system set-up.

1. Place the column in a holder and level it.

Caution: make sure the column is vertical.

2. Remove the top cap carefully and slowly. Pinch it tightly so that air can enter the top of the column as it is being removed. Reduce disturbance of the gel.
3. Remove the lower luer-slip cap.
4. Add 2 mL of PBS as soon as the manufacturer's storage solution reaches the column top filter.
5. Add up to 10 mL of PBS.
6. After PBS elution, stop elution by re-placing the bottom luer-slip cap.
7. Set a microcentrifuge tube holder below the column with the prepared low-binding 1.5 mL microcentrifuge tubes.

Suggestion: mark the microcentrifuge tubes with a pen to clearly see when the eluate reaches the 500 μL volume fraction.

8. Pipette out any remaining buffer above the top filter.

Caution: be careful to not disrupt the top filter.

9. Add 900 μL of plasma sample on the top of the column top filter.

Caution: avoid bubbles.

10. Remove the bottom luer-slip cap and collect fractions (F) of 500 μL of eluate into the microcentrifuge tubes up to $F10_{(4.5–5.0\ mL)}$.

Optional: other fractions of interest might be collected.

11. Add 2 mL of PBS when the sample reaches the column top filter.

Caution: wait until all sample enters the top-filter to avoid unintentional sample dilution.

12. Continue adding PBS up to 10 mL while collecting fractions.

Critical: do not allow the column to run dry. The top filter must stay wet. If the column runs dry, it may not function correctly.

13. Store $F8_{(3.5–4.0\ mL)}$, $F9_{(4.0–4.5\ mL)}$, and $F10_{(4.5–5.0\ mL)}$ on ice and discard the unwanted fractions. Proceed with EV Sample Preparation as soon as possible.

Suggestion: $F1–4_{(0.0–2.0\ mL)}$ might be stored to use as negative control (void volume).

14. Start Cleaning the SEC Column as soon as the 10 mL of PBS from step 12 is loaded into the column.

EV Sample Preparation

1. Vortex F8, F9, and F10 to detach EVs that may have adhered to the tube walls.
2. Pool the 3 fractions into a low-binding 2.0 mL microcentrifuge tube. Vortex to mix fractions.
3. Transfer the following volumes to new low-binding microcentrifuge tubes, properly identified:
   - 75 μL for EV size and concentration analysis (NTA)
   - 120 μL for total protein quantification
   - 5 μL for APOB100 quantification.
4. The remaining volume can be used for downstream transcriptional applications.
5. Store samples at −80°C until further processing.

Cleaning the SEC Column

1. Add 2 mL of Triton X-100 0.1% as soon as the PBS reaches the column top filter.
2. Add Triton X-100 0.1% up to 20 mL.
3. Add 2 mL of PBS as soon as Triton X-100 0.1% reaches the column top filter.
4. Add PBS up to 30 mL.

Optional: to confirm whether resin is completely clean, pipette on a 96-well plate 250 μL of the eluted PBS (in triplicate) and 250 μL of fresh PBS (in triplicate) and read their absorbance at 280 nm on a spectrophotometer.

Optional: register the time for 5 mL of buffer to flow through the column. A change in flow rate can suggest a blocked or dirty column.

Suggestion: if you are planning to run several samples, you can run these in parallel using more than 1 column. We recommend starting the SEC run using a second column during the first column cleaning step.

Column Re-use

After column cleaning, a new sample can be run by repeating the protocol from point 6 of the sample fractioning section. Re-use the column 5× maximum.

Storage of the SEC Column

Option 1: if you intend to run more samples in the following 1–2 days.

1. Close the column down lid and add 2.5 mL of PBS.
2. Close the top lid of the column and store it at RT (18–25°C).

Option 2: if you will not run more samples in the following 1–2 days.

1. Add 2 mL of 20% ethanol as soon as the PBS reaches the column top filter.
2. Add 20% ethanol up to 10 mL.
3. Once the ethanol approaches the column top filter, close the column down lid and add 2.5 mL of 20% ethanol. Close the top lid of the column.
4. Store the column at RT (18–25°C) for up to 3 months or at 4–8°C up to the expiration date.

Caution: storing columns at 4°C may increase bubble formation.

EV Characterization

EV Size and Concentration Analysis by NTA

Sample preparation.

1. Thaw EV samples on ice.

Caution: samples should be run as soon as possible after thawing to avoid sample aggregation. Thaw each sample at a time.

2. Vortex to detach EVs that may have adhered to the tube walls and to dissociate possible EV aggregates that may have been formed.
3. Dilute 20 μL of sample in 980 μL of H_2O. Spin the sample. Repeat the experiment 2 times for triplicates.

Optional: samples can be filtered through a 0.8-μm filter to remove possible aggregates and avoid clogging the device tubes.

Caution: filtration will lead to volume loss.

Table 1. Preparation of Bovine Serum Albumin (BSA) Standards for Protein Quantification

	PBS (μL)[a]	BSA (μL)[a]	[BSA] (μg/mL)
A (intermediate stock)	900	100 (2 mg/mL stock)	200
B	960	240 of A	40
C	600	600 of B	20
D	600	600 of C	10
E	600	600 of D	5
F	600	600 of E	2.5
G	600	0	0

[a]Volumes recommended to run assays in triplicate.

Sample loading and analysis.

1. Submerge the syringe in the plasma sample and pull the plunger on the syringe to draw about 1 mL of the sample.
2. Load the sample into the chamber according with the manufacturer's instructions.
3. Define image settings: make sure that the beam is centered in the field of view on the screen, adjust the camera level to be able to distinguish all particles without a saturation of scattering higher than 20%, and adjust the focus to visualize the center of the particles.

Note: this protocol was optimized with a camera level between 13–15. Other settings might be used but must be optimized and reported.

4. Use a syringe pump with constant flow injection. We recommend capturing 5 videos of at least 30 s from each sample.

Note: the flow rate of the syringe pump must be adjusted in a way that a single particle takes approximately 5–10 s to cross the whole field of view. For homogeneous EV populations, record 5 videos of 30 s. For heterogeneous EV populations, at least 5 videos of 60 s are recommended.

5. Adjust the detection threshold and process the data.

Note: this protocol was optimized with a threshold of 3. The detection threshold will depend on the software version. Ideal thresholds will cause the particle center to be marked with red crosses. If there are less than 10 particles per frame, the particle center will be marked with blue crosses instead.

Caution: for reliable measurements, 20–100 particles/frame are recommended for NanoSight NS300.

Critical: keep constant conditions between samples.

Total Protein Quantification

1. Thaw EV samples on ice.
2. Vortex to detach EVs that may have adhered to the tube walls and to dissociate possible EV aggregates that may have formed.
3. Prepare 500 μL of the sample diluted 5–7 times.

Note: this volume is recommended for triplicate assays.

4. Prepare bovine serum albumin (BSA) standards as shown in Table 1.
5. Prepare the working reagent according to the manufacturer's instructions.

Suggestion: perform the assay in triplicate.

6. Pipet 150 μL of each standard per well.
7. Pipet 150 μL of each sample per well.
8. Pipet 150 μL of the working reagent up and down.
9. Mix the plate thoroughly.
10. Incubate samples for 150 min at 37°C in the dark.
11. Cool at RT for 5 min and read absorbance at 562 nm on a microplate reader
12. Subtract the average 562 nm absorbance reading of the blank standard (solution G) replicates from the 562 nm reading of all other individual standard and sample replicates.
13. Generate a 2–40 μg/mL BSA standard linear curve and calculate protein concentrations by interpolation.

APOB100 Quantification

Sample preparation.

1. Thaw the EV samples on ice.
2. Vortex to detach EVs that may have adhered to the tube walls and to dissociate possible EV aggregates that may have formed.
3. Prepare the samples.

Note: the suggested dilution for EV samples obtained with this protocol is 1:1,000–1:5,000. For example, to prepare a 1,000-fold diluted sample, add 1 μL of EV sample into a tube with 999 μL of assay diluent. Mix thoroughly.

ELISA assay. 1. Pipet 100 μL of the sample per well.

Suggestion: perform the assay in triplicates.

2. Follow the manufacturers' instructions.

Biomarkers Research

RNA Extraction and Concentration for Transcriptional Analysis

1. Thaw the EV samples on ice.
2. Vortex to detach EVs that may have adhered to the tube walls and to dissociate possible EV aggregates that may have formed.
3. Transfer the EV sample to a 15 mL centrifuge tube and follow the Urine Cell-Free Circulating RNA Purification Mini Kit manufacturer's instructions.

Suggestion: add β-mercaptoethanol to your lysis buffer to guarantee a more efficient RNA extraction.

Critical: before RNA elution, it is important to guarantee that the spin column membrane is dry, since residual ethanol may interfere with

Table 2. Protocol Troubleshooting

Problem	Comments/Suggestions
SEC	
Clogs/bubbles visible in the matrix of the SEC column	Temperature changes impact the matrix of SEC columns. Allow gradual changes and let it equilibrate to RT (18–25°C) during more time (2–3 days).
Changes in flow rate, changes of color at the top of the column after flushing, or appearance of an evident space between top-filter and gel surface	Column integrity might be compromised. Use a new column.
EV Size and Concentration Analysis (NTA)	
Low/high particles/frame	Optimize sample dilution by testing serial dilutions. For reliable measurements, 20–100 particles/frame are recommended for NanoSight NS300.
Total Protein Quantification	
Sample absorbance is below or over the detection range	Optimize the sample dilution by testing serial dilutions.
APOB100 Quantification	
Sample absorbance is below or over the detection range	Optimize the sample dilution by testing serial dilutions.

downstream reactions. We recommend performing the last centrifugation with open lids to ensure no ethanol will be carried over during the following step.

4. Elute in 50 μL RNase free H_2O.

Caution: even though the RNA extraction kit provides an elution buffer, we recommend eluting in RNase-free H_2O instead to proceed with the RNA concentration procedure and avoid any incompatibility with downstream procedures.

5. Repeat the elution step by adding 50 μL of RNase-free H_2O onto the column to obtain a final eluted volume of 100 μL.
6. Concentrate RNA in as low as 8 μL using the protocol for concentrating RNA samples highly enriched with small RNA and following the manufacturer's recommendations.

Timing

- Blood collection and processing: 20 min
- Plasma preparation: 15 min
- SEC: 25 min
  - o Sample fractioning: 20 min
  - o EV sample preparation: 5 min
  - o Cleaning the SEC column: 45–60 min
- EV size and concentration analysis (NTA): 30 min
- Total protein quantification: 3 h–3 h and 30 min
- APOB100 quantification: 7 h
- RNA extraction and concentration for transcriptional analysis: 1 h 30 min–2 h

Troubleshooting

Difficulties that may arise during the protocol execution and potential solutions are summarized in Table 2.

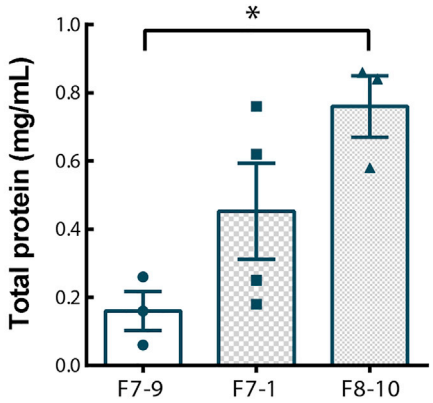
Anticipated Results

Here, we present a fully described SEC protocol to isolate EVs from human plasma for transcriptional analysis. The obtained EV population was characterized in accordance with MISEV2018 guidelines.[33] We first focused on individual SEC fraction analysis to define EV-enriched fractions that could be further pooled to increase yield without compromising purity. Each fraction was analyzed concerning total protein amount, presence of EV and cellular markers, particle number and size, morphology, plasma contaminants, and RNA concentration and profile (Figures S1–S8). The obtained results pinpointed $F7_{(3.0–3.5\ mL)}$, $F8_{(3.5–4.0\ mL)}$, $F9_{(4.0–4.5\ mL)}$, and $F10_{(4.5–5.0\ mL)}$ as the most promising EV-enriched fractions. From F11 on, we detected a western blot band profile of Calnexin, a cellular marker, that was similar to the one observed in HEK293 cells (Figures S2 and S3), suggesting cellular contamination. In opposition, $F7_{(3.0–3.5\ mL)}$, $F8_{(3.5–4.0\ mL)}$, $F9_{(4.0–4.5\ mL)}$, and $F10_{(4.5–5.0\ mL)}$ had no signs of cellular contamination and contained increasing particle NTA concentrations from $F7_{(3.0–3.5\ mL)}$ to $F10_{(4.5–5.0\ mL)}$, with sizes mainly below 150 nm (Figures S2–S5). While no proteins were detected in $F7_{(3.0–3.5\ mL)}$ and $F8_{(3.5–4\ mL)}$ by microBCA, $F9_{(4.0–4.5\ mL)}$ and $F10_{(4.5–5.0\ mL)}$ contained $2.23 \times 10^{10} \pm 8.39 \times 10^{9}$ and $8.28 \times 10^{9} \pm 2.04 \times 10^{9}$ particles/μg of protein, respectively (Figures S1 and S5). According to MISEV2018,[33] albumin and apolipoproteins A1/2 (APOA1/2) and B (APOB) are the best negative markers to evaluate contamination with non-EV structures when working with plasma. Less than 1% of plasma albumin and APOA1 are detected in these fractions. Increasing levels of APOB100 are observed from $F7_{(3.0–3.5\ mL)}$ to $F10_{(4.5–5.0\ mL)}$ (Figure S6). F7 to F10 individual fractions are mainly constituted by small RNAs (Figures S7 and S8).

Based on data from individual fractions, F7 to F9 [$F7–9_{(3.0–4.5\ mL)}$], F7 to F10 [$F7–10_{(3.0\ –\ 5.0\ mL)}$], and F8 to F10 [$F8–10_{(3.5–5.0\ mL)}$] were pooled and characterized to determine which pool would be more suitable for transcriptional analysis. Our data showed that all pools have less than 1 mg of protein/mL, as determined by microBCA. $F7–9_{(3.0–4.5\ mL)}$, $F7–10_{(3.0–5.0\ mL)}$, and $F8–10_{(3.5–5.0\ mL)}$ have $3.48 \times 10^{9} \pm 1.25 \times 10^{9}$, $2.29 \times 10^{9} \pm 1.12 \times 10^{8}$, and $1.19 \times 10^{10} \pm 7.38 \times 10^{9}$ particles/μg protein, respectively. Albumin is present in less than 0.5% of its total amount in plasma samples (Figures 3A and 3B). This reinforces increased SEC efficacy in separating EVs from plasma proteins[20,34,42] and contrasts with other techniques (e.g., ultracentrifugation, precipitation-based techniques, and immunocapture) in which significant contaminations with soluble plasma proteins have been observed.[17,34,42]

EV markers (Flotilin-1, LAMP2, CD81, ICAM, CD63, EpCAM, ANXA5, and TSG101) were detected in all pools (Figures 4A and 4B). The cellular marker GM130 was barely detected in the three pools, but Calnexin was detected in some $F7–9_{(3.0–4.5\ mL)}$ and $F7–10_{(3.0–5.0\ mL)}$ samples. On the contrary, Calnexin was scarcely detected

A



B

	Albumin			ApoA1			ApoB100		
	EV-enriched pools								
	F7-9	F7-10	F8-10	F7-9	F7-10	F8-10	F7-9	F7-10	F8-10
µg/mL ± SEM	5.29 ± 3.36	60.30 ± 21.49	52.64 ± 1.79	28.31 ± 2.20µ	236.70 ± 64.26	373.90 ± 132.50	286.30 ± 95.96	858.90 ± 226.80	1323.00 ± 47.30
	Plasma								
µg/mL ± SEM	33122 ± 4831	39026 ± 3964	42835 ± 1554	32098 ± 26514	63143 ± 18678	81437 ± 5328	4379 ± 351.4	4853 ± 534.8	5196 ± 580.1
% relative to plasma									

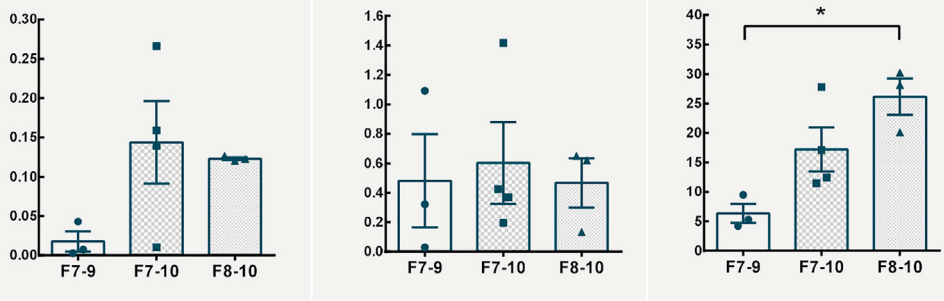


Figure 3. Quantification of Protein and Plasma Contaminants in Pooled EV-Enriched SEC-Eluted Fractions

Human plasma samples were run into a SEC column and $F7_{(3.0–3.5\ mL)}$, $F8_{(3.5–4.0\ mL)}$, $F9_{(4.0–4.5\ mL)}$, and $F10_{(4.5–5.0\ mL)}$ were collected and pooled into $F7–9_{(3–4.5\ mL)}$ (n = 3), $F7–10_{(3–5\ mL)}$ (n = 4), and $F8–10_{(3.5–5\ mL)}$ (n = 3). (A) Total protein was quantified by microBCA. (B) Percentage of albumin, APOA1, and APOB100 relative to plasma. Data are presented as mean ± SEM. One-way ANOVA test followed by a Tukey's multiple comparisons test; *$p \leq 0.05$.

in $F8–F10_{(3.5–5.0\ mL)}$ samples (Figure 4A). These data suggest that cellular contamination occurs, in a non-consistent manner, in F7-containing samples.

SEC's major limitation is its lower capacity to separate EVs from similar-size lipoproteins.[13,14,20,25,31] High-density lipoproteins (HDLs) are mainly constituted by APOA1, whereas APOB100 is the main constituent of low-density lipoproteins (LDLs) and very low-density lipoproteins (VLDLs). APOB100 levels comprised 6.34 ± 1.61%, 17.19 ± 3.74%, and 26.14 ± 3.09% of the total plasma in $F7–9_{(3.0–4.5\ mL)}$, $F7–10_{(3.0–5.0\ mL)}$, and $F8–10_{(3.5–5.0\ mL)}$, respectively. $F7–9_{(3.0–4.5\ mL)}$, $F7–10_{(3.0–5.0\ mL)}$, and $F8–10_{(3.5–5.0\ mL)}$ had $1.08 \times 10^9 \pm 7.62 \times 10^7$, $1.67 \times 10^9 \pm 2.70 \times 10^8$, and $7.28 \times 10^9 \pm 4.38 \times 10^9$ particles/µg of APOB100, respectively. Negligible levels of APOA1 were found (Figure 3B). These data indicate LDLs/VLDLs as major co-eluted plasma lipoproteins and may account for particle number overestimation as NTA does not distinguish between EVs and similarly sized structures.[15,33,43] Therefore, our protocol included APOB100 quantification to assess for lipoprotein contamination.

Concerns related to EV fraction contamination with LDLs/VLDLs are associated with the presence of RNA species carried by those, that are

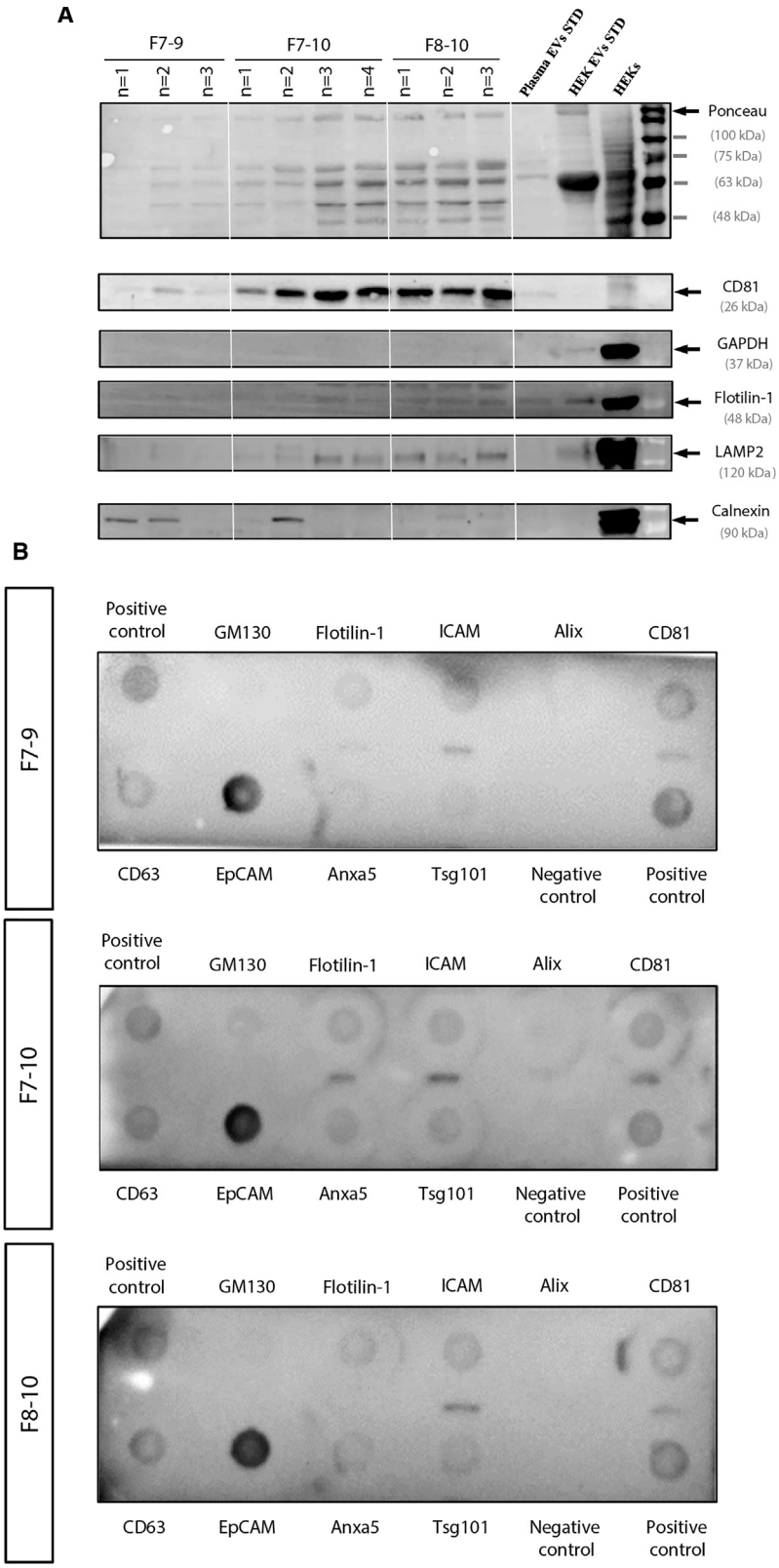


Figure 4. EV and Cellular Markers in Pooled EV-Enriched Fractions

Human plasma samples were run into a SEC column and $F7_{(3.0–3.5\ mL)}$, $F8_{(3.5–4.0\ mL)}$, $F9_{(4.0–4.5\ mL)}$, and $F10_{(4.5–5.0\ mL)}$ were collected and pooled into $F7–9_{(3–4.5\ mL)}$ (n = 3), $F7–10_{(3–5\ mL)}$ (n = 4), and $F8–10_{(3.5–5\ mL)}$ (n = 3). (A) Western blot of EV-associated (CD81, GAPDH, Flotilin-1, and LAMP2) and cellular (Calnexin) markers. Uncropped images are shown in Figure S10. (B) Dotblot of additional EV/cellular markers. Eight EV markers (CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5, and TSG101), a cellular marker (GM130), two positive controls (human serum EVs), and a negative control (blank spot) were evaluated in $F7–9_{(3.0–4.5\ mL)}$, $F7–10_{(3.0–5.0\ mL)}$, and $F8–10_{(3.5–5.0\ mL)}$ samples.

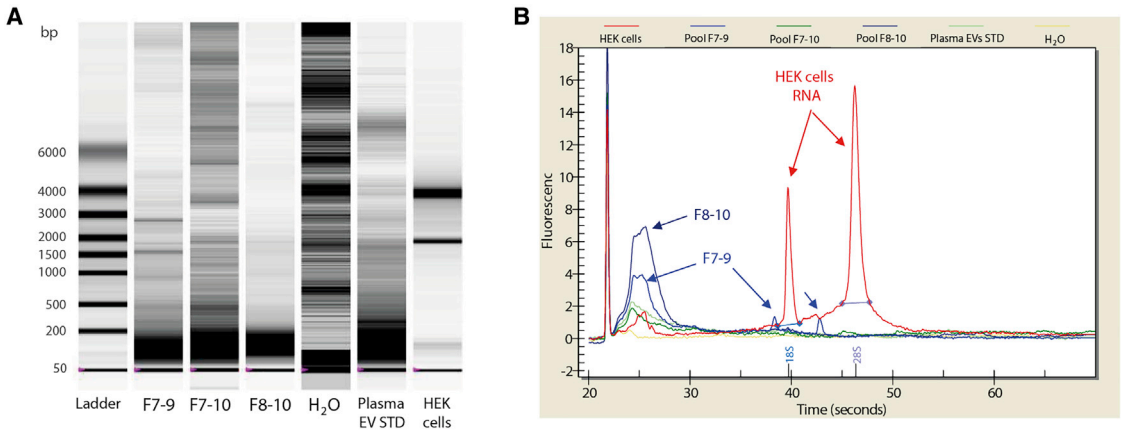


Figure 5. RNA Profile of Pooled EV-Enriched Fractions

Human plasma samples were run into a SEC column, and the RNA profile of F7–$9_{(3.0–4.5\ mL)}$ (n = 3), F7–$10_{(3.0–5.0\ mL)}$ (n = 4), and F8–$10_{(3.5–5.0\ mL)}$ (n = 3) samples was evaluated by automated electrophoresis. (A) Representative virtual RNA electrophoresis gel. (B) Representative RNA electropherogram. See the individual electropherograms in Figure S9.

not EV-specific.[44,45] However, LDL particles were shown to carry RNAs to a lesser extent than HDL particles (≈10-fold less). The majority of LDL-miRNAs are minimally detected at <10 copies/μg LDL and have a signature closely aligned with EV-miRNA signatures,[44,45] suggesting that LDL contamination might be less critical for EV transcriptional research than HLD contamination. Further experiments comparing the RNA expression profile of lipoprotein-depleted plasma EV samples with samples purified using this method would be useful to better characterize the extent of this contamination derived from lipoproteins.

To our knowledge, to this date, no stand-alone EV isolation method results in complete removal of plasma lipoproteins,[14,34,40,43,46] and isolation of EVs from lipoproteins seems possible only by combining multiple isolation techniques.[15,32] However, such approaches compromise yield, are labor intensive, are more prone to errors, and induce higher variability due to the associated multiple-step procedures.[15,17,18,32,47] In the absence of an ideal EV isolation method, it is crucial to balance the type and availability of starting material, required yield for downstream applications, and impact of co-isolated non-EV components (EVs purity) for each specific application.

A current challenge in the field is the definition of quality standards and thresholds of EV preparations for each application. It has been argued that a highly pure EV population should show a ratio equal to or above 3×10^{10} EVs/μg of protein.[11] However, this threshold should be

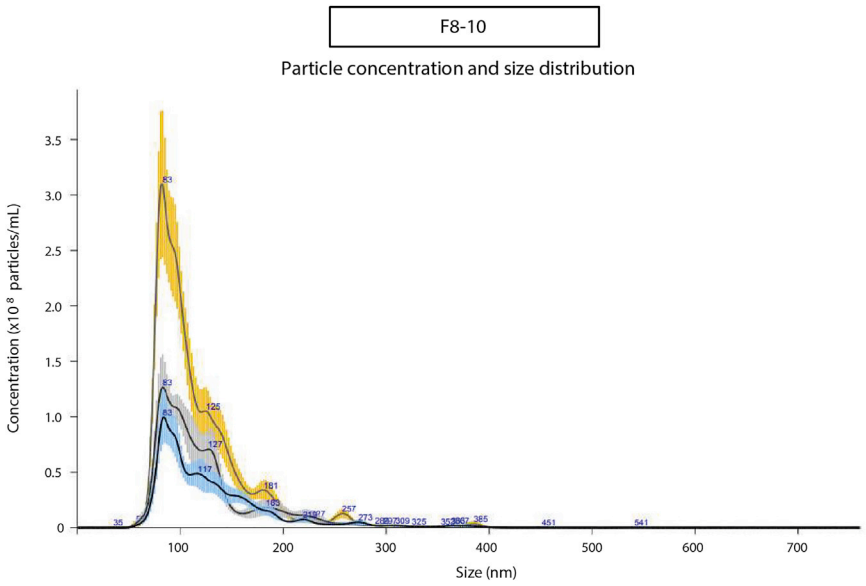
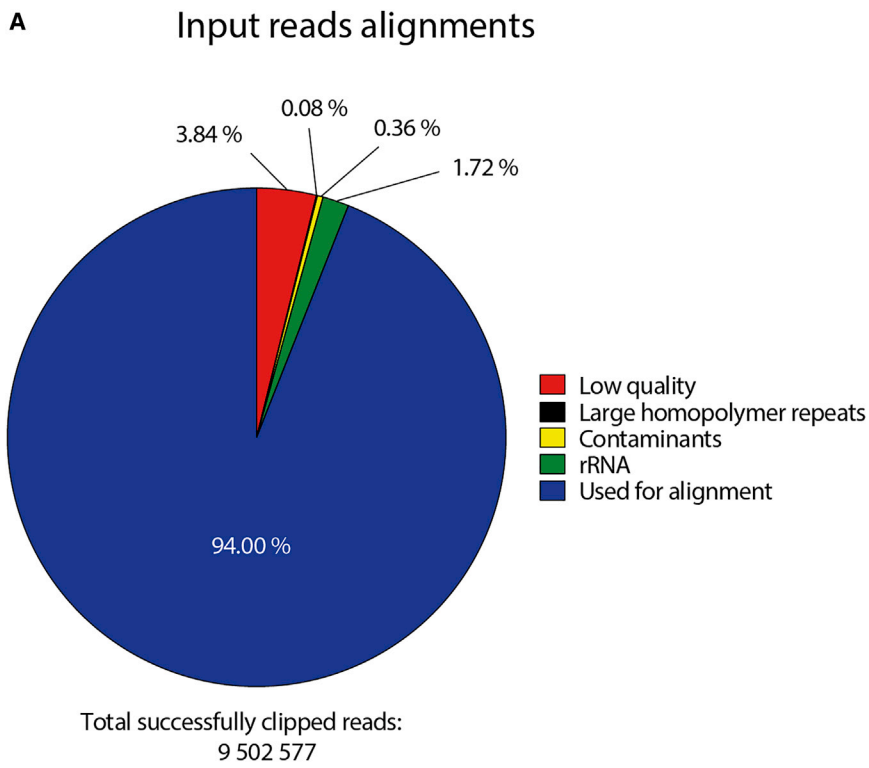


Figure 6. Particle Concentration and Size Distribution of F8–$10_{(3.5–5.0\ mL)}$

Particle concentration and size distribution of F8–$10_{(3.5–5.0\ mL)}$ was assessed by nanoparticle tracking analysis. 3 independent biological samples are shown.



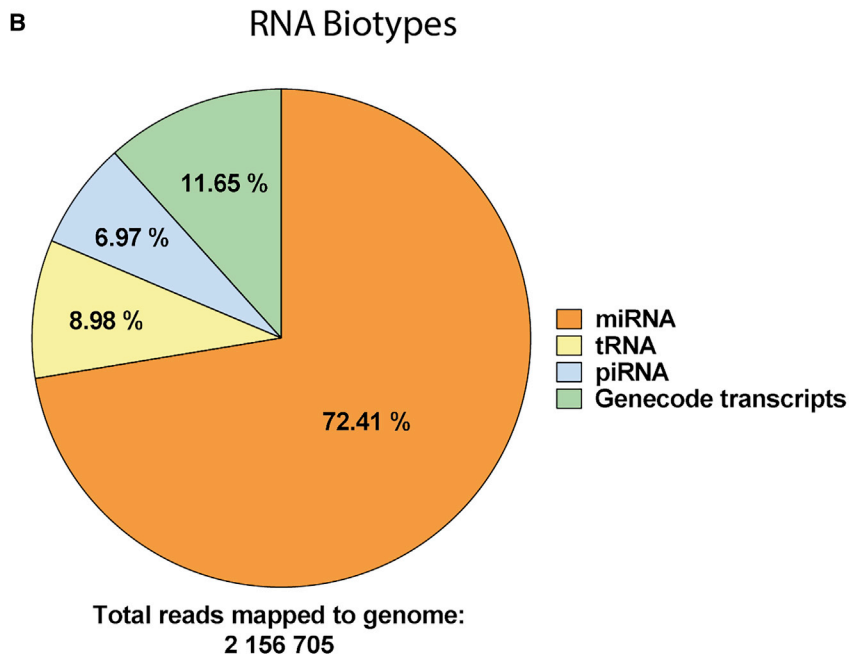


Figure 7. Small RNA Sequencing of F8–10$_{(3.5–5.0\ mL)}$
Human plasma samples (n = 12) were run into a SEC column and small RNA sequencing was performed in F8–10$_{(3.5–5.0\ mL)}$. Reads were aligned and mapped to the human genome after adaptor clipping and quality filtering. (A) Fraction aligned reads normalized by total number of input reads. Data presented as percentage mean. (B) Relative biotype distribution of small RNAs normalized to the total reads mapped to the genome.

interpreted with caution as particle/protein ratios depend on EV sources and isolation methods.[12] When working with complex biological fluids, such as plasma, it is virtually impossible to achieve such purity.[11] Indeed, Stranska et al. [34] obtained a particle/protein ratio of around 1.5×10^9 using 2 mL of plasma and 10 mL qEV columns in comparison with a 0.5×10^9 ratio when using a membrane-affinity spin column method. Our results show considerably higher particle/protein ratios, suggesting great potential for several downstream applications.

Using this protocol, RNA yields of 2,932.00 ± 473.4 pg/μL, 2,192.00 ± 327.1 pg/μL, and 3,085.00 ± 722.4 pg/μL are obtained for $F7–9_{(3.0–4.5\ mL)}$, $F7–10_{(3.0–5.0\ mL)}$, and $F8–10_{(3.5–5.0\ mL)}$, respectively, as assessed by fluorescence-based RNA quantitation. These fractions contain mainly small RNA species (<200 bp; Figures 5A and 5B), which is in agreement with the EV-RNA profile described in literature.[48] In some $F7–9_{(3.0–4.5\ mL)}$ samples, however, we detected potential rRNAs (Figures 5A and 5B; Figure S9). Even though fragmented rRNAs might be present in EV samples,[49] intact rRNAs have not yet been found.[1,50] This supports the cellular contamination derived from $F7_{(3.0–3.5\ mL)}$, which seems to be critical in introducing variability, probably due to upstream blood sample processing. Indeed, if remaining cells or cellular debris are present, these would be expected to elute in the first fractions due to their higher size. Given that all studies we found in the literature using qEV columns for EV isolation from human plasma included $F7_{(3.0–3.5\ mL)}$ in the final EV pool,[3,22,26–30] we recommend caution in results interpretation and stress the importance of using standardized protocols for blood collection and processing.

SEC-eluted individual and pooled fractions were characterized using concentrated samples (see Supplemental Information) as, for some experiments (e.g., western blot), samples were too diluted for signal detection. In accordance with the obtained data, we further validated a simpler protocol using non-concentrated samples as described in the suggested workflow (Figure 1). Non-concentrated $F8–10_{(3.5–5.0\ mL)}$ yielded $7.54 \times 10^9 \pm 1.22 \times 10^8$ NTA particles, with a mean size of 115.7 ± 11.12 nm and a mode size of 83.13 ± 4.72 nm (Figure 6), with 38.06 ± 4.59 μg of protein and 3.09 ± 0.7 ng of RNA, from 900 μL of plasma. This results in a ratio of $1.19 \times 10^{10} \pm 7.38 \times 10^9$ particles/μg of protein and $7.28 \times 10^9 \pm 4.38 \times 10^9$ particles/μg of APOB100.

The protocol suitability for downstream transcriptional applications was assessed by small RNA sequencing. $F8–10_{(3.5–5\ mL)}$ samples, from healthy individuals (n = 12), were sequenced using an Illumina NextSeq 500 platform as described by El-Mogy et al.[51] Reads were aligned and mapped to the human genome after adaptor clipping and quality filtering according to the exceRpt pipeline.[52] The total input reads mean was 15,695,124, with 9,502,577 reads being successfully clipped. From these, 94.00% were used for alignment, with a minimum percentage of reads failing quality filters (3.84%) and only 12% of reads being aligned to human rRNA. Upon RNA biotypes classification, miRNAs were the largest RNA biotype present in $F8–10_{(3.5–5\ mL)}$ samples (72.41%), in accordance with what has been observed in previous studies (Figure 7).[53–55]

Conclusions

We describe a SEC-based protocol to isolate EVs from human plasma using a qEV column and a sample input of only 900 μL of plasma. This protocol provides enough EV yield for transcriptional analysis, particle size and concentration evaluation, total protein quantification, and measurement of the APOB100 major contaminant. The presented workflow (Figure 1) is fast, easy to implement, can be adapted to different applications in the EV field, and has potential for future application in clinics.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.07.012.

AUTHOR CONTRIBUTIONS

L.S.G. and M.M.S., study design and plan, data collection, analysis, interpretation and manuscript writing; C.H., data collection, analysis, interpretation, discussion, and manuscript review; M.M.P., data collection, analysis, interpretation and discussion; T.M.R. and H.G., data collection and analysis (NTA experiments); R.J.N., study design, data interpretation, discussion and manuscript review; L.P.A., study design, data interpretation, discussion, and manuscript review and approval.

CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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