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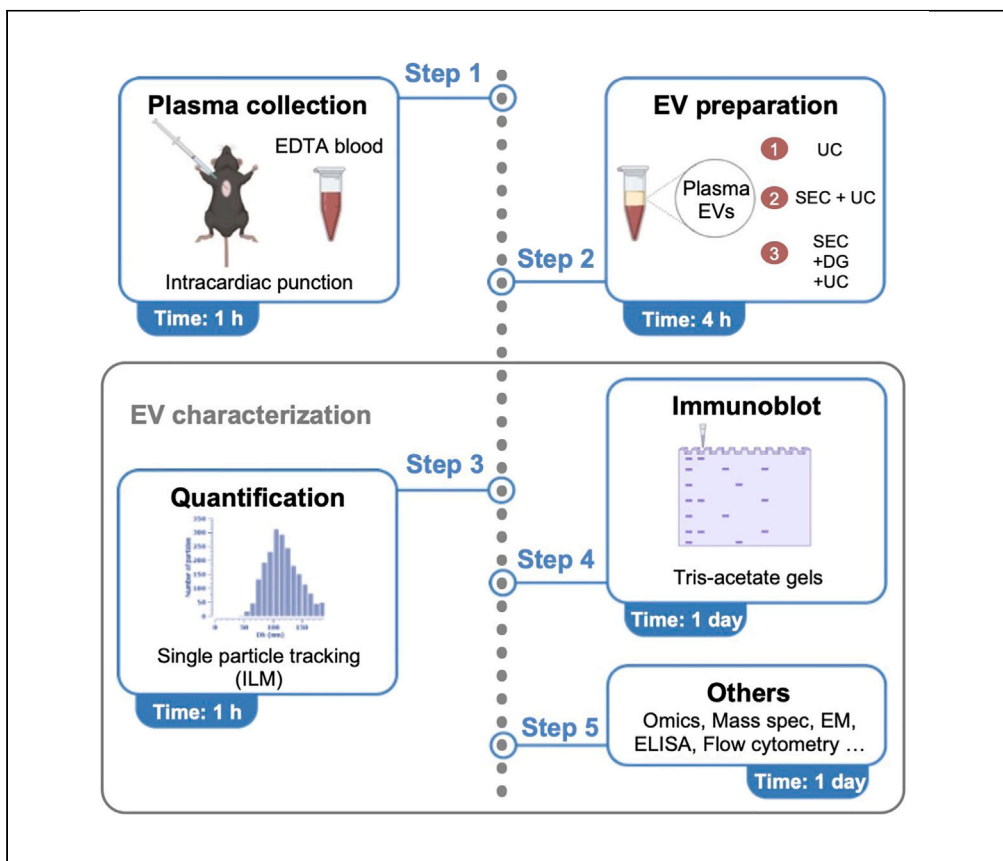
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Protocol

Isolating plasma extracellular vesicles from mouse blood using size-exclusion chromatography, density gradient, and ultracentrifugation



Circulating extracellular vesicles (EVs) could serve for the surveillance of diverse pathological conditions. We present a protocol for enriching and isolating plasma EVs from mouse blood. We describe steps for employing ultracentrifugation, size-exclusion chromatography, and density gradients, required for further quantitative and qualitative analysis. We detail the procedure for retrieving optimal volume of blood while preserving its integrity and avoiding hemolysis. We also describe the preparation of EVs from this complex fluid containing soluble proteins, aggregates, and lipoprotein particles.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optimized collection of mouse plasma to separate extracellular vesicles (EVs)

Separation of EVs with chromatography, ultracentrifugation, and density gradient

Comparison of the yield and purity between three different EV separation procedures

Analysis of the protein content of plasma EVs with Tris-acetate western blots

André-Grégoire et al., STAR
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Protocol

Isolating plasma extracellular vesicles from mouse blood using size-exclusion chromatography, density gradient, and ultracentrifugation

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SUMMARY

Circulating extracellular vesicles (EVs) could serve for the surveillance of diverse pathological conditions. We present a protocol for enriching and isolating plasma EVs from mouse blood. We describe steps for employing ultracentrifugation, size-exclusion chromatography, and density gradients, required for further quantitative and qualitative analysis. We detail the procedure for retrieving optimal volume of blood while preserving its integrity and avoiding hemolysis. We also describe the preparation of EVs from this complex fluid containing soluble proteins, aggregates, and lipoprotein particles. For complete details on the use and execution of this protocol, please refer to André-Grégoire et al. (2022).¹

BEFORE YOU BEGIN

The protocol below details the different steps to isolate and characterize EVs from mouse blood. It is noteworthy that a similar protocol can be applied to human blood samples.

In addition to mouse agreement and housing (refer to the 'institutional permissions' section), several materials and buffers need to be anticipated (including ultracentrifuge and other specific lab equipment).

Institutional permissions

This protocol requires prior ethical approvals for animal welfare structures and national authorities responsible for animal experimentation. The procedure described here was approved by the French Government (#24400-2020022713064016 v2).

Mouse blood collection for EV separation is performed via intra-cardiac puncture to maximize recovery of both plasma and EVs. The procedure therefore involves euthanasia of the animals and should be carefully planned according to the experimental context to ensure relevance in terms of time and expected quantity (for instance, in the case of xenografted mice).

Preparation of reagents and equipment

© Timing: 2 h



Table 1. Composition of density gradient working buffer (pH 7.4)

Reagent	Final concentration	Amount
Sucrose	0.25 M	42.79 g
Tris-HCl	60 mM	4.725 g
EDTA (0.5 M)	6 mM	6 mL
Milli-Q ddH ₂ O	N/A	qsf 500 mL
Total	N/A	500 mL

1. Cool down the bench top centrifuge and ultracentrifuge with MLA-130 or SW41TI rotors and buckets at 4°C.
2. Prepare 0.22 μm filtered PBS to eliminate particles that could interfere with downstream analysis of EVs using a 0.22 μm bottle-top filter unit and an autoclaved 500 mL glass bottle. Alternatively, use commercial sterile particle-free PBS.
3. Prepare density gradient working buffer (pH 7.4) as presented in [Table 1](#) and published methods.^{2–5}

Note: Filter-sterilized density gradient working buffer can be stored at 4°C for 6 months.

- a. Add sucrose, Tris-HCl, EDTA, and 450 mL of Milli-Q ddH₂O to a clean 500 mL glass bottle and homogenize the buffer using a magnetic stirrer.
 - b. Adjust pH to 7.4 with HCl and NaOH solutions.
 - c. Transfer the buffer to a 500 mL volumetric flask and adjust the volume to 500 mL with Milli-Q ddH₂O.
 - d. Filter through a 0.22 μm bottle top filter into a new sterile 500 mL glass bottle and store at 4°C.
4. Prepare density gradient homogenization buffer (pH 7.4) as presented in [Table 2](#) and published methods.^{2–5}

Note: Filter-sterilized density gradient homogenization buffer can be stored at 4°C for 6 months.

- a. Add sucrose, Tris-HCl, EDTA, and 450 mL of Milli-Q ddH₂O to a clean 500 mL glass bottle and homogenize the buffer using a magnetic stirrer.
- b. Adjust pH to 7.4 with HCl and NaOH solutions.
- c. Transfer the buffer to a 500 mL volumetric flask and adjust the volume to 500 mL with Milli-Q ddH₂O.
- d. Filter through a 0.22 μm bottle-top filter into a new sterile 500 mL glass bottle and store at 4°C.

△ CRITICAL: To perform density gradient ultracentrifugation, an adapted rotor with swinging buckets is mandatory.

5. Prepare 3M Tris-acetate buffer pH 7.0 (15×) for immunoblot gels.

△ CRITICAL: Due to the low quantities of starting material, the performance of immunoblot is crucial. For this reason, we strongly recommend using Tris-acetate gels to improve transfer quality. In addition, gradient gels are preferred to resolve low and high molecular

Table 2. Composition of density gradient homogenization buffer (pH 7.4)

Reagent	Final concentration	Amount
Sucrose	0.25 M	42.79 g
Tris-HCl	10 mM	788 mg
EDTA (0.5 M)	1 mM	1 mL
Milli-Q ddH ₂ O	N/A	qsf 500 mL
Total	N/A	500 mL

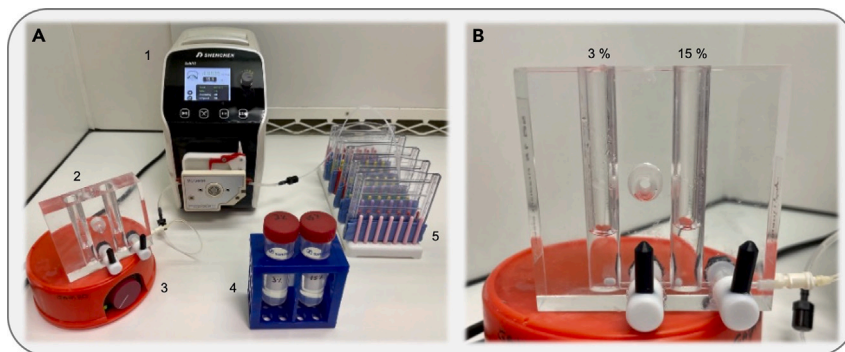


Figure 1. Tris-acetate gradient gel set-up

(A) Set-up with 1: Peristaltic flow rate pump. 2: Gradient maker. 3: Magnetic stirrer. 4: Acrylamide pre-mix solutions of 3 and 15%. 5: Empty gel cassettes.

(B) Gradient maker loaded with 3 and 15% acrylamide solutions.

weight proteins from one single sample. Tris-acetate gradient gels are available commercially (for instance NuPAGE Tris-Acetate mini gels 10 wells from Thermo Scientific, reference EA0375D) or can be homemade as described below from our step-by-step protocol adapted from published methods.⁶

- a. Dissolve 36.33 g Tris-base in 40 mL Milli-Q ddH₂O + 10 mL ice-cold acetic acid using a magnetic stirrer.
- b. Slowly adjust pH to 7 with ice-cold acetic acid.
- c. Transfer the buffer to a 100 mL volumetric flask and adjust the volume to 100 mL with Milli-Q ddH₂O.
- d. Store at 4°C.
6. Prepare Tris-acetate gels (Figures 1A and 1B). Volumes are indicated for 5 gels.
 - a. Prepare 3% and 15% acrylamide solutions as follows (Table 3).
 - b. Dispose of 5 empty gel cassettes on an appropriate support.
 - c. Switch on the peristaltic flow rate pump connected to the gradient maker (flow rate: 4.92 mL/min) and the magnetic stirrer.

Note: The leftover volume of the 3% acrylamide solution will be used for the stacking gel.

- d. Prepare the first running gradient gel: dispose of 2.8 mL of 3% acrylamide solution in the left column and 2.8 mL of 15% acrylamide solution in the right column of the gradient maker, and add 30 μL of ammonium persulfate 10% (APS) and 1 μL of tetramethylethylenediamine (TEMED) in each (final concentration: 0.1% APS and 0.03% TEMED). Immediately open both columns to start filling the gel cassette with the premixes.
- e. Once the two premixes have been transferred to the cassette, promptly rinse the tubing with water to avoid any leftover drops to polymerize.
- f. Carefully load 500 μL of 2-propanol (diluted at 50% with Milli-Q ddH₂O) on the top of the casted gradient gel to line up the surface before polymerization.
- g. Repeat steps d-f for the 4 other empty cassettes.

Table 3. Composition of acrylamide pre-mix solutions for Tris-acetate gels

	3%	15%
3M Tris-acetate buffer (pH 7.0)	1.88 mL	1.08 mL
Acrylamide 40%	2.12 mL	6 mL
Milli-Q ddH ₂ O	24 mL	8.92 mL
Total	28 mL	16 mL

- h. Let the 5 gels polymerize at 16°C–25°C for at least 10 min.
 - i. Remove the 2-propanol by gently tilting the cassettes, rinse the gel surfaces with Milli-Q ddH₂O, and dry with filter paper.
 - j. Cast the stacking gel: add to the remaining 14 mL of 3% acrylamide solution 140 μL of APS 10% and 14 μL of TEMED (final concentration: 0.1% APS and 0.1% TEMED), and immediately add this stacking solution onto the 5 polymerized gradient gels.
 - k. Quickly insert combs with the desired number of wells.
 - l. Let the gels polymerize at 16°C–25°C for at least 10 min.
 - m. For storage, prepare a sealable plastic bag containing 2 mL Tris-acetate SDS Running Buffer (20×) and 38 mL of Milli-Q ddH₂O. Tris-acetate gels can be stored for up to one month at 4°C.
7. Prepare denaturation SDS sample buffer (2×) under a chemical fume hood.
- a. Take 2 mL of buffer away from a new SDS sample buffer (2×) bottle.
 - b. Add 2 mL of 2β-mercaptoethanol and mix.
 - c. Store at 4°C for several months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alix (dilution 1:1,000)	BioLegend	Cat#634502; RRID: AB_2162471
ALB/albumin (F-10) (dilution 1:1,000)	Santa Cruz Biotechnology	Cat#sc-271605; RRID: AB_10647230
Apolipoprotein B (dilution 1:1,000)	Aalto Bio Reagents	Cat#BB2285
Calreticulin (dilution 1:1,000)	Cell Signaling Technology	Cat#122238; RRID: AB_2688013
CD9 (dilution 1:1,000)	System Biosciences	Cat#ExoAB-CD9A-1; RRID: AB_2687469
GM130 (dilution 1:1,000)	Abcam	Cat#ab52649; RRID: AB_880266
HSP70 (dilution 1:1,000)	System Biosciences	Cat#ExoAB-Hsp70A-1; RRID: AB_2687468
Goat anti-rabbit IgG (H + L), mouse/human ads-HRP (dilution 1:5,000)	SouthernBiotech	Cat#4050-05; RRID: AB_2795955
Goat anti-mouse IgG1, human ads-HRP (dilution 1:5,000)	SouthernBiotech	Cat#1070-05; RRID: AB_2650509
Rabbit anti-goat IgG (H + L)-HRP (dilution 1:5,000)	SouthernBiotech	Cat#6160-05; RRID: AB_2796231
Biological samples		
Mouse blood	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Rompun 2%	Bayer HealthCare - Animal Health	N/A
Imalgene 1000	Boehringer Ingelheim	N/A
OptiPrep (iodixanol) density gradient medium	STEMCELL Technologies	Cat#07820
Sucrose	Merck	Cat#S7903
Tris-HCl	Calbiochem	Cat#648313
Tris-base	VWR Chemicals	Cat#28808.294
Density gradient working buffer	This paper, protocol from already published manuscripts ²⁻⁵	N/A
Density gradient homogenization buffer	This paper, protocol from already published manuscripts ²⁻⁵	N/A
EDTA 0.5 M	Invitrogen	Cat#15575020
Novex Tris-glycine SDS sample buffer (2×)	Invitrogen	Cat#LC2676
Tris-acetate buffer 3M	This paper, adapted from Cubillos-Rojas et al. ⁶	N/A
Acetic acid, glacial	Alfa Aesar	Cat#36289
Ammonium persulfate (APS)	Merck	Cat#A3678-100G
Acrylamide/Bis-acrylamide, 40% solution	Merck	Cat#A7802
TEMED	Eurobio	Cat#GEPTM18-0T
2-Mercaptoethanol	Carl Roth	Cat#4227.1
NuPAGE antioxidant	Invitrogen	Cat#NP0005

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NuPAGE Tris-acetate SDS running buffer (20×)	Invitrogen	Cat#LA0041
Ponceau S staining solution	Thermo Scientific	Cat#A40000279
Tween 20	Euromedex	Cat#2001-B
Ethanol absolute	VWR Chemicals	Cat#20821.296
2-Propanol	VWR Chemicals	Cat#20842.298
Deposited data		
EV-TRACK	evtrack.org	EV230981
Experimental models: Cell lines		
L-929 mouse fibroblasts	ATCC	NCTC clone 929 [L cell, L-929, derivative of strain L]; RRID: CVCL0462
Patient-derived glioblastoma stem-like cells GSC9	Harford-Wright et al. ⁷	N/A
Experimental models: Organisms/strains		
WT C57BL/6Jrj males (4–10 weeks)	Janvier Labs	Cat#C57BL/6 J-M
WT BALB/c nude females (BALB/cAnNRj-Foxn1 nu/nu, 6–8 weeks) GSC9 engrafted	Janvier Labs	Cat#SM-BALNU-F
Other		
qEVOoriginal/70 nm Gen 2 column - 5 pack	Izon	Cat#ICO-70
2.2 mL open-top thinwall polypropylene tube	Beckman Coulter	Cat#347357
13.2 mL, open-top thinwall polypropylene tubes	Beckman Coulter	Cat#331372
MLA-130 fixed-angle rotor	Beckman Coulter	Cat#367114
SW 41 Ti swinging-bucket rotor	Beckman Coulter	Cat#331362
Optima XPN-80 ultracentrifuge	Beckman Coulter	Cat#A95765
Optima Max-XP tabletop ultracentrifuge	Beckman Coulter	Cat#393315
Amicon Ultra-2 centrifugal filter unit 10 kDa MWCO	Merck Millipore	Cat#UFC201024
Filtermax vacuum filtration system 0.22 μm	TPP	Cat#99505
Duran laboratory bottles, with caps	Merck	Cat#Z305197
Empty gel cassettes 1 mm	Invitrogen	Cat#NC2010
Gradient maker 15 mL	Hofer Instruments	Cat#SG15
LabN1 peristaltic flow rate pump	Shenchen	Cat#LABN1-II/MC12
Magnetic stir bar	Heathrow Scientific	Cat#HS120547
Magnetic stirrer	N/A	N/A
XCell II blot module	Invitrogen	Cat#EI9051
XCell SureLock mini-cell	Invitrogen	Cat#EI0001
Electrophoresis power supply	Consort	Cat#EV3020

STEP-BY-STEP METHOD DETAILS

Plasma collection

⌚ Timing: 1 h

The first step involves blood collection to retrieve the optimal volume of plasma in a quick and preserved fashion.

1. Mouse preparation.
 - a. Anesthetize the mouse with ketamine and xylazine (in a dedicated room for sampling and euthanasia).

Note: Deep anesthesia is reached with 150 μL per 10 g of animal of a cocktail of ketamine 100 mg/kg and xylazine 10 mg/kg, injected intraperitoneally. This deep anesthesia in living animals is needed to collect blood, as undesired blood coagulation starts immediately at death.

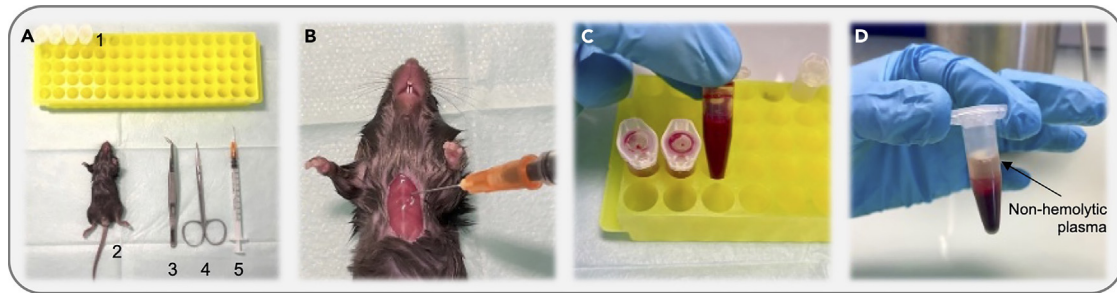


Figure 2. Step-by-step recovery of mouse plasma by intracardiac puncture

(A) Surgical field with anesthetized mouse and all materials required for intracardiac puncture step. 1: microtubes containing 30 μ L EDTA 0.5 M. 2: anesthetized mouse with moistened hair and skin with ethanol 70%. 3: Semken tweezers curved. 4: surgical scissors. 5: syringe with 25G needle flushed with EDTA 0.5 M.

(B) Skin incision as buttonhole and puncture site located on the left side between the first and the second ribs.

(C) Homogenized total blood in EDTA 0.5 M microtubes, sitting at 16°C–25°C for less than 2 h.

(D) After centrifugation at 1,000 g for 15 min at 4°C, supernatant plasma is separated as a transparent yellow upper phase.

- b. Wait for at least 10 min to allow sedation.
- c. Moisten hairs with ethanol 70%.
- d. Incise the skin to make a buttonhole facing the upper part of the thoracic cage at the level of the first ribs (Figure 2A).
2. Intracardiac puncture.
 - a. Prepare 1.5 mL microtubes with 30 μ L EDTA 0.5 M (5% of the mean expected blood collection volume) and a 1 mL syringe with 25G or 26G needle flushed with the EDTA just before plasma collection. A dry EDTA collecting tube can be used as well (commercial purple tube).

Note: The type of blood collection tube (EDTA, citrate, heparin, etc.) impacts platelet activation and EV recovery, and therefore must be carefully selected. ACD-A (acid citrate dextrose), EDTA, and citrate tubes offering the best performances according to a recent benchmarking study by Dhondt et al.⁸

- b. Localize the puncture area: Depending on mouse strain, it is usually between the first and second ribs on the left side toward the axis (Figure 2B).
- c. At the beginning of the puncture, apply a gentle suction as the syringe penetrates the muscle layer.

Note: This generates a vacuum effect enabling the blood to promptly ascend in the syringe once the needle reaches the adequate depth in the heart. Thus, this technique allows harvesting quickly “fresh and clean” blood with minimal intervention on the animal (i.e., without opening up the thoracic cavity).

- d. Slowly suck up 500 μ L to 1 mL of blood from the heart.
- e. Transfer quickly the collected blood into the EDTA tube.

Note: tubes can sit at 16°C–25°C for a maximum of 2 h, whether several blood samples need to be collected.

- f. Immediately after blood collection, euthanize the mouse according to approved procedures.

Note: The expected mean volume of collected blood is 600 μ L. While an occasional experimenter can recover 300 μ L, an advanced operator can expect to recover around 1 mL of blood. As plasma accounts for 60% of total blood volume, it is expected up to 600 μ L of

plasma per mouse. Thus, the described protocol here may allow individual immunoblot analysis for example. However, plasma from replicate animals can be pooled if higher volumes are required.

3. Separation of plasma from total blood.
 - a. Centrifuge EDTA tubes at 1,000 g for 15 min at 4°C to enable separation of plasma from blood cells and platelets.

Optional: Residual platelets in plasma can release elevated amounts of confounding EVs following freezing and thawing.^{9,10} To maximize platelet separation, a second centrifugation step $\geq 1,000$ g can be performed.

- b. Pipet carefully the supernatant, transfer it into a new collection tube, and snap-freeze in liquid nitrogen.
- c. Store at -80°C for further isolation or analysis.

△ CRITICAL: The plasma color has to be transparent yellow rather than red, which indicates hemolysis and poor quality of puncture.

▮▮ Pause point: Mouse plasma can be stored at -80°C for several months to years before EV separation.

Extracellular vesicle preparation

⌚ Timing: 4–24 h

In this step, we present three alternative strategies that can be used to separate and enrich EVs from mouse plasma with different specificity and yield depending on desired applications. As described here, all EV separations can be performed upon plasma thawing, following i) differential ultracentrifugation (UC), ii) size exclusion chromatography (SEC) combined with UC, or, iii) SEC combined with density gradient (DG) and UC.

Note: Extracellular vesicles should be separated from other plasma constituents with overlapping physical properties, consisting mainly of lipoprotein particles and non-EV protein complexes from plasma.¹¹ The applied procedure should take into account the yield and purity required for downstream analysis.⁹ While the sequential, combinatory implementation of separation methods improves EV separation from non-EV constituents, it also decreases the yield (Figure 3). This can be particularly critical for biological samples with small input volumes, such as mouse plasma.

4. Plasma pre-processing: Thaw at least 200 μL of plasma on ice (can be enough for options 1 and 2; should be as close as possible to 600 μL for option 3).

Note: Plasma from replicate mice can be pooled to increase volume and improve sensibility/yield.

5. (Option 1) Differential ultracentrifugation.
 - a. Dilute 200 μL (or more if available) of freshly thawed plasma in ice-cold particle-free PBS to a final volume of 2 mL.
 - b. Transfer diluted plasma in a 2 mL microtube and centrifuge at 2,000 g for 10 min, 4°C, to pellet large unwanted particles.

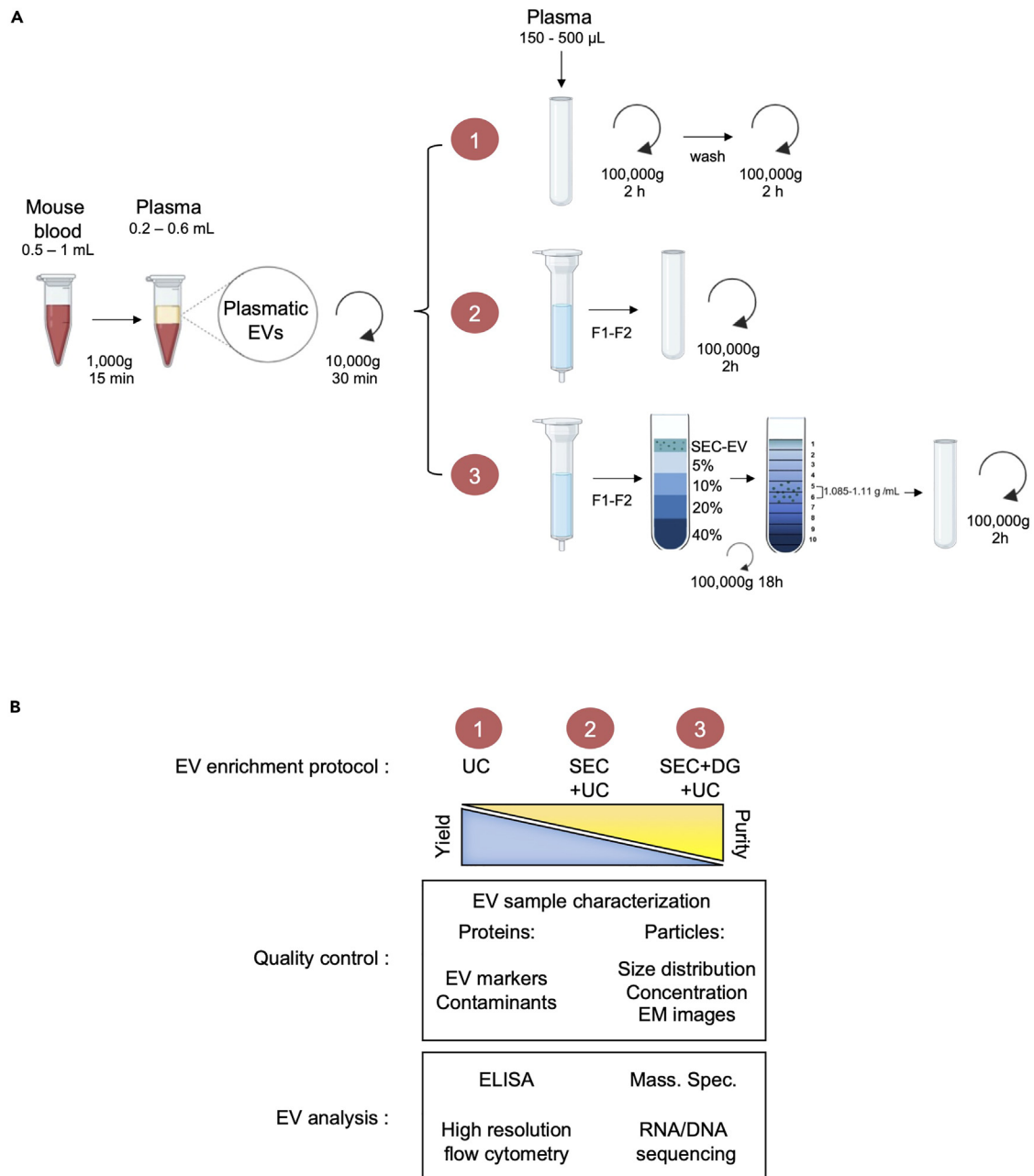


Figure 3. Overview of the different EV enrichment options to recover circulating EVs from mouse plasma

(A) Separation steps to isolate EVs from mouse plasma: three optional procedures are depicted: 1: differential ultracentrifugation, 2: Size exclusion chromatography and ultracentrifugation, 3: Size exclusion chromatography followed by density gradient and ultracentrifugation.

(B) The three optional enrichment protocols i.e., differential ultracentrifugation (UC), size exclusion chromatography and ultracentrifugation (SEC+UC), and, size exclusion chromatography followed by density gradient and ultracentrifugation (SEC+DG+UC) allow either high recovery or high purity EV preparations, suitable for EV characterization and further dedicated analysis.

- Pipet the supernatant and transfer into a new 2 mL microtube and centrifuge at 10,000 g for 30 min, 4°C, to pellet large EVs (discard pellet or save as the 10 K fraction).
- Pipet the supernatant and transfer into a 2 mL open-top polypropylene tube and centrifuge at 100,000 g for 2 h, 4°C, to pellet small EVs (maximum acceleration: 0; maximum deceleration: 0).

- e. To wash small EVs:
 - i. Carefully discard supernatant and resuspend the pellet (invisible) in 1 mL of ice-cold particle-free PBS.
 - ii. Fill the tube up to 2 mL with particle-free PBS.
 - iii. Perform additional centrifugation at 100,000 g for 2 h, 4°C (maximum acceleration: 0; maximum deceleration: 0).

△ **CRITICAL:** Salts contained in PBS buffer may scatter light and possibly interfere with particle analysis methods. As aforementioned, we recommend the use of 0.22 µm-filtered PBS or commercial sterile particle-free PBS to wash and resuspend EVs. The absence of confounding particles in PBS should be controlled before any particle quantification.

- f. Carefully discard the supernatant by pipetting it out or by slow aspiration from the opposite side of the tube.

Note: To make sure not to disturb the pellet, never pipette or aspirate close by the pellet side (fixed-angle rotor).

- g. Resuspend the pellet (invisible) in a low volume of ice-cold particle-free PBS (30–100 µL).
- h. Proceed to quality control and EV characterization. Please see 'expected outcomes' section.

▮ **Pause point:** At that time, isolated EVs can be frozen at –80°C until analysis. Freezing and long-term storage impact EV integrity and downstream characterization,¹² we recommend performing EV analysis on fresh preparations when possible. Temporary storage should be at 4°C during workday, as most steps described in this protocol (except SEC column and particle characterization done by ILM).

△ **CRITICAL:** Although commonly implemented, differential UC is known to separate EVs with low specificity, recovering a significant amount of non-EV particles and protein complexes.^{11,13,14} Additionally, high-speed pelleting enables a limited recovery of EVs and may impact their integrity.⁹

6. (Option 2) Size exclusion chromatography.

- a. Centrifuge at least 200 µL and up to 600 µL of freshly thawed plasma at 10,000 g for 30 min, 4°C, to pellet large particles.
- b. Meanwhile, equilibrate a size exclusion chromatography column at 16°C–25°C with particle-free PBS following the manufacturer's instructions https://files.izon.com/hubfs/Manuals, Technical Notes and Customer Support/qEV columns/qEVoriginal_Gen_2_User_Manual-I-CO-DQ-001.pdf.

Note: While commercial columns might help standardization and reproducibility, such as Izon qEVoriginal/70 nm used here, SEC columns with similar properties can also be hand-made, based on this published method.³

- c. Collect 500 µL of plasma supernatant to transfer onto the SEC column (if starting with 200 µL of plasma, collect 150 µL of supernatant and adjust to 500 µL with particle-free PBS).
- d. Elute the column by adding ice-cold particle-free PBS on the top once the sample enters the column.
- e. Discard the void volume (2.9 mL) and collect the two first 500 µL EV-enriched SEC fractions 1 and 2 (F1 and F2). Automate might help standardization and reproducibility (such as Izon AFC).

Optional: EV-enriched SEC fractions can be used directly to monitor particle and protein elution as described [expected outcomes](#) section.

- f. Pool F1+F2 EV-enriched SEC fractions to obtain 1 mL.
- g. Recovered EVs can be concentrated, if required, using ultracentrifugation (steps i. to iv.) or ultrafiltration (step v.):
 - i. Dilute pooled EV-enriched SEC fractions to 11 mL with cold particle-free PBS in a 13.2 mL thinwall polypropylene tube.
 - ii. Pellet EVs by ultracentrifugation at 100,000 g for 2 h, 4°C.
 - iii. Carefully discard the supernatant by pipetting it out or by slow aspiration from the opposite side of the tube.

Note: To make sure not to disturb the pellet, never pipette or aspirate close by the center of the tube, and leave a drop of supernatant.

- iv. Resuspend the final pellet in 30–100 µL of cold particle-free PBS for further storage and analysis. Alternatively, lyse the pellet in 30 µL of boiling 2× denaturation SDS sample buffer for immunoblotting.
- v. Concentrate EV-enriched fractions by ultrafiltration using 2 mL Amicon concentrators with regenerated cellulose and 10 kDa MWCO.

△ **CRITICAL:** The molecular weight cutoff and membrane composition of the ultrafiltration devices both impact the recovery of EVs and should be carefully selected.¹⁵

- h. Proceed to quality control and EV characterization. Please see the ‘[expected outcomes](#)’ section.

▮ **Pause point:** At that time, isolated EVs can be frozen at –80°C until analysis. Freezing and long-term storage impact EV integrity and downstream characterization,¹² we recommend performing EV analysis in fresh preparations when possible.

7. (Option 3) Size exclusion chromatography and density gradient.

- a. Perform SEC separation as described in step 6 a-f of this protocol to obtain 1 mL of SEC fractions F1 and F2. If needed, adjust to a final volume of 1 mL with cold particle-free PBS.

Note: The density gradient protocol described hereafter has been adapted from the already described protocol^{4,5} to be used with SW41Ti rotor and 13.2 mL thinwall polypropylene tubes.

- b. Prepare iodixanol (OptiPrep, 50% w/v) working solution (Table 4) by mixing five volumes of iodixanol (OptiPrep, 60% w/v) stock solution with 1 volume of previously prepared working buffer (Table 1).

Note: Iodixanol (OptiPrep) working solution should be freshly prepared, kept on ice, and protected from light.

- c. Prepare 5%, 10%, 20%, and 40% iodixanol solutions as described in Table 5 using previously prepared homogenization buffer (Table 2), and iodixanol working solution (Table 4).

Table 4. Iodixanol (OptiPrep) working solution

Number of gradients	Iodixanol (OptiPrep, 60% w/v) stock solution (mL)	Working buffer (pH 7.4) (mL)
1	5	1
2	10	2
3	15	3
4	20	4
5	25	5
6	30	6

Table 5. Density gradient iodixanol solutions of 5%, 10%, 20%, and 40%

Iodixanol solution	5%	10%	20%	40%
Working solution (mL)	1	2	4	8
Homogenization buffer (pH 7.4) (mL)	9	8	6	2

Note: The iodixanol solutions should be freshly prepared and kept on ice while building the gradient. Indicated volumes of iodixanol solutions can be used to prepare 3 gradients of 10.5 mL (sample volume included) as described hereinafter.

- d. In a new 13.2 mL thinwall polypropylene tube, dispose gently 3 mL of 40% iodixanol solution (Figures 4A and 4B).
- e. Gently tilt the tube horizontally and add dropwise 2.5 mL of 20% iodixanol solution, followed by 2.5 mL of 10%, and 1.5 mL of 5% iodixanol solutions.

Note: For a video illustration of layering density gradient iodixanol fractions, please see the published STAR Protocols.³

- f. Carefully add dropwise 1 mL of pooled EV-rich SEC fractions generated at step 7a.

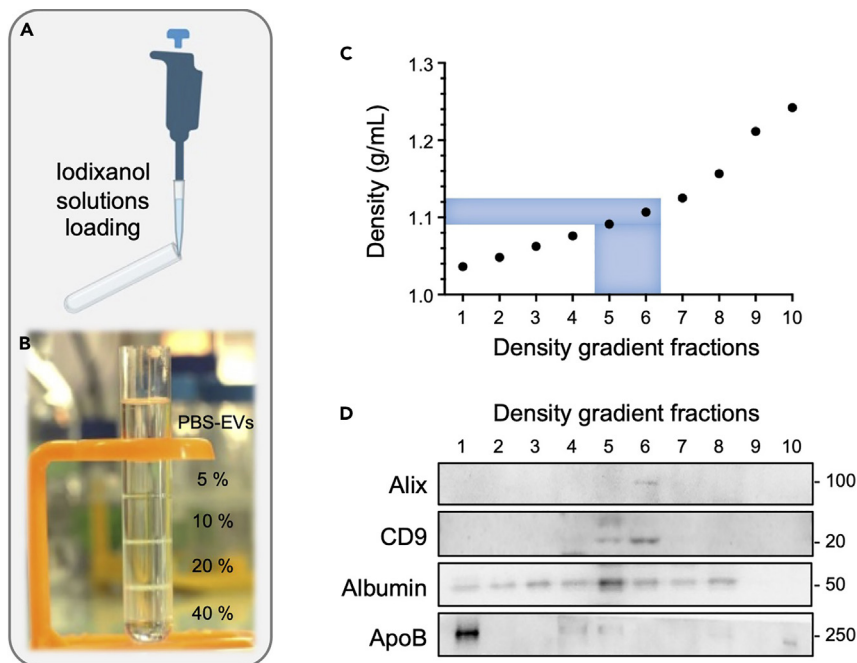


Figure 4. Mouse plasma EV separation by density gradient

(A) Loading of iodixanol solutions in an ultracentrifugation tube.

(B) Layers of 5, 10, 20, and 40% iodixanol solutions in a 13.2 mL ultracentrifugation tube on which 1 mL of SEC fractions F1+F2 (EV enriched fractions) were loaded on the top.

(C) Density of each density gradient fractions of 1 mL collected from the top was measured at 340 nm for iodixanol absorbance, as described by Dhondt et al.³

(D) Immunoblot analysis of each density gradient fraction of 1 mL (1 to 10 from the top) processed from 500 μL of mouse plasma with size exclusion chromatography, density gradient, and ultracentrifugation (SEC+DG+UC), and resolved on a Tris-acetate gel. Luminal Alix and transmembrane CD9 were analyzed as EV markers. Apolipoprotein ApoB and plasma protein albumin served as contamination controls. Primary antibodies were incubated at 1:1000 in PBS-Tween 5% milk for 12–18 h at 4°C. Secondary HRP-conjugated antibodies were diluted at 1:5000, incubated for 1 h at 16°C–25°C, and revealed by chemiluminescence.

- g. Centrifuge density gradients at 100,000 g for 18 h, 4°C (maximum acceleration: 0; slow deceleration: 9).

△ **CRITICAL:** A slow deceleration is required at the end of the centrifugation to preserve the density gradient formed during the 16–18 h run (Figure 4C). A smooth handling of the tubes following the centrifugation is also essential to avoid disturbing the gradient and recover EV in their expected density fractions.

- h. Meticulously collect from the top 1 mL density gradient fractions with a P1000 pipet in new 1.5 mL microtubes.

△ **CRITICAL:** Pipet fractions from the center of the meniscus using a smooth and precise P1000 pipet, maintaining the immersed part of the tip at 1–2 mm below the surface of the liquid to avoid mixing gradient fractions.

Note: For a video illustration of density gradient fractions collection, please see the *STAR Protocols* from Dhondt et al.³

Optional: Measure the density of collected gradient fractions using fractions collected from a blank PBS-loaded density gradient prepared at step 7b–e. Density can be determined using a plate reader by measuring the absorbance of iodixanol at 340 nm (Figure 4C). For detailed instructions, please see the *STAR Protocols* of Dhondt et al.³

- i. EVs accumulate at a density of ≈ 1.085 – 1.110 g/mL, corresponding here to fractions 5 and 6 collected from the top. These EV-rich fractions were analyzed by western blot (Figures 4C and 4D).
- j. Pool EV-enriched density gradient fractions and dilute them in a new 13.2 mL thinwall polypropylene tube to 11 mL with cold particle-free PBS.
- k. Pellet EVs by ultracentrifugation at 100,000 g for 2 h, 4°C.
- l. Carefully discard the supernatant by pipetting it out or by slow aspiration from the side of the tube, and resuspend the final pellet in 30–100 μ L of cold particle-free PBS. Alternatively, lyse the pellet in boiling 2 \times denaturation SDS sample buffer for immunoblotting.
- m. Proceed to quality control and EV characterization. Please see the ‘[expected outcomes](#)’ section.

Note: Due to the low yield, we do not advise freezing EV preparations that have been resuspended in PBS at this step.

EXPECTED OUTCOMES

This protocol illustrates how to collect large volumes of murine plasma to study circulating EVs in individual mouse blood, with suitable separation and detection techniques. In this section, we describe recommendations for quality control and analysis of isolated EVs, discussing tips and options. Minimal characterization of EV preparations must be performed at the particle and protein level to ensure that EVs were separated from known confounding proteins and particles that may result in artifacts in downstream analysis.^{9,16}

The first step consists in determining the particle concentration and size distribution. First, particle concentration is impacted by storage conditions including time and temperature and should ideally be measured on fresh EV preparations,^{12,17} whatever the method described in the ‘[extracellular vesicle preparation](#)’ section. The measure of separated EVs reflects the initial concentration of circulating EVs in the bloodstream, termed as vesiclemia.¹⁸ Nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), interferometric light microscopy (ILM), or high-resolution flow cytometry can be used to characterize the particle content of EV preparations (count and size). Here, ILM

(Videodrop, Myriade) was used to measure particle concentration and size distribution in EV samples freshly prepared from 500 μL of individual mouse plasma following the three protocol options (Figure 5A). As expected, from the same initial volume of murine plasma, particle concentration is higher with differential ultracentrifugation (high yield but low purity) compared to size exclusion chromatography followed by density gradient and/or ultracentrifugation (high purity but low yield, Figure 5A). Non-EV particle/aggregate contaminants, such as the apolipoproteins which probably pollute the counting, are appreciated by ApoB immunoblot (Figure 5B). Regarding particle size distribution, to date, ILM hardly recorded particles under 80 nm, leading to a mean hydrodynamic diameter that might be higher than with other single particle tracking techniques (Figure 5A).

△ CRITICAL: Particle characterization methods hardly discriminate EVs from confounding particles with overlapping physical properties, such as lipoprotein particles highly abundant in plasma.¹¹ Results should therefore be cautiously considered and controlled.

Additionally, morphological analysis can be performed by electron microscopy (EM). To date, EM and atomic force microscopy (AFM) are the only available techniques to observe and appreciate small EV structures. EM requires native, freshly separated EVs. Indeed, sample preparation and storage strongly affect EV shape and integrity. We highly recommend SEC or SEC+DG (followed by ultrafiltration if needed to concentrate EVs) and avoid high-speed ultracentrifugation if possible. Someone should expect cup-shaped structures on EM close-up images and double-lipid membranes on cryo-EM images, for example, associated with minimal protein aggregates on wide-field images.

Next, to perform protein characterization by immunoblot, we recommend gradient Tris-acetate gels to detect on one single EV preparation, from the plasma of one animal, two or three specific EV markers (with ideally one transmembrane and one cytosolic) and one negative protein marker of contamination.⁹ As aforementioned, these Tris-acetate gels (prepared in the 'before you begin' section) allow for optimization of the protein resolution and detection with a low amount of biological material. Following the protocols described here from 500 μL of plasma, EV markers Alix, Hsp70, and CD9 are robustly detected. However, the apolipoprotein ApoB is also visible in UC preparation, whereas CD9 levels were maintained together with lower ApoB detection following SEC + UC (Figure 5B). Adding the density gradient step (option 3 protocol) reaches the detection limit in these immunoblot conditions (Figures 4D and 5B). In this last condition, we can nonetheless appreciate fractions 5 & 6 of expected EV density, with Alix and CD9 co-expression and minimal ApoB detection (Figures 4D and 5B). Last, albumin (the most abundant free protein in plasma) binds to these EVs (Figure 4D), to a lesser extent than with only the UC procedure (Figure 6).

A challenging point of this protocol was to analyze EVs from individual mice with a limited volume of collected plasma. In Figure 6, we show a representative immunoblot obtained from only 150 μL of plasma, compared to a more comfortable volume of 500 μL . UC and UC + SEC allow the detection of the three specific EV markers, namely CD9 transmembrane protein, and Alix and Hsp70 core protein from the cytosolic compartment. Plus, there was no detectable GM130 and calreticulin, used as protein markers of cellular components, as well as minimal plasma soluble protein Albumin (Figure 6).

Note: Please refer to the [key resources table](#) for the recommended antibodies. Primary antibodies were diluted at 1:1000 in PBS-Tween 5% milk and membranes were incubated for 12–18 h at 4°C. Secondary antibody HRP-conjugated were diluted at 1:5000, incubated for 1 h at 16°C–25°C, and revealed by chemiluminescence.

To perform protein quantification by ELISA, this candidate approach does not necessarily require the purest sample and can be performed on freshly prepared or frozen, native or lysed EVs, from differential ultracentrifugation (protocol option 1). Circulating EVs can be detected through ELISA kits, that target tetraspanins, for CD63,^{1,19} or proteins of interest, like VEGF.²⁰

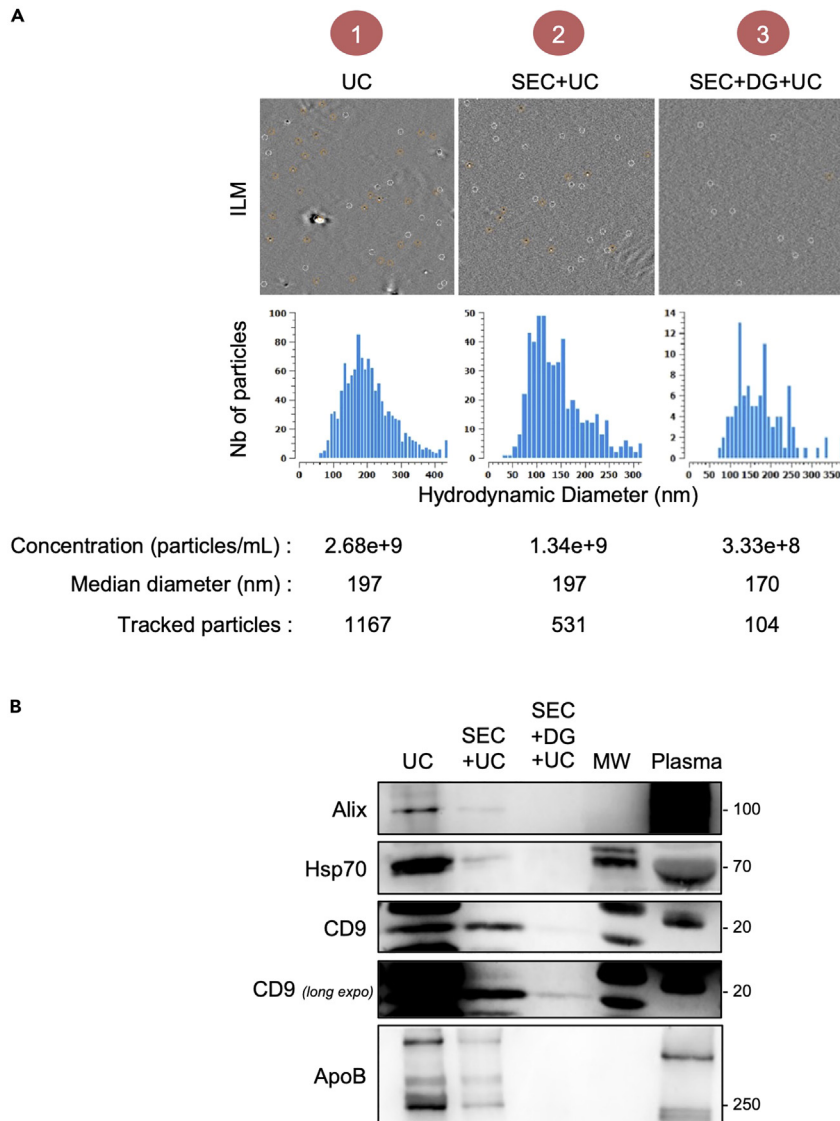


Figure 5. Particle and protein characterization of mouse plasma EVs separated by UC (option 1), SEC+UC (option 2), or SEC+DG+UC (option 3)

4-year-old frozen plasma from three mice bearing human GSC-derived orthotopic tumors¹ was thawed on ice and pooled to a final volume of 1500 μ L. Pooled plasma was divided into three identical samples of 500 μ L that were each processed with separation protocol options 1, 2, or 3, as depicted in Figure 3A. EVs obtained by UC, SEC+UC, and SEC+DG+UC were recovered with a final 100,000 g pellet resuspended in 100 μ L of cold particle-free PBS.

(A) 7 μ L of each fresh EV preparation was used to measure the particle concentration and size distribution by interferometry light microscope (ILM, Videodrop, Myriade).

(B) Each sample used for ILM measurement was recovered, placed back in the corresponding UC tube, diluted to 11 mL with particle-free PBS, and pelleted again at 100,000 g for 2 h. Supernatants were discarded and pellets were lysed in 35 μ L of boiling 2 \times denaturation SDS sample buffer. The total volume was loaded in Tris-Acetate gels and membranes were probed with primary antibodies at 1:1000 in PBS-Tween 5% milk for 12–18 h at 4°C. Luminal/cargo (Alix, Hsp70) and membrane (CD9) EV markers, as well as plasma lipoprotein contaminant (ApoB) were analyzed. Secondary HRP-conjugated antibodies were diluted at 1:5000, incubated for 1 h at 16°C–25°C, and revealed by chemiluminescence.

Note: Grafts of human cells in immunocompromised host mice might enable detection and discrimination of EVs from human origin, as examined with cancer EV markers such as glypican-1²¹ and human CD63.¹ Similar tracking could be envisioned with the use of tagged EV-expressing cells.

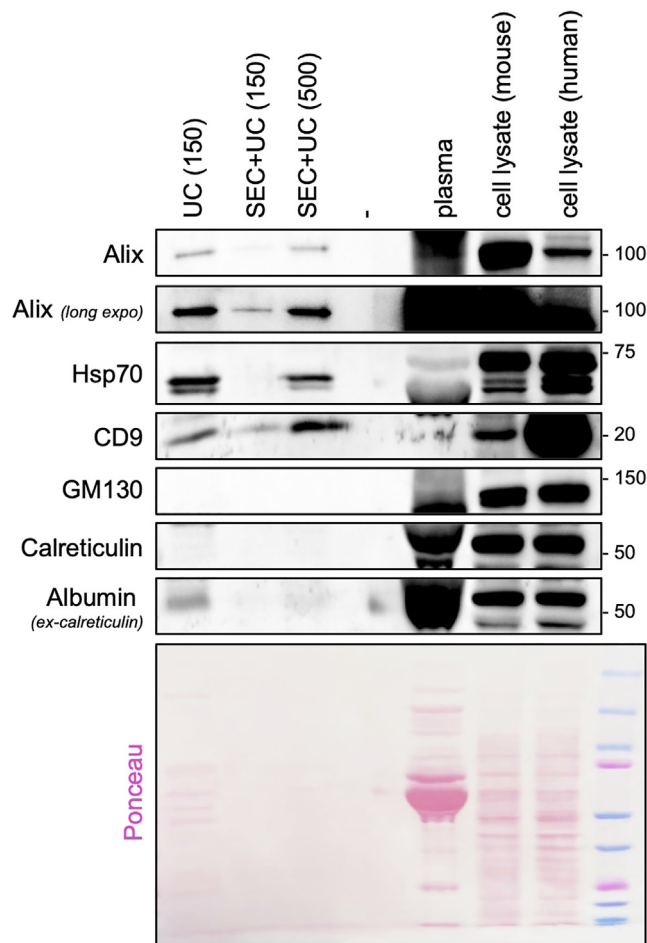


Figure 6. Protein characterization of circulating EVs separated from 150 or 500 μ L of mouse plasma

4-year-old frozen plasma from two control mice was thawed on ice and pooled to a final volume of 1 mL. Pooled plasma was divided into three identical samples of 150 or 500 μ L and completed with PBS to a final volume of 500 μ L when needed. EVs were enriched by ultracentrifugation (UC, protocol option 1) or size exclusion chromatography combined with ultracentrifugation (SEC+UC, protocol option 2). Pellets were lysed in 35 μ L of 95°C pre-warmed 2 \times denaturation SDS sample buffer. The entire volume was loaded in Tris-Acetate gels and membranes were probed with primary antibodies at 1:1000 in PBS-Tween 5% milk for 12–18 h at 4°C. Alix, Hsp70, and CD9 EV markers were analyzed. GM130 and calreticulin markers of cellular components, and plasma albumin protein served as negative controls. Secondary HRP-conjugated antibodies were diluted at 1:5000, incubated for 1 h at 16°C–25°C, and revealed by chemiluminescence. 1 μ L of plasma lysed in lysis buffer and total cell lysates from mouse fibroblast cell line L929 and human patient-derived glioblastoma cells served as positive controls.

For proteomic analysis requiring mass spectrometry and other omic studies (lipidomic, transcriptomic, etc.), a high purity of the sample is needed and a density gradient coupled with SEC (protocol option 3) might allow size and density exclusion of contaminants, based on published methods.^{3,4} Alternatively, flow cytometry, nucleic acid analysis, or other techniques for pre-determined targets of interest might require a compromised method between yield and purity (Figure 3B) coupling UC + SEC (protocol option 2). For DNA and RNA analysis, a special caution to contaminants that may be sources of nucleic acids has to be taken into consideration. Additional controls with RNase or DNase need to be properly planned.⁹

LIMITATIONS

One of the main limitations of the protocol is both the quality and quantity of harvested plasma. As a mouse of 20 g has approximately 1.2–1.5 mL of blood (60–80 μ L/g), the technical puncture is critical

for plasma volume to be maximized. Practicing the gesture and/or pooling animal samples has to be anticipated to avoid unsuccessful protocol depending on the sensitivity of downstream analysis and characterization.

The purity of harvested EVs can also narrow the interpretation of the results. Indeed, the separation of plasma EVs from overlapping contaminants (lipoproteins, large protein complexes) is challenging and may require the combination of several separation methods, resulting in a low yield. Given the low volume of mouse plasma, extended protocols, such as SEC+DG+UC, may result in insufficient yield for downstream analysis (Figure 5). While less specific approaches, *i.e.*, UC and SEC+UC, allow higher yield of EVs, co-separated contaminants may alter/modify the results. This must be carefully controlled and documented. Thus, the separation method should be selected in agreement with the goal of the study and by implementing recommended controls to characterize EV preparations and contaminants.

Removal of chemical additives (iodixanol/sucrose) that could interfere with EV functional assays²² but also methods potentially impairing EV integrity, as UC pelleting,²³ are important points to consider for downstream analysis and functional assays.

An additional limitation is labor-intensive protocols regarding the options chosen for EV enrichment. Whereas UC (option 1) and SEC+UC (option 2) last for 4 and 5 h, respectively, with several samples running easily at the same time, SEC+DG+UC (option 3) requires almost 24 h with a more limited number of samples. Gradients may indeed need to be built and collected individually, adding substantial time and effort. Automates are however available to limit the workload of density gradient layering and fractions collection, and ultimately they help standardization and reproducibility.²⁴

TROUBLESHOOTING

Problem 1

The experimenter is not comfortable with performing intracardiac puncture for mouse blood recovery (related to Step 2).

Potential solution

Alternate methods, such as retro-orbital blood draw, can be performed under general anesthesia by collecting blood with capillary tubes from the retro-orbital venous sinus. This generally however allows the collection of lower blood volume.

Problem 2

Not enough blood plasma is recovered from one mouse for EV separation (related to Step 2).

Potential solution

Pool plasma collected from replicate animals to perform EV separation. Pooling of replicate animals can also enable researchers to compare EV separation methodologies, as illustrated in Figures 5 and 6.

Problem 3

Low amounts of particles are measured after resuspending the ultracentrifugation EV pellet (related to Step 5).

Potential solution

EV pellets recovered with a high-speed centrifugation stick to plastic, and need to be thoroughly resuspended before particle analysis. If little particles are detected, it may be due to insufficient resuspension of the pellet. For immunoblotting, EVs can be lysed in the UC tube by adding 2× denaturation SDS sample buffer ideally pre-warmed at 95°C to optimize EV lysis.

Problem 4

Particle analysis of EV-enriched SEC fractions F1+F2 shows a suboptimal recovery of EVs (related to Step 6).

Potential solution

A proportion of EVs may be lost in late SEC fractions. A solution may be to adapt the collected volume for EV-enriched SEC fractions and control for the presence of EV markers and protein contaminants.

Problem 5

No limits are visible between density layers after building the density gradient (related to Step 7).

Potential solution

Although the presence of a visible limit between density fractions supports the optimal layering of the gradient, its absence does not overtly affect the performance of the gradient. Training for slow and limber deposit can help improve its quality. The correct layering can be controlled by running a blank PBS-loaded density gradient and measuring the absorbance at 340 nm of individually collected fractions, based on published methods.³

Problem 6

No EV markers or proteins of interest are detected by immunoblot after loading the maximum sample volume per well (related to 'expected outcomes').

Potential solution

This may happen when low amounts of EVs are separated by a given method. A solution might be to increase the volume of EVs loaded per well. This can be achieved by doubling the sample volume: after a short run of 5 min maximum at 150 V to allow the initial sample to enter the stacking gel, load again the same lysed sample in the same well. This will maximize the amount of EVs loaded in each lane.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julie Gavard (julie.gavard@inserm.fr).

Materials availability

This protocol is not associated with newly generated materials.

Data and code availability

This protocol does not report original code. All data generated or analyzed during this protocol are included in this article.

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230981).¹⁶

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AUTHOR CONTRIBUTIONS

G.A.G. and J.G. designed the experimental approaches and coordinated the project. G.A.G. provided murine and human samples. G.A.G. and Q.R. performed the experiments, interpreted the data, prepared the figures, and wrote the manuscript. J.G. supervised the work, interpreted the data, and edited the text. All authors approved the submitted manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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