Human plasma extracellular vesicle isolation and proteomic characterisation for the optimization of liquid biopsy in multiple myeloma

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Abstract

Cancer cells secrete small membranous extracellular vesicles (EVs) which contain specific oncogenic molecular cargo (including oncoproteins, oncopeptides and RNA) into their microenvironment and the circulation. As such, EVs including exosomes (small EVs) and microvesicles (large EVs), represent important circulating biomarkers for various diseases, including cancer and its progression. These circulating biomarkers offer a potentially minimally invasive and repeatable targets for analysis (liquid biopsy) that could aid in the diagnosis, risk stratification and monitoring of cancer. Although their potential as cancer biomarkers has been promising, the identification and quantification of EVs in clinical samples remains challenging. Like EVs other types of circulating biomarkers (including cell-free nucleic acids, cf-NAs; or circulating tumor cells, CTCs) may represent a complementary or alternative approach to cancer diagnosis. In the context of multiple myeloma (MM), a systemic cancer type that causes cancer cells to accumulate in bone marrow, biomarkers for diagnosis and monitoring remain undefined. The tumor heterogeneity of make this disease difficult to be monitored using conventional testing (e.g. sequential serological testing and bone marrow biopsies) and the utility of liquid biopsy and circulating tumor-derived EVs a promising approach. In this protocol we describe the isolation and purification of EVs from peripheral blood plasma (PBPL) collected from healthy donors and patients with MM for a biomarker discovery strategy. Our results demonstrate detection of circulating EVs from as little as 1 mL of MM patients' PBPL. High resolution mass spectrometry (M)S-based proteomics promises to provide new avenues in identifying novel markers for detection, monitoring, and therapeutic intervention of disease. We describe biophysical characterisation and quantitative proteomic profiling of disease-specific circulating EVs may provide important avenues to or the development of cancer diagnostics in MM.

1. Introduction

Multiple Myeloma (MM) is a blood cancer that originates from the clonal expansion of plasma cells in the bone marrow (BM) [1-3]. MM remains incurable despite advances in its treatment due to high rates of relapse and drug resistance [4,5]. Risk stratification remains a major challenge in plasma cell dyscrasias where current recommendations for therapy are observation for asymptomatic patients (smoldering MM; pre-malignant condition Monoclonal Gammopathy of Undetermined Significance, MGUS), with the initiation of therapy only at the time of the emergence of symptomatic disease [6-9]. Therefore, a better understanding of the molecular characteristics that define the risk of progression to symptomatic MM would provide a framework for early initiation of systemic treatment [10-15].

Liquid biopsies represent less invasive diagnostic alternatives or additions to single site tissue biopsies, being able to capture the spatial and temporal tumor heterogeneity - a major limitation of tissue biopsies [16-20]. The latter are often invasive or not feasible due to patient compliance/capacity (e.g. comorbidities, logistics) or tumor (e.g. location, size, type/subtype) characteristics [21-26]. Conversely, liquid biopsies are innovative tools in precision medicine and cancer diagnostics with the ability to detect, monitor and characterize tumors in a minimally invasive and repeatable way [16-20,27-31]. It is well established that tumor cell-derived proteins, nucleic acids and extracellular vesicles (EVs), enter the circulation and reach distant sites where they establish a favorable microenvironment for tumor expansion [32-35]. These circulating factors represent useful biomarkers for cancer diagnosis with studies highlighting their prognostic and predictive significance with important clinical implications [36-40]. Liquid biopsies have the potential to detect low-abundant biomarkers from complex biofluids like blood, making them exceptional candidates for early detection and monitoring of cancers (diagnosis and/or residual disease) or for risk stratification [41-44,16,17]. Highly sensitive and selective omics (including genomics and proteomics) technologies and strategies have been developed to overcome the inherent challenge posed by the low-abundance of tumor-derived circulating factors [28,19,31,45-48]. Importantly, next-generation sequencing (NGS) technologies and mass spectrometry (MS)-based proteomics, with the aid of advanced bioinformatic tools, have been successfully utilized for cancer biomarker discovery [46,49,43,50-57].

Circulating cf-NA (cf-DNA/RNA) together with CTCs are the most developed biomarkers detected by liquid biopsy, with increasing evidence that combined analyses (i.e., combination of cf-NA and CTC), rather than single-source strategies (cf-NA or CTC), represent the key for increasing specificity of cancer detection [27,16,18-20,58]. The interest for EVs as liquid biopsies is also increasingly, expanding through pre-clinical and clinical evaluation in both solid tumors and haematological malignancies including MM [59-68,42,47].

EVs are particles delimited by a lipid bilayer and cannot replicate as they do not contain a functional nucleus [69,70]. EVs are secreted by almost all cell types including cancer cells and have been shown to play an important role in cell-to-cell communication by horizontal transfer of their contents (lipids, proteins, nucleic acids) [71-75]. They are able to influence pathological as well as normal homeostatic cellular processes by reprogramming signalling pathways to modulate the function and activity of target cells [76-79]. EVs have the capacity to signal at distant sites and reprogram organs conducive towards a metastatic microenvironment [80-82]. Moreover, EVs share common surface markers with their cell/tissue of origin, designating them as a promising source for biomarker discovery and diagnostics [83,84,40]. EV subtypes including large (~50-1300 nm) and small (30-150 nm) originate from different cellular compartments; plasma membrane- and endosomally-derived, respectively [69,70]. Omic technologies represent key strategies for the study of EV cargo [46,55,76,85,86] with several recent data suggesting a critical role for EVs in the context of cancer liquid biopsy [41,43,47,63]. A role for large EVs as predictive and prognostic biomarkers has also been suggested in MM [59,60], while data on small EVs are still exploratory and requires further investigation [62,64-68].

In a rapidly growing field, a major challenge is related to the isolation, purification and characterization of EVs. Several position statements from the International Society for Extracellular Vesicles (ISEV) and the Extracellular RNA Communication Consortium (ERCC) have been issued, highlighting the importance of standardization of sample collection and EV isolation and characterization approaches and reporting metrics [70,87]. It is important to consider the starting material, the downstream application and the end use, as a guide to choose the most suitable isolation method wherein balance between purity, cost and time must be achieved [70,88]. The isolation of small EVs from blood represents one of the most demanding applications. Blood is a complex biofluid that contains highly abundant proteins (HAP; e.g. albumin) which are often co-isolated and impair the enrichment of low abundant particles such

as small EVs [89-91]. While several strategies have been used for EV isolation [92-98], commercially available kits represent valuable tools for blood derived EVs with readily translational implications when compared to time-consuming methods such as ultracentrifugation which are more suitable for *in vitro* studies. Commercially available kits are based on different isolation modalities such as immunoaffinity capture, precipitation, membrane-based affinity and resin [92,93,95-97]. Here, we provide a detailed isolation and purification protocol together with methods for EV characterization which are employed to optimize the pre-analytical and isolation/purification steps prior to mass spectrometry proteomics to define the composition of EV cargo (**Figure 1**). Methodologies including immunoblotting, nanoparticle tracking analyses (NTA) and electron microscopy are of critical importance for EV characterization [70,94,98]. These techniques show that small EVs derived from 1 mL of fresh PBPL and isolated utilizing a commercial kit (resin-based) are homogeneous in terms of size and morphology, and enrichment of EV markers (Alix, PDCD6IP TSG101, and tetraspanins CD63 and CD81) in comparison to whole blood.

Of critical importance in the study of blood-derived EVs is the demonstration of the source of EVs of interest. Platelet-derived vesicles (microparticles, MPs and exosomes) represent a significant proportion of the source of EVs in blood [99-101]. The levels of protein expression of platelet EV markers (e.g. CD41, CD62) may indicate the amount of 'contamination' of the isolated EV sample by platelets EVs. Specific protocols for blood collection and PBPL preparation are tailored for minimizing the activation of platelets with subsequent release of platelet EVs in the sample of interest [102-104]. Immunocapture has also been successfully utilized to exclude this population from EVs of interest (e.g. $CD41^{-}$) [105]. *In vitro/ex vivo* [106,107] models utilising cell lines (human myeloma cell lines, HMCL) and primary (MM and stromal) cells are also suggested not only for EV functional studies but also to complement the validation of the PBPL-EV findings. Side-by-side comparison of EVs with source material (cells/tissue) [70] utilizing omic strategies may provide important insights in the specific EV cargo enrichment. The use of blood collected from healthy controls as a comparator is strategic as it implements the strength of MM derived data when a normal background (reference) is defined.

2. Materials

2.1 PBPL isolation

1. Blood obtained from healthy donors, MGUS, and MM patients utilizing 2 x 10 mL STRECK RNA Complete BCT™ tubes (see **Notes 1-4**).
2. 1.5 mL centrifuge low protein binding tubes (see **Note 5**).
3. Sterile pipette tips with filters.
4. Pipettes, stripettor, pipettors.
5. Allegra X-15R refrigerated benchtop centrifuge with SX4750 swinging-bucket rotor for large scale preparation.
6. Eppendorf 5424R refrigerated benchtop centrifuge with FA45-24-11 fixed angle rotor (24 × 1.5/2.0 mL) for small scale preparation.

2.2 Large EVs isolation

1. Fresh/Frozen PBPL (see **Note 6**).
2. Sterile/filtered phosphate-buffered saline (PBS).
3. 1.5 mL centrifuge low protein binding tubes (see **Note 5**).
4. Eppendorf 5424R refrigerated benchtop centrifuge with FA45-24-11 fixed angle rotor (24 × 1.5/2.0 mL).

2.3 Small EVs isolation

2.3.1 CD63 Exo-FLOW capture kit

1. CD63 Exo-FLOW capture kit (# EXOFLOW300A-1) from System Biosciences (SBI) (**see Notes 7-9**) containing Streptavidin Magnetic Beads, biotinylated capture antibody (CD63), bead wash buffer, exosomes stain buffer, Exo-FITC universal exosome stain and exosome elution buffer.
2. ExoQuick™ precipitant (see Sections **2.3.2**, **3.3.2**).
3. 100-200 μg EV protein.
4. 1.5 mL centrifuge tubes.
5. Vortex mixer.
6. Rotating shaker.
7. Magnetic stand.
8. Canto II Flow Cytometer.

9. FACSAria Flow sorter (see **Note 8**).

2.3.2 ExoQuick ULTRA kit

1. ExoQuick ULTRA kit (#EQULTRA-20A-1) from SBI containing proprietary precipitant and purification columns, collection tubes, buffer A and B (**see Note 10**).
2. Thrombin Plasma prep for Exosome precipitation (see **Note 11**).
3. 1.5 mL centrifuge tubes.
4. Eppendorf 5424R refrigerated benchtop centrifuge with FA45-24-11 fixed angle rotor (24 × 1.5/2.0 mL).
5. Rotating shaker.

2.3.3 exoEasy kit

1. exoEasy kit (#76064) from Qiagen containing proprietary membrane-columns and buffers (see **Note 12**).
2. Conical tubes.
3. Syringe filters for excluding particle larger than EVs of interest (see **Note 12**).
4. Allegra X-15R refrigerated benchtop centrifuge with SX4750 swinging-bucket rotor for large scale preparation.

2.3.4 Purification Mini Kit

1. Purification Mini Kit (#57400) from Norgen Biotek Corporation containing proprietary resin, mini filter spin columns, elution tubes and buffers (**see Note 13**).
2. 15 mL conical tubes.
3. Nuclease-free water.
4. Sterile pipette tips with filters.
5. Allegra X-15R refrigerated benchtop centrifuge with SX4750 swinging-bucket rotor for large scale preparation.
6. Eppendorf 5424R refrigerated benchtop centrifuge with FA45-24-11 fixed angle rotor (24 × 1.5/2.0 mL) for small scale preparation.
7. Vortex mixer.

2.4 Albumin depletion (see **Note 14**)

1. Pierce™ Albumin depletion kit (see **Note 15**) containing resin, buffers and spin columns.
2. Sterile pipette tips with filters.
3. 1.5 mL centrifuge low protein binding tubes.
4. Eppendorf 5424R refrigerated benchtop centrifuge with FA45-24-11 fixed angle rotor (24 × 1.5/2.0 mL).

2.5 EV lysate preparation

1. 10X RIPA buffer: 0.22% (w/v) Beta glycerophosphate, 10% (v/v) 4-Nonylphenol (branched, ethoxylated), 0.18% (w/v) Sodium orthovanadate, 5% (w/v) Sodium deoxycholate, 0.38% (w/v) EGTA, 1% (v/v) Sodium lauryl sulfate, 6.1% (w/v) Tris-base, 0.29% (v/v) EDTA, 8.8% (w/v) Sodium chloride, 1.12% (w/v) Sodium pyrophosphate decahydrate; pH 7.5 (see **Notes 16, 17**).
2. 25X protease inhibitors (PI) cocktail (see **Note 18**).
3. 1.5 mL centrifuge tubes.
4. Eppendorf 5424R refrigerated benchtop centrifuge with FA45-24-11 fixed angle rotor (24 × 1.5/2.0 mL).

2.6 Protein Quantitation (see Note 19)

1. Mirco BCA Protein assay kit.
2. Bovine Serum Albumin (BSA) as standard protein. BSA purity > 98%.
3. Working reagent (WR): Mix 25 parts Reagent MA, 24 parts Reagent MB, 1 part Reagent MC. 150 µL for each standard/sample.
4. 1% (w/v) sodium dodecyl sulfate (SDS) in ultrapure water.
5. EV lysate sample for measurement.
6. 1.5 mL centrifuge tubes.
7. Flat-bottom, clear 96-well plates.
8. Incubator (37 °C).
9. Microplate reader with 562 nm filter.

2.7 Western blot analysis

1. EV lysate preparations: ~7-20 µg protein.

2. 4-15% MiniProtean TGX Precast Gels; 4-15% MiniProtean TGX Stain-Free Protein Gels.
3. TTBS solution: Tris-buffered saline (TBS) with 0.05% (v/v) Tween-20.
4. Blocking solution: 5% (w/v) skim milk powder in TTBS.
5. 4X SDS-PAGE sample buffer: 0.2 M Tris-HCl; 0.4 M DTT; 277 mM SDS, 8.0% (w/v) SDS; 6 mM Bromophenol blue; 4.3 M Glycerol; pH 6.8.
6. Heat block.
7. Precision Plus Dual Colour Protein Standard.
8. SDS-PAGE electrophoresis buffer: 0.1% (v/v) SDS, 25mM Tris-base, 190 mM glycine, pH 8.3. Do not titrate pH.
9. Transfer buffer: 25mM Tris-base, 190 mM glycine, 20% (v/v) methanol.
10. Immobilon-P PVDF blotting membrane (0.45 µm pore size).
11. 100 % methanol for activating PVDF membranes.
12. Whatman® cellulose blotting papers (3 mm grade).
13. Frozen cooling unit.
14. Mini-PROTEAN Tetra cell system.
15. Mini Trans-Blot Cell.
16. Ready-to-use Ponceau Red Staining solution.
17. Primary antibodies in TTBS: 1:500 (mouse anti-TSG101, BD Biosciences, #612697); 1:1000 (mouse anti-Alix, Cell Signaling Technology, #2171S; mouse anti-CD81, Santa Cruz Biotechnology, #7637; rabbit anti-CD63, Abcam, #ab134045; rabbit anti-Integrin alpha 2b, Cell Signaling Technology, #13807S; mouse anti-P-selectin, Santa Cruz Biotechnology, #8419; rabbit anti-Albumin, Abcam, #ab207327; rabbit anti-GAPDH, Cell Signaling Technology, #8884).
18. Secondary antibodies, HRP conjugated, in TTBS: 1: 2000 (anti-rabbit, Dako, #P0217); 1: 1500 (anti-mouse, Dako, #P0447)
19. Supersignal™ West Pico PLUS ECL reagent.
20. Orbital shaker.
21. Imager for chemiluminescence, e.g. ChemiDoc™ Touch Imager and software for data analysis.

2.8 Nanoparticle Tracking Analysis

1. **NanoSight NS300 system** or **Zetaview PMX-120 system**
2. EV preparation (~1-2 µg protein)

3. Ultra-pure water
4. Disposable 1mL syringe for sample loading

2.9 Transmission electron microscopy

1. EV preparation (~1-2 µg protein).
2. Sterile/filtered PBS.
3. Fixing solution: 1% (v/v) glutaraldehyde.
4. Carbon coated copper 400 mesh grids (#GSCU400CC) from ProScitech.
5. 2% (w/v) aqueous uranyl acetate.
6. Gatan CCD camera coupled to a Jeol JEM-2100 electron microscope (80 kV).

2.10 Proteomics: sample preparation

2.10.1 In-solution reduction, alkylation and digestion

1. EV lysate preparations (+/- depletion with albumin, ~5-10 µg protein).
2. 1.5 mL Protein LoBind Tubes or Protein LoBind deep 96 well Plates (1000 µL).
3. 1 M Tetraethylammonium bromide (TEAB) stock solution in LC-MS grade water.
4. 50 mM TEAB, pH 8.0, in LC-MS grade water. For pH adjustment use 1 M hydrochloride in LC-MS grade water.
5. 100 mM TEAB, pH 8.0, in LC-MS grade water. For pH adjustment use 1 M hydrochloride in LC-MS grade water.
6. 100% (v/v) acetone (LC grade). Stored at -20 °C.
7. 90% (v/v) acetone in water (LC grade). Stored at -20 °C.
8. Lysis buffer: 1% (w/v) SDS, 50 mM TEAB, pH 8.0.
9. 500 mM DTT stock solution in 100 mM TEAB, pH 8.0. Prepare fresh before the reduction step.
10. 1 M iodoacetamide (IAA) stock solution in 100 mM TEAB, pH 8.0. Prepare fresh before the alkylation step.
11. Trypsin, sequencing grade. Store lyophilized or frozen at -80 °C.
12. 1.5% (v/v) Formic acid (LC-MS grade).
13. Water (LC-MS grade).
14. ThermoMixer with 1.5 mL microfuge tube capacity.
15. Thermostat oven or incubator at 37 °C.
16. pH strip, range 1-14.
17. Sonicator with microtip, e.g. Minsox S-4000, 600W.

2.10.2 StageTip sample cleanup

1. Empore SDB-RPS (Styrene Divinyl Benzene–Reversed Phase Sulfonate) solid-phase extraction disks for StageTip preparation.
2. SDB-RPS StageTip loading buffer: 1% (v/v) trifluoroacetic acid (TFA) in acetonitrile (ACN).
3. SDB-RPS StageTip wash buffer 1: 1% (v/v) TFA in ACN.
4. SDB-RPS StageTip wash buffer 2: 0.2% (v/v) TFA in 5% (v/v) ACN.
5. SDB-RPS StageTip elution buffer: 20 µL of NH_4OH in 4 mL of 60% (v/v) ACN. Elution buffer must be prepared fresh (within 1 h of use) because the pH will begin to increase due to its high volatility, thereby reducing its elution strength.
6. Water (LC-MS grade).
7. Vacuum centrifuge (lyophilizer).
8. MS loading buffer: 0.07% (v/v) TFA in LC-MS water. This buffer is stable for >6 months at RT.

2.11 Fluorometric peptide assay

1. Quantitative Fluorometric Peptide Assay kit.
2. MS loading buffer (see step 8 in **Section 2.10.2**).
3. Peptide samples for analysis.
4. Fluorescence compatible 96 well microplate (black).
5. Fluorescent plate reader compatible with Ex 390 nm / Em 475 nm.

2.12 UHPLC-MS/MS

1. MS loading buffer: 0.07% (v/v) TFA in LC-MS water.
2. Transparent MS sample vials:300µL capacity and snap ring vial with PP insert.
3. UHPLC System coupled to a Q Exactive HF-X Hybrid Quadrupole-Orbitrap MS System.
4. Load column: Acclaim PepMap100 C18, 5 µm beads with 100 Å pore-size.
5. Analytical/separation column: 50-cm fused-silica emitter reversed-phase PepMapRSLC C18, 75 µm inner diameter, 2 µm resin with 100 Å pore-size.
6. Mobile Phase A: 0.1% formic acid.
7. Mobile Phase B: 0.1% formic acid in acetonitrile.

2.13 Data analysis

1. MaxQuant software.
2. Microsoft Office Excel.
3. Perseus software (*91*) from Max-Planck Institute of Biochemistry, Munich, Germany.

3. Methods

3.1 PBPL isolation

1. Whole blood, collected using STRECK RNA tubes, is immediately transferred to the laboratory (same site) avoiding agitation and allowed to sit at room temperature for 30 min (see **Notes 1-4**).
2. Centrifuge at 1,800 x *g* for 10 min at 4 °C to separate plasma.
3. Carefully transfer the upper plasma phase to low protein binding tubes (see **Note 5**), without disturbing the intermediate buffy coat layer which contains white blood cells and platelets. Normally up to 4-5 mL plasma can be recovered from 10 mL whole blood.
4. Centrifuge at 2,000 x *g* for 15 min at 4 °C (see **Notes 20, 21**).
5. Carefully transfer the cleared supernatant to new low protein binding tubes without disturbing the pellet (see **Note 5**).
6. Store aliquots in low protein binding tubes at −80 °C.
7. Depending on EV isolation method, 0.5 to 4 mL aliquots (fresh or frozen) are utilized for EV isolation (see **Note 6**).

3.2 Large EVs isolation (see Note 22)

1. Centrifuge plasma at 10,000 × *g* for 30 min at 4 °C to pellet large EVs.
2. Resuspend the pellet in 1 mL of sterile/filtered PBS.
3. Centrifuge at 10,000 × *g* for 30 min at 4 °C to obtain the washed large EVs pellet.
4. The pellet can be resuspended in either SDS sample buffer for PAGE analysis (i.e., Sections **3.6-3.7**), or sterile PBS for other downstream applications (i.e., Sections **3.8-3.10**).

3.3 Small EVs isolation

3.3.1 CD63 Exo-FLOW capture kit

1. Briefly vortex the bead slurry and then load 40 µL of bead slurry solution into a 1.5 mL tube per sample.
2. Place tubes on magnetic stand for 2 min.
3. Carefully remove the supernatant making sure to not disturb the magnetic bead pellets.
4. Remove the samples from magnetic stand and add 500 µL of Bead Wash buffer.

5. Invert the tubes a few times, place samples on magnetic stand for 2 min and discard the buffer. Wash steps are repeated one more time and all liquid removed. Beads are on the side of the tube.
6. After removing tubes from magnetic stand, add 10 µL of CD63 biotinylated capture antibody, using the pipette tip to move the beads to the bottom of the tube, and mix by pipetting up and down 3 times.
7. Place tubes on ice for 2 hrs, flicking the tubes every 30 min to gently mix.
8. Add 200 µL of Bead Wash buffer, mix by flicking and place samples on magnetic stand for 2 min.
9. Carefully remove the supernatant making sure to not disturb the magnetic bead pellets.
10. Add 500 µL of Bead Wash buffer after removing the samples from magnetic stand. Beads are washed by inverting 2-3 times and flicking the tubes a few times.
11. Place samples on magnetic stand for 2 min and discard the buffer. Repeat wash steps for a total of 3 washes and remove all liquid.
12. Resuspend capture antibody-beads with 400 µL of Bead Wash buffer per sample.
13. Add 100 µL (100-200 µg of protein) of ExoQuick™-precipitated EVs (see **3.3.2**) to each bead sample for a total volume of 500 µL.
14. Incubate on a rotating rack at 4 °C overnight for capture.
15. Place samples on magnetic stand for 2 min.
16. Carefully remove the supernatant making sure to not disturb the magnetic bead pellets.
17. Add 500 µL of Bead Wash buffer after removing the samples from magnetic stand. Beads are washed by inverting 2-3 times and flicking the tubes a few times.
18. Place samples on magnetic stand for 2 min and discard the buffer. Repeat wash steps for a total of 2 washes and remove all liquid.
19. Add 240 µL of Exosome Stain Buffer and 10 µL of Exo-FITC exosome stain for a final volume of 250 µL per sample, and place tubes on ice for 2 hrs (flicking the tubes every 30 min to gently mix).
20. Place samples on magnetic stand for 2 min.
21. Carefully remove the supernatant making sure to not disturb the magnetic bead pellets.
22. Add 500 µL of Bead Wash buffer after removing the samples from magnetic stand. Beads are washed by inverting 2-3 times and flicking the tubes a few times.
23. Place samples on magnetic stand for 2 min and discard the buffer. Repeat wash steps for a total of 3 washes and remove all liquid.

24. Resuspend samples in 300 µL of Bead Wash buffer for flow cytometry/sorting (avoid vortexing prior to loading into FACS instrument).
25. For EV Stain removal and elution (if desired), place samples on the magnetic stand for 2 min and remove buffer.
26. Add 300 µL of Exosome Elution Buffer and invert samples a few times. Vortexing is not recommended. Mix by flicking.
27. Incubate on a rotating rack or shaker at 25 °C for 2 hrs.
28. Place samples on magnetic stand for 2 min.
29. Carefully transfer the supernatant containing eluted EVs to a fresh tube, making sure to not disturb the magnetic bead pellets. Discard the beads after use.

3.3.2 ExoQuick ULTRA kit

1. Add 5 µl thrombin to 500 µl of plasma (see **Notes 10, 11, 23**) followed by gentle mixing (flick the tube).
2. Incubate at RT for 5 min.
3. Centrifuge at 9,400 x *g* for 5 min. Transfer the supernatant to a new tube.
4. Add appropriate volume of ExoQuick™ (i.e. 67 µl for each 250 µl of plasma) and mix by inverting or flicking the tube.
5. Incubate on ice for 30 min and then centrifuge at 3,000 x *g* for 10 min (RT or 4 °C) to pellet the EVs from the solution.
6. Aspirate and discard the supernatant, making sure not to disturb the pellet containing EVs. Resuspend the pellet in 200 µL of provided Buffer B, proceeding to protein quantitation (see Sections **2.5, 2.6, 3.5, 3.6** and **Note 24**).
7. Add 200 µL of provided Buffer A.
8. Loosen the screw cap, snap off the bottom closure and place the purification column into a collection tube (save the bottom closure for step 11). Centrifuge the column at 1,000 x *g* for 30 sec to remove storage buffer.
9. Discard the flow-through and replace back the column into the collection tube and wash twice by applying 500 µL of Buffer B on top of the resin.
10. Centrifuge at 1,000 x *g* for 30 sec. Discard the flow-through.
11. Prime the column by applying 100 µL of Buffer B on top of the resin. The entire content from steps 6-7 (400 µL or up to 4 mg of total protein content) is then added (plug the bottom of the column with the bottom closure, see Step 8) and mixed at RT on a rotating shaker for 5 min.

12. Loosen the screw cap, remove the bottom closure and immediately transfer the column to a 2 mL Eppendorf tube.
13. Centrifuge at 1,000 x *g* for 30 sec to obtain purified EVs.
14. EVs are aliquoted depending on intended use, and either used immediately or stored at -80 °C.

3.3.3 exoEasy kit

1. All steps should be performed at RT.
2. Filter 2-4 mL of plasma (see **Notes 12, 23, 25**) to exclude particles larger than 0.2 μm (for small EVs isolation).
3. Add 1 volume of buffer XBP to 1 volume of sample and mix well by gently inverting the tube 5 times.
4. Load mixture onto the exoEasy spin column and centrifuge at 500 x *g* for 1 min.
5. Discard flow-through and place column back into the same collection tube. Add 10 mL of buffer XWP, and residual buffer from the column is removed by centrifuging at 5,000 x *g* for 5 min. Discard the flow-through and the collection tube.
6. Place the spin column into a fresh collection tube.
7. Add 400 μl of buffer XE to the membrane, incubated for 1 min, and centrifuge at 500 x *g* for 5 min. Collect the eluate containing EVs.
8. Re-apply the EV containing eluate to the spin column membrane, incubate for 1 min, and centrifuge at 5,000 x *g* for 5 min.
9. Aliquot depending on intended use (see **Notes 26, 27**), and either use immediately or store at -80 °C.

3.3.4 Purification Mini Kit

1. All steps should be performed at RT.
2. Add 3 mL of Nuclease-free water to 1 mL of plasma (see **Notes 13, 23, 28**).
3. Add 100 μl of ExoC buffer.
4. Add 200 μl of Slurry E (mix well Slurry E prior to use).
5. Vortex sample for 10 sec and incubate for 5 min.
6. Vortex for 10 sec and centrifuge at 930 x *g* (2,000 rpm) for 2 min. Discard the supernatant.
7. Add 200 of μl ExoR buffer, mix well by vortexing for 10 sec, and incubate at RT for 5 min.

8. Vortex the sample for 10 sec, centrifuge at 500 rpm for 2 mins, and transfer the supernatant to a Mini Filter Spin column assembled with an elution tube.
9. Centrifuge at 3,400 x *g* (6,000 rpm) for 1 min.
10. EV containing eluate is collected and aliquoted depending on intended use, and either used immediately or stored at -80 °C.

3.4 Albumin depletion (see Notes 14, 15, 29)

1. Resuspend well the resin by shaking the resin bottle.
2. Transfer 400µl of the slurry (corresponding to 200 µl settled resin volume) into a spin column (loosely cap the column).
3. Twist off the bottom closure of the spin column and place the spin column into a collection tube.
4. Centrifuge at 12,000 × g for 1 min to remove excess liquid. Discard the flow-through and place the spin column back into the same collection tube.
5. Add 200 µl of Binding/Wash Buffer into the spin column. Centrifuge at 12,000 × g for 1 min and discard the flow-through. Place the spin column into a new collection tube.
6. Load (maximum) 50 µl of albumin-containing EV sample onto the resin and incubate for 1-2 min at RT. Centrifuge at 12,000 × g for 1 min. Collect the flow-through.
7. Re-apply the flow-through to the spin column, and incubate for 1-2 min at RT to ensure maximal albumin binding. Thus, centrifuge at 12,000 × g for 1 minute, retaining the flow-through.
8. Add 50µl of Binding/Wash Buffer for each 200µl of resin used and centrifuge at 12,000 × g for 1 min to wash the resin (to release unbound proteins). Retain the flow-through. Place spin column into a new collection tube.
9. Step 8 is repeated one more time and the 3 EV albumin-depleted fractions are combined (see **Notes 30, 31**).

3.5 EV lysate preparation (see Notes 16-18)

1. Add RIPA buffer and PI cocktail (final concentrations 1X) to EV samples.
2. Vortex sample for 15 sec and incubate for 1 hr at 4 °C (vortexing every 10 min).
3. Centrifuge at 16,000 x *g* for 15 min at 4 °C to remove insoluble debris.

4. Collect the supernatants and store at -30 °C (up to 6 months) or proceed to protein quantitation (see section 3.6).

3.6 Protein Quantitation (see Note 19)

1. A set of standard dilutions with BSA is prepared starting with 200 µg/mL, performing dilutions in 1% SDS solution to 0.5 µg/mL, and placed at RT (1% SDS solution can crystallize on ice).
2. EV lysates are thawed on ice.
3. Load in a clear flat-bottom 96-well plate 150 µl of EV eluent/lysis solution (blank), 150 µl of each standard BSA dilution, and 150 µl of diluted EV lysate sample (2 µl in 148 µl of 1% SDS solution).
4. Add 150 µl of the WR to each well and mix the plate (sealed with adhesive film) thoroughly on a plate shaker for 30 sec. Plate is covered, incubated at 37 °C for 2 hrs and then cooled to RT.
5. Absorbance is measured at 562 nm on a plate reader.
6. Determine the protein concentration of each EV sample using the BSA standard curve (plotting the average Blank-corrected 562 reading for each BSA standard vs its concentration in µg/mL). Multiply by the diluting factor.

3.7 Western blot analysis (see **Notes 32-35**)

1. Mix EV samples (7-20 µg protein) with SDS-PAGE sample buffer at a final concentration of 1X, heat for 5 min at 95-100 °C, and cool on ice before proceeding to electrophoresis which is performed at constant 140-180 V for 45-60 min (until the dye reaches the reference line).
2. Activate PVDF membranes: soak in 100% methanol for 30 sec and then in ice cold transfer buffer.
3. Following electrophoresis, perform a tank (wet) blotting procedure: Sandwich gel and membrane between sponge and blotting paper (sponge/2x blotting paper/gel/membrane/2x blotting paper/sponge) and clamp tightly after ensuring no air bubbles have formed between the gel and membrane using a roller.
4. Insert the cassette/sandwich into the tank (the black side of the cassette should face the black side of the central core) together with the frozen cooling unit. Complete transfer at 90-100 V for 60-90 min (protein transfer is confirmed by the presence of

the protein standard on the membrane and further confirmed by Ponceau Red staining).

5. Block membranes with blocking buffer for 1 hr at 4 °C. Care should be taken not to touch and disrupt the membrane (only the immediate corners/edges).
6. Probe membranes with primary antibodies overnight in TTBS.
7. Incubate with appropriate secondary antibody for 1 hr.
8. All antibody incubations are carried out using gentle orbital shaking at RT.
9. Western blots are washed six times in TTBS for 5 min after each incubation step and subsequently visualised using ECL reagents, developed using ChemiDoc™ Touch Imager, and analysed with the aid of Image Lab software.

3.8 Nanoparticle Tracking Analysis (see Notes 32, 36)

3.8.1 NanoSight NS300 system

1. Dilute EV sample to a final volume of 1 mL (final concentration 1 µg/mL in ultra-pure water, for example 1:1000 dilution, depending on the concentration of EVs).
2. Load sample onto NS300 flow-cell top plate using syringe pump. Avoid the introduction of air bubbles into the chamber.
3. Operation settings: Detection threshold = 10; Flow rate = 50; Temperature = 25 °C (Standard measurement).
4. Camera level and screen gain need adjustment until particles are visible.
5. Conditions for video capture are as follows: Number of capture = minimum 3 (for triplicate); capture duration = 60 sec.
6. For acquisition, change base file name to preferred destination folder.
7. Click ‘Create’ and ‘Run script’ to begin capture and analysis with NTA software, ensuring that detection threshold is set to include as many particles as possible with the restriction that 20-100 red crosses are counted while only <10% are not associated with distinct particles. Dilution and analysis can be repeated as required ensuring particle counts within 20-100 particle/frame.
8. Set report details as prompted and NTA report can be found in destination folder: PDF graphs and batch summaries show size distribution profile data and statistics; CSV files contain raw data for further processing; AVI files contain video capture.

3.8.2 Zetaview PMX-120 (see Notes 32, 36)

1. Perform automated quality control measurements including cell quality check and instrument alignment, and focused, conductivity and electrical field measurements.
2. Perform calibration using Polystyrene particles with a known average of 100 nm.
3. Dilute EV sample in ultra-pure water ensuring particle counts within 100-200 particle/frame (depending on the concentration of EVs, for example 1:1000 dilution).
4. Set the instrument parameters to a temperature of 25ºC, a sensitivity of 90, a shutter speed of 100, Scattering Intensity 4.0.
5. Load samples into the cell, and the instrument measures at 11 different position through the cells and capturing 60 frames per position.
6. Data is analysed using in-build ZetaView software 8.02.31, and after automated analysis of all 11 positions and removal of any outlier positions, the mean median and mode (indicated as diameter) size, as well as the concentration of the sample, are calculated by the optimized machine software.

3.9 Transmission electron microscopy (see **Notes 27, 37-39**)

1. Fix EV preparations (~1-2 µg protein) in 1% (v/v) glutaraldehyde
2. Layer preparations onto Carbon coated copper 400 mesh grids, and allow to dry at RT.
3. Wash grids twice with water for 5 min, and stain with 2% (w/v) uranyl acetate in distilled water for 10 min.
4. Imaging is performed at an acceleration voltage of 80 kV using a Gatan CCD camera coupled to Jeol JEM-2100 electron microscope (80 kV).
5. Typically, 10-20 fields of view are obtained.

3.10 Proteomics: sample preparation

3.10.1 In-solution reduction, alkylation and digestion

1. On ice, normalize EV samples to 10 µg in 50 µL lysis buffer (see **Note 40**) in protein LoBind tubes/plates.
2. Prepare fresh 500 mM DTT stock, vortex briefly until dissolved (see **Note 41**).
3. For sample reduction, pre-heat ThermoMixer to 50 °C and add 1 µL DTT stock to each tube (10 mM final concentration), vortex and microfuge briefly. Incubate all samples in the thermomixer at 50 °C for 45 min (350 rpm). Keep the remaining DTT stock on ice for quenching (see step 7).

4. Cool down all samples to RT and reset the ThermoMixer to 25 °C to avoid non-specific alkylation reaction.
5. Prepare fresh 1 M IAA stock and ensure minimal light exposure of IAA. Vortex briefly until dissolved.
6. For sample alkylation, add 1 µL IAA stock to each tube (final concentration 20 mM IAA), vortex and microfuge briefly. Incubate samples in the ThermoMixer at 25 °C for 30 min (350 rpm) in the dark.
7. To quench the alkylation reaction, add 1 µL DTT stock to all samples (final concentration 20 mM), exposing to light.
8. Add 500 µL ice-cold acetone to all samples, vortex briefly. Store the samples in -20 °C freezer overnight. Samples can be stored at -20 °C for up to three days.
9. Prior to tryptic digestion centrifuge all samples at 16,000 × *g* for 10 min at 4 °C. Carefully discard supernatant without disturbing the protein pellet.
10. Resuspend protein pellets in 50 µL 90% (v/v) ice-cold acetone, vortex briefly.
11. Centrifuge all samples at 16,000 × *g* for 10 min at 4 °C. Carefully discard supernatant without disturbing the pellet. Air-dry the samples under the fume hood for 5 min.
12. Resuspend protein pellets in 50 µL 50 mM TEAB, pH 8.0 (see **Note 42**).
13. Add 0.2 µg equivalent trypsin enzyme (1:50 enzyme-to-substrate ratio) to each sample. It is often easier to make the trypsin working solution (calculate adding 50 µL per sample; 50 mM TEAB, pH 8.0, on ice), add required amount of trypsin to the trypsin working solution, and aliquot to each resuspended protein pellet (see **Note 42**).
14. Set the ThermoMixer to 37 °C and incubate all samples for 18 hrs at 350 rpm.
15. Acidify all samples with 0.75 µL formic acid (final concentration 1.5% (v/v)) to pH 2, test with pH strips.
16. Centrifuge all samples at 16,000 × *g* for 5 min. Transfer supernatant to a protein LoBind tubes if insoluble precipitate is spotted at the bottom of the tube.
17. Place all samples in -80 °C freezer until frozen. Peptides can be stored in -80 °C for an extended period before lyophilization. Skip this step if StageTip Cleanup is required.

3.10.2 StageTip cleanup

1. SDB-RPS StageTips are prepared as described[108], using Empore solid-phase extraction disks. Prepare one SDB-RPS StageTip for each sample.
2. We use two plugs of SDB-RPS material for the respective StageTips, punched out by using a blunt-end 14-gauge syringe to pierce the ends of 200 µL pipette tips (see **Note 43**).
3. Add 30 µL of SDB-RPS loading buffer to the top of each sample. Place the StageTips into centrifuge adapters and centrifuge the sample through to dryness (1,500 × *g*, for ~1 min at RT).
4. Wash the SDB-RPS StageTips using 30 µL of SDB-RPS wash buffer 1 to each sample and centrifuge for ~1 min. Repeat.
5. Transfer each peptide sample to the top of an SDB-RPS StageTip.
6. Place the StageTips into same centrifuge adapter and centrifuge the sample through to dryness (1,500 × *g* for ~5-8 min at RT) (see **Note 44**).
7. Wash the StageTips with 100 µL of SDB-RPS wash buffer 1 and centrifuge the sample through to dryness (1,500 × *g* for ~5-8 min at RT). Repeat with SDB-RPS wash buffer 2.
8. Elute the peptides by adding 60 µL of SDB-RPS elution buffer of the StageTip, and centrifuge through to dryness (1,500 × *g* for ~5 min at RT). Collect the eluates into clean protein LoBind tubes.
9. Immediately place the tubes into an evaporative concentrator and concentrate under vacuum at 45 °C to dryness. The time to dryness will depend on the number of samples being processed. This typically takes 40-60 min; however, after the first 30 min have elapsed, samples should be checked at intervals of 10 min and concentrated until no liquid remains.
10. Reconstitute peptide by adding 12 µL of MS loading buffer and either shaking for 2 min at 2,000 rpm or optionally incubating for 5 min in a sonicating water bath on low power, then centrifuge the samples at 2,000 × *g* for 1 min at RT.

3.11 Fluorometric peptide assay (see **Note 45**)

1. Prepare peptide standards from the Assay Kit starting with 1000 µg/mL and performing serial dilutions in MS loading buffer down to 7.8 µg/mL as per the manufacturer's instructions (8 standards plus MS loading buffer as blank).
2. Load the 10 µL of each standard/blank into the black 96 well plate.

3. Load 2 µL of each sample into the plate and make up to 10 µL by adding 8 µL MS loading buffer.
4. Add 70 µL of Fluorometric Peptide Assay Buffer to each well.
5. Add 20 µL of Fluorometric Peptide Assay Reagent to each well.
6. Incubate at RT for 5 min.
7. Measure fluorescence using Ex/Em at 390 nm/475 nm.
8. Use the standard curve to calculate the concentrations of your samples, ensuring to subtract blank (abs) from all standards/samples (abs).

3.12 UHPLC-MS/MS

1. Perform Ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis of trypsin digested exosome protein samples at a flow rate of 300 nL min^{-1} by using the following UHPLC gradient[109].

Time interval (min)	Gradient (% buffer B)
0	2
95	28
98	80
100	80
104	2
110	2

2. Use the following MS parameters on the **Q Exactive HF-X** for protein identification.

Instrument	
Polarity	Positive
S-lens/ion-funnel RF level	45
Capillary temperature	300 °C
Full MS	
Microscans	1
Resolution	60,000
Automatic gain control target	3×10^6 ion counts
Maximum ion time	128 ms
Scan range	300–1650 m/z
dd-MS^2 (data dependent)	
Microscans	1
Resolution	30,000
Automatic gain control (AGC) target	1×10^5 ion counts
Maximum ion time	60 ms
Loop count	25
Isolation window	1.3 m/z
Isolation offset	0

Fixed first mass	120 m/z
Normalized collision energy	25
Spectrum data type	Profile
Data Dependent settings	
Minimum AGC target	1.2×10^4 ion counts
Apex trigger	-
Charge exclusion	Unassigned, 1, ≥6
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	30 s

739

740 **3.13 Data analysis**

741 1. Analyse raw proteomics data using MaxQuant (see **Note 46**) and perform downstream
742 bioinformatics analysis using the Perseus platform (described below). However, several
743 other suitable programs are freely or commercially available that can be used to process
744 proteomics data (see **Note 46**).
745 2. Specify the parameters used for searching label-free proteomics data using MaxQuant.
746 Default settings in MaxQuant are typically optimal and can normally be left unchanged,
747 with a few exceptions such as defining experiment, modifications, and enzyme as
748 described below. Typical parameters are outlined in the table below.

Setting	**Value**
Raw data	
Parameter group	All samples: Group 0
Experiment	Enter unique name, suffixed by biological replicate (e.g., 'Control_1')
Fraction	All samples: 1
PTM	FALSE
Group-specific parameters	
Type	Standard
Multiplicity	1
Labels	None
Variable modifications	Oxidation (M), Acetyl (protein N-term)
Digestion mode	Specific
Enzyme	Trypsin/P
Label free quantitation (LFQ)	TRUE, maxLFQ
Maximum missed (cleavages)	2
Database	Specify protein database (i.e., reference proteome) in FASTA format
Global parameters	
FASTA files	Specify protein sequence databases
Fixed modifications	Carbamidomethyl (C)
Second peptide	TRUE

Protein FDR, peptide spectral match	0.01 / 0.01
Match between runs	TRUE
Match time window	0.7 min
Alignment time window	20 min
Advanced site intensities	Yes

3. Perform data analysis using Microsoft Office Excel and Perseus software (see **Note 46**).
4. Use ProteinGroup.txt file for protein grouping and identification, with contaminants (CON_) and reverse (REV_) identifications manually removed.

4. Notes

1. Human clinical samples are treated as potentially infectious. Therefore, proper biosafety measures are taken when processing these samples with adherence to Federal, State and local regulations [Alfred Health and Monash University Occupational Health and Safety (OHS) Policy and Procedures]. For example, clinical samples are to be processed in facilities that meet PC2 facility and procedural requirements.

2. Viscosity of the fluids, as well as their fat and protein content, age, gender, use of medications, are among several factors that can affect the amount, purity and content of biofluid-derived EVs. Other important factors include the method of blood collection, anticoagulants, agitation of samples leading to release of platelet derived MPs, storage time, volume of starting material [70,110].

3. Blood collection must only be performed by personnel trained in phlebotomy/venepuncture. An evacuated tube system is usually utilized. Unless otherwise indicated patients are not fasting. Tubes are collected following a specific tube sequence as per local hospital protocol, being Full Blood Examination (FBE) tubes the first ones.

4. Accessible biobanks from our group include PBPL samples derived from blood collected in both EDTA (BD Vacutainer Venous Blood Collection tubes) and STRECK RNA tubes. Plasma is preferable over serum because the latter employs coagulation, which has been shown to be accompanied by the release of EVs, particularly from platelets [110]. EDTA is generally preferred over heparin as the latter is difficult to remove and can interfere with platelet activation and downstream applications. Moreover, heparin reduces the exosomes yield [111-113]. STRECK RNA tubes contain a preservative which minimizes degradation of white and red blood cells and stabilizes cf-RNA and EV counts for up to 7 days when stored at RT. Typically, we isolate EVs from fresh STRECK-RNA derived PBPL within 4 hrs. In a clinical setting, timing might represent a limiting step. The use of STRECK RNA tubes allows the storage of stabilized samples at RT without the need for immediate processing. EDTA activates platelets causing degranulation and release of microparticles that can contaminate the EV sample, requiring sample processing within few hrs. Furthermore, small EVs isolated from STRECK RNA tubes display a more

typical morphology with lower background compared to EDTA-derived EVs when analysed by TEM (unpublished data). Frozen (EDTA)-samples remain a good source of study material where other options are not available, bearing in mind that samples might be exposed to several freeze-thaw cycles, and processed using centrifugation steps that were not intended for EV studies [114-117].

5. Low protein binding tubes are utilized for all PBPL and EV processing steps to minimize protein sample loss.

6. When frozen PBPL samples are utilized for EV isolation, samples are quickly thawed at 37 °C in a water bath or dry heating system, followed by a centrifugation at 5000 x *g* for 15 min at 4 °C to remove cryoprecipitates [118].

7. Although mostly used for cellular analysis, flow cytometry is currently one of the most popular techniques used to study EVs, in particular large EVs [92,97]. Dedicated flow cytometers have been shown to be capable of resolving particles consistent with biological vesicles to <30 nm. Imaging flow cytometry (iFCM) by Imagestream is a method combining flow cytometry with imaging, and all signals using iFCM are collected through microscope objectives and quantified based on images detected by charge-coupled device (CCD) cameras. iFCM has been reported to be suitable and sensitive enough to detect and enumerate both large and small EVs [119]. Recent guidelines reporting Minimum Information about Flow Cytometry in EV studies (MIFlowCyt-EV) have been published [120]. It is highly recommended to report critical information related to sample staining, EV detection and measurement and experimental design in manuscripts that report EV-related flow cytometry data.

8. Exo-FLOW takes advantage of capture beads (immunocapture, see **Note 9**) that enable the analysis of stained EVs by flow cytometry. Furthermore, EVs can be released from the capture beads, enabling their utilization for downstream applications after imaging/sorting.

9. Although providing highly purified populations of EVs [92,94], immunocapture selects sub-types of EVs based on the use of specific markers. To date, universal EV markers

have not been identified. Therefore, immunocapture is not recommended in the context of new EV-based projects or when EVs sub-populations have not been clearly identified.

10. The kit contains the precipitation reagent ExoQuick™ and purification columns (the resin characteristics are proprietary). The company indicates that the additional step using the purification columns should reduce carry-over of albumin by 75% and immunoglobulins by 40%. Two separate kits are provided for isolating EVs from plasma/serum or CM with indicated minimum starting volumes of 250 µL for PBPL/serum and 5 mL for CM.

11. Highly purified thrombin is used to defibrinate plasma, making the resulting supernatant compatible with the ExoQuick™ EVs precipitation. The suggested starting PBPL volume is 500 µL.

12. The membrane properties of the exoEasy kit are not disclosed. The method does not distinguish EVs by size or cellular origin, and is not dependent on the presence of a particular epitope. Thus, samples need to be filtered or centrifuged to remove cells, cell debris and other particles, and to select the EV of interest. The same kit can be used for both plasma/serum (up to 4 mL) or CM (up to 32 mL).

13. The purification is based on Norgen's proprietary resin and does not require the use of precipitation reagents. This specific kit is intended for the isolation of small EVs from small volumes (up to 1 mL) of plasma/serum. Other kits are available for use with CM or different volumes.

14. If the downstream application involves proteomic studies, an albumin depletion step should be taken into consideration [121]. In fact, albumin represents >50-60% protein content in plasma and can co-isolate with EVs and impair proteomic analysis by mass spectrometry, with subsequent lower detection of EV-related cargo proteins. Enrichment for low-abundant proteins of interest together with fractionation of peptides obtained after proteolytic digestion and depletion of highly abundant proteins, represents a critical pre-processing step which has been shown to improve MS-based proteomic analysis. The same considerations should be made when using conditioned medium to isolate EVs and

the use of serum-free medium or EV-depleted medium is highly recommended[46,57,85,122,123]. One should optimise this approach in depleting abundant proteins such as albumin.

15. The resin provided in this kit is a high capacity, immobilized Cibacron Blue dye agarose resin, which is optimized for the binding of human serum albumin. Each aliquot of 200 µL settled resin can process up to 50 µL of albumin containing sample. Check manufacturer's instructions notes for details regarding sample type, volume, and pH.

16. It is important to check if the downstream application requires native, non-denatured proteins, thus avoiding the use of denaturing agents (i.e., DTT).

17. EVs isolated utilizing commercially available kits are often eluted in the provided buffers. To avoid further dilution of the sample we use small volumes of highly concentrated lysis buffers.

18. Protease Inhibitor (PI) cocktails are commercially available and added if the lysates are to be stored and utilized at a later stage.

19. For protein quantitation different methods based on sample quantity and buffer compatibility may be employed. It is important to perform protein quantification on EV and cell lysates prior to immunoblotting, or proteomic profiling to ensure normalised protein content.

20. Obtaining strictly platelet-free plasma (PFP) is one of the major challenges of EV isolation methods from blood. The second centrifugation step employed to isolate PBPL ensures the generation of PFP from platelet-poor plasma. It is important to apply the same protocol to all samples that are to be compared [103,104,110].

21. Platelet-derived MPs are small vesicles released from platelets upon activation and/or mechanical stimulation. MPs represent the vast majority of EVs in circulation and are thought to originate from both circulating platelets and platelet precursors, which reside

in the bone marrow [124]. The expression levels of MP markers (i.e. Integrin alpha 2b or CD41, P-selectin or CD62) can be evaluated by immunoblotting and may indicate the level of contamination of EV samples by platelet-derived particles [104,112]. Immunoaffinity capture approaches may benefit from specific selection of the EV population of interest by excluding MPs ($CD41^-$) [105].

22. Large EVs or shed microvesicles (sMVs) are distinct in their biogenesis and marker expression composition to small EVs [69,70,85,94]. If researchers are interested in the isolation of large EVs, then this fraction can be easily isolated using a direct ultracentrifugation approach. Following 10,000 x *g* centrifugation, the sMV pellet is resuspended in 1 mL of PBS and 10,000 x *g* centrifugation performed to obtain the washed sMV fraction (resuspend in 50-100 µL PBS). Further use of density-based separation can purify this EV type [94].

23. The volume of the starting material is indicated in the instruction manuals of the commercially available kits. Optimization might be performed to define the volume of choice for the specific study.

24. Evaluating the protein concentration after the precipitation step is important for defining the amount of EVs to be used for the purification step (up to 4 mg protein). We lyse a small aliquot of EV eluted in buffer B (5-10 µL) and proceed to protein quantitation. We observe a ~10-fold reduction in protein content after the purification step in PBPL-EV samples, and a ~5-fold reduction after the purification step in CM-EV samples (unpublished data).

25. We evaluate the presence of EVs in the final eluted sample by immunoblotting to confirm the expression of several EV markers (CD63, CD81, Alix, TSG101), relative to whole blood (volume matched).

26. The above described commercial kits provide eluted EVs in proprietary buffers. The latter are not always compatible with downstream applications such as functional studies. It is important to perform a mock control to ensure full compatibility of the buffers with cell culture with no change in morphology, density or viability. For example, the constituents of the elution buffer provided with the exoEasy kit are inorganic salts that are not

compatible with our HMCLs cultures. A further ultracentrifugation step is required (100,000 x *g* for 1 hr) and pellet is resuspended in PBS. Buffer exchange (e.g. by ultrafiltration) may represent another option.

27. The exoEasy elution buffer is not compatible with TEM for which we resuspend EVs in PBS after centrifugation (100,000 x *g* for 1 hr). The precipitation reagent ExoQuick interferes with the electron beam producing images with a high background and low contrast, affecting the visualization of EVs [125].

28. The minimum input volume is 50 µL. We use 1 mL to maximize the use of the kit and to isolate a higher amount of small EVs for downstream applications (genomics and proteomics).

29. Our data generated using MS show that albumin is still present (33% of peptides identified and MS/MS coverage obtained) after EV isolation utilizing the Norgen kit with a total of 253 proteins detected/identified (unpublished data). Thus, we add the additional albumin depletion step for proteomics applications (see **Note 14**) in order to overcome the albumin protein/peptide coverage detected using mass spectrometry. Preliminary data (unpublished) confirm an increase in protein detection (390 proteins vs 253) after depletion of albumin (22% in combined fractions 1 to 3, see **Note 30**).

30. The final wash steps can be optimized depending on intended application. Additional wash steps may be performed or different wash buffer volumes may be used. Our immunoblot data demonstrate an efficient depletion of albumin with predicted variability between samples derived from different donors/patients and within same samples processed at different times (unpublished data). We observe expression of EV markersonly in the first 3 fractions, therefore we do not collect further fractions.

31. For 2D PAGE or mass spectrometry analysis, albumin-depleted samples must be precipitated, dialysed or desalted to remove interfering salts.

32. Immunoblotting may represent the first step to characterize EV samples and to select samples for further characterization with NTA and EM which are usually more expensive (both) and time-consuming (EM) techniques [70]. Based on immunoblotting, NTA and

EM characterization, is possible to evaluate the optimal isolation method for proceeding with downstream analysis. It is important to consider that the data obtained by different methods can vary significantly and even the settings of measuring devices can considerably influence the corresponding results. Flow cytometry represents another important tool for EV characterization. Although developed for cellular analysis, EV-dedicated flow cytometers have been shown to be capable of resolving particles consistent with biological vesicles to < 50-100 nm (see **Note 7**).

33. To characterize EVs, it is important to demonstrate the expression of common EV proteins using immunoblotting [70,94]. The expression of EV markers is usually evaluated both in EV lysates and lysates of the cells of origin. It is also recommended to evaluate EV markers in small EVs compared to large EVs which are distinct not only based on size but also functionality. Commonly used markers are: Alix, TSG101, CD63, CD81. The use of loading control markers such as GAPDH, calnexin or tubulin, is also recommended to confirm the absence of cellular contamination [69,70]. The expression levels of the EV markers represent a good indicator not only of the presence of EVs in a chosen sample but also of the purity of the sample. For example, we observe a lower expression of EV markers in frozen samples when compared to fresh samples or a lower expression of EV markers in samples isolated utilizing ExoQuick ULTRA and exoEasy kits when compared to the Norgen kit, for PBPL, or OptiPrep™, for CM (data not shown). Immunoblotting is also utilized for evaluating the expression levels of abundant proteins such as albumin and for determining the level of platelet contamination in PBPL samples (see **Notes 14, 20, 21**).

34. In the context of EVs, EVs biology/functions and omics technologies (i.e., genomics, transcriptomics, and proteomics) it is critical to generate an *in vitro* model [106,107]. We use Human Myeloma Cell Lines (HMCLs) [126]. Culture medium for EV isolation is collected as follows:
    (a) All cells are cultured in a humidified incubator at 37 °C with 5% CO2 and used until 20th passage. HMCLs are grown at a density of 2-2.5 x 10^5 cells/mL in 2D 175 cm^2 flasks. Cells should be grown to reach 70-80% confluency. The advantage of utilising as many cells as possible ensures a concentrated CM and enrichment of EVs. HMCL are cultured in serum-free medium or EV-depleted culture medium (CM).

(b) For large-scale EV preparation, the use of two-compartment bioreactor CELLine tank systems can be employed to achieve continuous culture and high EV yields (in mg quantities). The morphology, phenotype and function of these exosomes has been shown to be identical to traditional flask culture methods [94].

(c) Wash confluent cells three times with 30 mL of serum-free RPMI-1640 media or sterile PBS and culture for 24 h in 35 mL of serum-free medium of EV-depleted medium (see **Note 17**). Cell viability should be assessed (trypan blue) before proceeding to cell wash and at CM collection (24 h).

(d) CM is collected under sterile conditions, transferred to 50 mL polypropylene centrifuge tubes and centrifuged at 4 °C (500 x *g* for 5 min followed by 2,000 x *g* for 10 min) to remove intact cells and cell debris. Note to retain the supernatant media and leave ~half a centimetre of liquid above the pellet. Separate tubes should be used following each centrifugation stage. This supernatant contains both soluble and membrane vesicle components.

(e) CM storage: Short-term on ice (within 3 days), long-term up to 6 months -20 °C.

35. Commercially available kits can be used (with optimization for CM volume needed) to isolate small EVs, although our (unpublished) data show that OptiPrep™ density gradient separation provides isolation of EVs with higher purity. See refs [85,94] for protocol.

36. NTA is a high-throughput technique utilized to determine the size and the concentration of EV samples. Despite its debatable accuracy, complementing EV characterization with NTA analysis is important to confirm the homogeneity of small EV samples (single peak at 50-200 nm). NTA can be used in a fluorescent mode making it capable of providing specificity for labelled particles. NanoSight and ZetaView are commonly used NTA devices. Differences in their hardware and software have been shown to affect measuring results: NanoSight provides size measurements of higher resolution while ZetaView provides a more accurate and repeatable analysis of EV concentration [127].

37. Small EVs that have been purified should be used to obtain high quality electron micrographs without storage at either -20 °C or -80 °C. Note that EM should be performed within one week of EVs purification.

38. For TEM analysis, small EVs are chemically fixed and negatively stained using uranyl acetate. Whole mounts are extremely useful for morphological analysis (e.g. size, shape, density). Nevertheless, the visualisation of EVs by TEM suffers mostly from two major challenges: lack of contrast and preservation of vesicle morphology. If the samples are not highly pure, it can be difficult to distinguish between proteins, small EVs and other vesicles. Some whole-mount preparations yield "cup-shaped" EVs, suggested to be an artefact caused by sample dehydration. Indeed, TEM preparation steps interfere with an important feature of EVs, that of size. Cryo-EM can be employed as an alternative as it does not use staining or chemical fixation procedures and samples are directly applied onto an EM grid, vitrified and visualized. Cryo-EM of vitrified whole cells or EVs enables observation of biological structures in a near-native state. Further, cryo-EM allows tomographic data collection and the ability for spatial visualization of more complex structures[70,94,128].

39. EM can be combined with immunolabeling techniques which enable the identification and localisation of immunological epitopes on the external surface of EVs [129].

40. For efficient reduction, alkylation and digestion, pH of the MS SDS lysis buffer should be adjusted between 7.9-8.0 immediately prior to use. This also applies to enzymatic (tryptic) digestion.

41. Avoid exposure to moisture when storing DTT. IAA should be protected from light during storage, stock preparation and alkylation reaction to prevent degradation of the IAA. To avoid non-specific alkylation, allow both sample and ThermoMixer to cool down to RT before adding IAA [130]. Several solutions (i.e., TFA and formic acid) are corrosive and should be prepared in a fume hood, with appropriate PPE. This buffer is stable for >3 months at RT.

42. We recommend diluting the trypsin solution in ice-cold 50 mM TEAB (pH 8) immediately before enzymatic digestion if dealing with small amounts of starting material. Other proteases can be used in addition to or replace trypsin (i.e. Lys-C, Arg-C). To ensure optimal peptide digestion, adjust the working pH and the temperature of reaction accordingly when using other proteases for protein digestion [131,132].

43. StageTips can be prepared in advance and stored in a covered pipette-tip box for several months at RT. Commercially available alternatives may be used; however, buffers may need to be adapted. Ensure that the discs are securely wedged in the bottom of the tip (i.e., no gap between inserts), without using too much force to compress StageTip material into the pipette tips as this increases the time required for sample/solvent to flow through. Slow-flowing StageTips can be used but may require higher centrifugation speeds for longer duration (see **Note 44**).

44. If a StageTip flow is slow or stops entirely during capture, lipid or other insoluble material may have been present before enrichment. This should be avoided, but can be resolved by centrifugation of peptide sample (2,000 × *g*, 15 min, RT) and careful transfer of supernatants to prepared StageTips.

45. Ensure compatibility of buffers and reagents with the peptide assay. If peptides have been tandem mass tag (TMT) labelled, a colorimetric (e.g., Life Technologies, #23275) alternative must be used being the fluorescence peptide assay not suitable.

46. Softwares such as current MaxQuant release (http://maxquant.org) for analysing raw proteomics data, installed on a suitable workstation or server. For in-depth details on MaxQuant setup and minimum system requirements, and analysis pipelines including Perseus (https://maxquant.net/perseus/) see [133-139].

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Figure 1. Protocol for isolation and characterization of PBPL-derived EVs

(a) Multiple myeloma cell clones secrete various factors into the local tumor microenvironment and circulation, including EVs, CTCs, and cf-DNA/RNA. Therefore, liquid biopsies are able to capture the spatial and temporal heterogeneity of tumors which is often underestimated by single site tissue biopsy. Blood, a complex bio-fluid, comprises HAP (e.g. albumin) and platelet derived EVs which represent major challenges in the pre-analytical and purification steps prior to omic approaches to define the composition of EV cargo.

(b) Strategy to isolate and characterize small EVs from blood is provided. Isolation of small EVs from 1 mL of PBPL utilizing a resin-based approach is shown (refer to Methods 3.1-3.4). Biochemical and biophysical characterization of EVs can include western blotting (EV marker proteins), nanoparticle tracking analyses (particle detection, particle size distribution) and transmission electron microscopy (EV size, morphology), proteomic-based profiling of EVs cargo, with analysis and informatic approaches depending on the question and strategy. It is important to deplete albumin or other highly abundant proteins which may be co-purified during EV isolation due to MS identification issues.

[EVs = extracellular vesicles; CTC = circulating tumor cells; cf-DNA/RNA = circulating cell free DNA/RNA; HAP = highly abundant proteins; PPP = platelet-poor plasma; PFP = platelet-free plasma; PBPL = peripheral blood plasma; MS = mass spectrometry; nLC = Nano-scale liquid chromatography; RT = room temperature]