Purification Protocols for Extracellular Vesicles

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Running Title: Purification of EVs

i. Summary

The following chapter contains a description of some of the standard methods used for the isolation of extracellular vesicles (EVs) from a variety of biological fluids, including cell culture media, urine, plasma and serum. The methods presented include ultracentrifugation, ultrafiltration, proprietary polymer-based reagents, size exclusion chromatography, density gradient separation and immunoaffinity capture. Ultracentrifugation methods use high speed centrifugation to pellet vesicles, whilst polymer-based reagents are added to the sample to facilitate vesicle precipitation using lower speeds. Ultrafiltration involves the concentration of vesicles from a large volume of biological fluid using a centrifugal filter unit. Size exclusion chromatography and density gradient separation are both designed to allow the separation of vesicles from other non-vesicular debris. Immunoaffinity capture methods use antibody-coated beads to selectively isolate vesicles displaying a surface marker of interest. Ultimately, the choice of purification method for an individual experiment is influenced by time, cost and equipment considerations, as well as the sample requirements for any downstream analyses.

ii. Key words

Extracellular vesicles, exosomes, microvesicles, isolation

1. Introduction

Exosomes and microvesicles are sub-micron membranous particles shed from cells into circulation, and are collectively referred to as extracellular vesicles (EVs). Exosomes have garnered particular interest and are small (approximately 30-100 nm), relatively homogenous and endosomally derived [1]. Microvesicles span a larger size range (100-1000 nm) and are directly shed from the plasma membrane [1]. EVs have been shown to participate in many normal and aberrant physiological processes [2]. For example, cancer cells have been shown to constitutively release exosomes into the circulation, and these promote several disease-associated processes including angiogenesis [3], establishment of the pre-metastatic niche [4] and mediation of organ-specific metastasis [5]. Additionally, there is substantial evidence that circulating EVs contain disease-associated mRNA,

miRNA and protein species, endearing them as a potential biomarker source [6,7]. As such, there is considerable interest in studying the physical and molecular characteristics of EVs in order to utilise them for diagnostic or therapeutic purposes. Such analyses first require that extracellular vesicles be enriched and isolated from the surrounding biological material, which is a complex mixture of cells and cellular debris, protein, nucleic acids and lipids [8]. Depletion of these background contaminants is especially critical where nucleic acid or protein analysis are to be performed, such that low abundance EV-associated species may be detected [9,8,10].

As exosomes have been the focus of much of the current EV research, EV isolation protocols are predominantly directed towards enrichment of these small (≤ 100 nm) vesicles (See Section 3.1). These isolation protocols vary greatly in their cost and equipment requirements, processing time, type and volume of input material and the purity of the isolated material (Table 1). For example, ultrafiltration and proprietary precipitation-based reagents allow rapid enrichment of all EVs in a sample, however, the purity of this material is generally low due to the presence of co-isolated protein contaminants [11]. Density gradient and immunoaffinity techniques are low throughput and require longer processing times, however, they produce more pure yields and may allow separation of EV subpopulations [12].

The following chapter will detail the standard protocols for six of the most commonly used techniques for isolation of vesicles from urine, cell culture media and plasma or serum samples. These standard protocols may be adapted for a variety of sample sources and input volumes. Ultimately, determination of the most appropriate protocol/s for an individual experiment will depend on the sample characteristics, as well as the nature and requirements of any downstream analyses.

2. Materials

All methods require standard 1x PBS prepared using deionized or ultrapure (e.g. MilliQ) water. It is recommended that PBS be filtered to 0.22μm to eliminate contaminants prior to use.

2.1 Ultracentrifugation

1. Ultracentrifuge with compatible fixed angle or swinging bucket rotor.
    a. Benchtop models: Beckman Coulter Optima MAX-XP, MAX-TL or similar
    b. Floor models: Beckman Coulter Optima L-series, XPN-series or XE-series

2. Polycarbonate, polypropylene or similar tubes capable of withstanding forces ≥ 100,000 x g and that are compatible with selected rotor and sample input volume. Table 2 shows some example centrifuge, rotor and tube configurations for a variety of volumes. All listed components are available from Beckman Coulter (CA, USA, see Note 1).

2.2 Ultrafiltration

1. Filter columns with 10-100 kDa molecular weight cutoff (MWCO), with capacity appropriate to sample volume, e.g:

    a. Amicon Ultra Columns (Merck Millipore, Germany), available in 0.5, 2, 4 and 15 mL capacities
    b. VivaSpin Centrifugal Concentrators (GE Healthcare Life Sciences, MA, USA) available in 0.5, 2,4, 6 and 20mL capacities

2.3. Proprietary polymer-based reagents

One of the following, compatible with the sample type of interest:

1. Invitrogen Total Exosome Isolation Kit (Life Technologies, USA) for cell culture media or for serum;

2. ExoSpin Exosome Purification Kit (Cell Guidance Systems, USA) for cell culture media/urine/ saliva and other low protein biological fluids, or for blood sera/plasma;

3. ExoQuick Exosome Precipitation Solution (System Biosciences, USA) for tissue culture and urine, or for biofluids (serum, ascites).

2.4 Size Exclusion Chromatography (SEC)

1. qEV Size Exclusion Column (Izon Science, UK) (See Note 2)

2. PBS with a bacteriostatic agent for column storage e.g 20% Ethanol, <0.05% w/v Sodium Azide

2.5 Density Gradient Centrifugation

1. Optiprep Density Gradient Medium (60% w/v Iodixanol, Sigma-Aldrich, MO, USA)
2. Tris-Sucrose Buffer: 0.25M Sucrose, 10mM Tris, pH 7.5
3. Ultracentrifuge with swinging bucket rotor (see Section 2.1)
4. Centrifuge tubes capable of withstanding forces ≥ 100 000 x g (see Section 2.1)
5. Spectrophotometer and compatible cuvettes (if required)

2.6 Immunoaffinity Isolation

1a. Exosome-Streptavidin Isolation/Detection Reagent (Thermo Fisher, MA, USA) and biotinylated antibody against vesicle surface marker of interest (see Note 3); or

1b. Exosome-Human CD9, CD81, CD63 or EpCAM Isolation Reagent (Thermo Fisher, MA, USA)

2. Magnetic Separator. DynaMag®-2 (Thermo Fisher, MA, USA)

3. Mixing/rocking device or sample shaker

4. Isolation Buffer: PBS + 0.1 % BSA, filtered to 0.22µm

5. Lysis buffer appropriate for downstream analysis

a. For downstream protein analysis. RIPA buffer: 150 mM sodium chloride, 1.0% Triton X-100 or NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0, protease inhibitor cocktail (prepare as per individual manufacturer specifications).

b. For downstream RNA analysis: TRIzol reagent (Thermo Fisher, MA, USA). See Note 4.

3.1 Ultracentrifugation

Ultracentrifugation is generally considered the 'gold standard' method for vesicle isolation, and is arguably the most commonly used method for this purpose [13]. This technique separates extracellular vesicles from other sample components based on their differential sedimentation rates, which are primarily affected by size and density [14]. The technique involves first pelleting cellular debris under slow (2 000 – 5 000 x g) centrifugation, then pelleting larger vesicles (apoptotic bodies and some larger microvesicles) at a faster (10 000 – 20 000 x g) centrifugation speed [15]. Small vesicles (exosomes and smaller microvesicles) can then be pelleted under very high speed (100 000 x g) centrifugation. It is worthwhile to note that due to the overlapping size profiles of exosomes (30-100 nm) and microvesicles (100-1000 nm), the separation of these two vesicle classes by differential centrifugation will invariably be incomplete, and the final pellet will contain a population of EVs secreted by different cellular mechanisms [14].

The following protocols have been adapted from those originally described by Thery et al. [16]. The standard ultracentrifugation protocol detailed below is suitable for cell culture media, urine and other low-protein fluids. For processing plasma and serum, there are minor differences to centrifugation conditions (see Note 5). At each step of the protocol, the alternate conditions for these high protein samples are noted inside square brackets [ ].

1**.** For plasma and serum samples, dilute sample 1:2 in PBS before beginning protocol.

2. Centrifuge sample at >1000 x g for 5-10 minutes at 4° [> 1 000 x g for 5-10 minutes] to remove residual cells. Discard pellet and transfer supernatant to new centrifuge tube (see Note 6).

3. Centrifuge sample at 2000-5000 x g at 4°C for 10-20 minutes [2 000 x g for 30 minutes] to remove dead cells and large debris. Discard pellet and transfer supernatant to new centrifuge tube.

4. Centrifuge sample at 10 000 x g for 30 minutes [12 000 x g for 45 minutes] to pellet small debris and larger vesicles. Transfer supernatant to ultracentrifuge tube/s (see Note 7). Retain pellet if analysis of larger vesicles is desired, else discard.

5. Centrifuge sample at 100 000 x g for 1-2 hours [110 000 x g for 2 hours]. Discard the supernatant, the pellet contains the small extracellular vesicles.

6. The pellet may be directly resuspended in a convenient volume of PBS (50-200µL) for downstream use.

6a. Optional wash step to remove contaminating proteins: resuspend pellet in 1 mL PBS and centrifuge for 1-2 hours at 100 000 x g as per step 5. For further depletion of contaminants, this wash step may be performed twice (see Note 8). Resuspend the final pellet in a convenient volume of PBS (50-200µL).

7. The isolated vesicles may be stored at -80 °C long term (up to one year).

3.2 Ultrafiltration

Ultrafiltration is commonly used as a first step to concentrate vesicles from a large volume of starting material (e.g. > 100 mL cell culture media or urine) into a small, more manageable volume (typically 1-2 mL) which can then be subjected to further purification protocols (e.g. ultracentrifugation, size exclusion chromatography). Typically, the molecular weight cut off (MWCO) for ultrafiltration columns used for vesicle concentration is 50 – 100 kDa. Using liposomes as a model vesicle system as described in [17], we have demonstrated successful vesicle concentration by ultrafiltration using filter MWCOs of 10, 50 and 100 kDa respectively (Figure 1).

1. Remove debris from sample as described the ultracentrifugation protocol (Section 3.1, steps 2-4). It is recommended to dilute plasma and serum at least 1:2 in PBS (or similar buffer) before filtration.
2. Assemble the ultrafiltration unit and place into/onto a flowthrough collection tube.
3. Load sample into the assembled ultrafiltration column. If sample volume is larger than column capacity, sample can passed through the column in multiple sequential aliquots to concentrate (see Notes 9 and 10).
4. Centrifuge column to draw fluid through filter. Tables 3 and 4 give the centrifugation conditions required for each capacity column with either a fixed angle or swinging bucket rotor. Begin by

centrifuging the column for 10 minutes, and adjust centrifugation time for subsequent runs as required to produce appropriate concentrate volume (see Note 11).

5. After each centrifugation step, discard column flow through. The vesicles will be retained in the concentrate above the filter.
6. Repeat step 3 and 4 until entire sample volume has been processed.
7. Recover concentrate either by directly pipetting from the filter unit (Vivaspin columns, 4 & 15 mL Amicon Ultra columns), or by inverting filter unit into a fresh collection tube and centrifuging at 1000 x g for 2 minutes (other Amicon Ultra columns).
8. The recovered concentrate can be subjected to further purification protocols as required (see Note 12). Concentrate may be stored long term (up to one year) at -80°C.

3.3 Proprietary Polymer-Based Reagents

There are several proprietary polymer based reagents which are commercially available for use in EV isolation. The exact mechanism of these reagents has not been reported, however, they are designed to facilitate precipitation of vesicles during low-speed (≤ 20 000 x g) centrifugation. These reagents are said to simplify the process of vesicle isolation and avoid the need for specialised ultracentrifugation equipment. Despite these advantages, there is some evidence to suggest that these reagents may co-precipitate non-vesicular debris to a higher degree than other techniques [11,18], and therefore may not be ideal where downstream proteomic or nucleic acid analysis is to be performed. The following section will focus on three of the most commonly used reagents: Invitrogen Total Exosome Isolation Kit (Life Technologies, CA, USA), ExoSpin Exosome Purification Kit (Cell Guidance Systems, MO, USA) and ExoQuick Exosome Precipitation Solution (System Biosciences, CA, USA).

1. Remove debris from sample as described by the manufacturer protocol or as described in the ultracentrifugation protocol (Section 3.1, Steps 2-4. See Note 13).
2. Add the appropriate volume of reagent to each sample (see Table 5).

3. Incubate sample at 4°C for the time period recommended by the manufacturer (see Table 5, and Note 14). It is not necessary to agitate the sample during incubation.
4. Centrifuge sample at 4°C at the speed and duration recommended by the manufacturer (see Table 4, and Note 15).
5. Remove and discard the supernatant. Resuspend the pellet in a convenient volume of PBS (50-200µL).

Steps 6-8 are for the ExoSpin kits only

6. Remove top and bottom caps from spin column. Equilibrate column by centrifuging at 50 g for 15-30 seconds, applying 200 µL PBS to column and repeating centrifugation.
7. Apply EV sample to prepared column and centrifuge at 50 x g for 60 seconds. Discard the eluate.
8. Place column into collection tube, apply 200 µL PBS and centrifuge at 50 x g for 60 seconds. The eluate contains vesicles.
9. Isolated vesicles may be stored at -80°C long term (up to one year).

3.4 Size Exclusion Columns

The following protocol is specifically for the qEV size exclusion column (Izon Science, UK). Size exclusion allows separation of sample components based on the differential rate of movement through a gel matrix of different sized components [19]. The smaller a component, the more it is able to penetrate into the gel matrix, the longer it is retained in the column and the later the elution time [19]. In the context of EV isolation, this means that the vesicles may be separated from smaller protein and nucleic acid contaminants [20]. The qEV column has been previously used for EV isolation, and is reported in the literature to produce yields depleted from contaminants and enriched in EV markers [9,21].

1. Remove debris from sample as described in the ultracentrifugation protocol (see Section 3.1, Steps 2-4). For samples with a low starting vesicle concentration (e.g. cell culture media, urine), concentrate vesicles to ≤ 1mL volume using one of the previously described methods (see Section 3.1-3.3).

2. To prepare column for use, secure in an upright position using a retort stand or similar. Ensure that there is sufficient space underneath the column to place collection tubes.
3. Remove the top cap of the column by pinching it inwards and lifting it slowly and carefully. Do not remove the bottom cap until ready to start collecting flow through.
4. Equilibrate the column by passing through at least 10 mL of buffer (PBS or similar). Apply this to the top of the column in multiple 1-2 mL aliquots, ensuring that the top of the column never runs dry (see Note 16).
5. Measure and record the time taken for 5 mL of buffer to pass through the column. This will serve as a reference to indicate when column needs to be cleaned. Typically this time is around 5 minutes for a clean column.
6. When ready to process the sample, replace the bottom cap and carefully remove any buffer from the top of the column. Pipette sample in, and remove bottom cap when ready to begin collecting.
7. The first 3 mL to elute from the column is the void volume, and this will contain molecules which are too large to enter the gel matrix (> 1 μm). This can be collected as 6 individual 0.5 mL fractions if desired, or as one 3 mL fraction.
8. The vesicles will begin to elute after the void volume has passed through. Collect a further 3 or 4 x 0.5 mL fractions and retain. Top up column with buffer as necessary during elution, but not until the sample has completely entered the column matrix. The vesicles should be in the first 1-1.5mL to elute after the void volume (see Note 17).
9. Wash column with 10 mL of buffer. Measure the time taken for 5 mL of buffer to pass through the column. If column is clean and not compromised, this should be comparable to the measurement taken before sample processing.
10. The presence of vesicles in each fraction can be determined by physical characterisation techniques such as tunable resistive pulse sensing (TRPS, see [22] for technique details) or nanoparticle tracking analysis (NTA, see [23] for technique details). Sample purity and protein contamination may be assessed by performing SDS PAGE with a Coomassie stain and/or a Western blot for vesicle markers (e.g. CD63, CD81, flotillin).

11. The vesicle-containing fractions may be pooled for further downstream analysis. Samples may be stored at -80 °C for up to one year.

3.5 Density Gradient Centrifugation

Density gradient centrifugation is a method which separates vesicles from other contaminants based on their buoyant density, which for exosomes is estimated to be 1.13-1.19 g/mL [1]. A discontinuous gradient is formed by layering solutions of different densities, and the sample overlaid on top. The column is centrifuged at high speed (100 000 x g) overnight (~16-18 hours) to separate out sample components. This method has been found to produce purer yields than ultracentrifugation alone, and can be used to 'clean up' preparations of crude vesicle concentrates produced by ultrafiltration, ultracentrifugation or other methods [24,18]. It has been noted, however, that this method cannot separate abundant high density lipoprotein (HDL) particles from extracellular vesicles in plasma and serum samples, and may therefore not be an ideal method for this sample type [20]. Further, this method is associated with a loss of vesicle yield during processing [8].

1. Prepare 40%, 20%, 10% and 5% iodixanol solutions by diluting the 60% w/v Optiprep stock solution with Tris-Sucrose buffer. Table 6 shows the volumes of stock solution and buffer required to produce 12 mL of each concentration.
2. Layer 3 mL of each of the 40%, 20% and 10% solutions and 2.5 mL of the 5% solution in a 13-15 mL ultracentrifuge tube (see Note 18).
3. Overlay 500 µL of the sample on top of the gradient. This should be a pre-processed concentrated sample, produced by ultracentrifugtion (see section 3.1), ultrafiltration (see section 3.2) or a polymer-based reagent (see section 3.3), and reconstituted to approximately 500µL with PBS or similar buffer.
4. Prepare a second blank gradient as per step 2, and overlay 500 µL of Tris-Sucrose buffer in place of the sample.
5. Centrifuge both sample and blank gradients at 100 000 x g for 16-18 hours, in a swinging bucket rotor (see Table 2) .

6. Following centrifugation, collect 12 x 1 mL fractions from top to bottom (i.e. with increasing density) from sample and blank gradients. Ensure that each collected fraction is clearly labelled.
7. Using the fractions from the blank gradient, calculate the density of each fraction by diluting the solution 1:10 000 and measuring the absorbance of the solution at 244 nm with a spectrophotometer (see Note 19).
8. Centrifuge the fractions collected from the sample gradient at 100 000 x g and 4^0C for 1-2 hours (see Section 3.1 for ultracentrifugation details). Resuspend each in 50-100 µL PBS.
9. The presence of vesicles in each fraction can be determined by physical characterisation techniques such as tunable resistive pulse sensing (TRPS, see [22] for technique details) or nanoparticle tracking analysis (NTA, see [23] for technique details). Sample purity and protein contamination may be assessed by performing SDS PAGE with a Coomassie stain and/or a Western blot for vesicle markers (e.g. CD63, CD81, flotillin).
10. Vesicle-containing fractions may be pooled for further downstream analysis. Samples may be stored at -80°C for up to one year.

3.6 Immunoaffinity Isolation

Immunoaffinity isolation allows the separation of EV subpopulations based on the expression of surface markers. Pre-concentrated EV samples are incubated with magnetic beads coated with an antibody against the target marker, allowing the specific pulldown of EVs expressing this marker on their surface. There are commercially available beads pre-coated with a general exosome surface marker (e.g. CD81, CD9, CD63), and these are designed to allow separation of exosomes from other co-isolated EVs. In addition, there are several reports of the purification of EVs expressing cell-specific target markers, allowing the isolation of a subpopulation of EVs derived from a particular cell type [25,12]. The advantage of this method is that it produces pure and homogenous EV yields [26]. This method by design, however, excludes the capture of potentially biologically relevant subpopulations which do not express the particular surface marker of interest.

Steps 1- 8 are for the Exosome-Streptavidin Isolation reagent only

1**.** Resuspend streptavidin-coated beads, either by vortexing for 30 seconds or by placing tube on a mixer/shaker for at least 10 minutes.

2. Transfer 1mL ($1x10^7$) of beads to a new tube (see Note 20 and 21).

3. Place the tube on a magnet separator for at least 1 minute. Aspirate the supernatant and discard, then remove tube from magnet.

4. To wash beads, resuspend in 1 mL isolation buffer, return tube to magnet and aspirate supernatant.

5. Remove tube from magnet, resuspend beads in 1 mL isolation buffer, and add an appropriate amount (typically ~4 µg) of biotinylated antibody. Mix well, then incubate for 30-60 minutes at room temperature under gentle agitation. The recommended speed for a shaker is 650 rpm.

6. Place tube on a magnet separator for at least 1 minute. Aspirate the supernatant and discard.

7. To wash beads, remove tube from the magnet, resuspend beads in 1 mL isolation buffer. Place tube on magnet for at least 1 minute, aspirate the supernatant and discard. Repeat twice, for a total of three washes.

8. Remove tube from the magnet, resuspend antibody-coupled beads in 1 mL isolation buffer.

Steps 9-16 apply to all reagents

9a. For Exosome-Streptavidin or Human CD63 Isolation reagent: Resuspend bead mixture by vortexing for 30 seconds or by placing on a mixer for at least 10 minutes. Transfer 100 µL of this mixture to a new round or flat-bottomed tube.

9b: For Exosome-Human CD9/CD81/EpCam Isolation reagent: Resuspend beads as per step 9a. Transfer 40µL of this mixture to a new round or flat-bottomed tube.

10. Reconstitute concentrated EV sample to a total volume of 100 µL using isolation buffer. The protein content of the sample should be approximately 25 µg, as determined by Bradford Assay or similar technique.

11. Add at least 500 µL of isolation buffer to the bead mixture and mix well. Place bead mixture on a magnet separator for at least 1 minute, aspirate the supernatant and discard.

12. Remove the bead tube from the magnet, and add 100 µL of reconstituted EV sample. Mix sample well, and incubate overnight (at least 18 hours) at 2-8 ^{0}C with gentle agitation (shaker at 650 rpm).

13. Centrifuge the sample tube for 3-5 seconds. For Exosome-Human CD81/CD9/EpCAM reagent, add 1 mL of isolation buffer and mix gently by pipetting. For Exosome-Streptavidin or Human CD63 Isolation reagent, add 300 µL isolation buffer and mix gently by pipetting. Do not vortex.

14. Place the sample on a magnet for at least 1 minute, aspirate supernatant and discard, taking extra care not to dislodge any beads from the side of the tube.

15. Remove tube from the magnet. For Exosome-Streptavidin or Human-CD63 reagent, add 400 µL isolation buffer and mix gently as above (see step 13). For Exosome-Human CD81/CD9 or EpCAM reagent, add 500 µL isolation buffer and mix gently as above (see step 13). Place tube on a magnet for at least 1 minute, aspirate supernatant and discard, again taking extra care not to dislodge beads.

16. Elute bound EVs from beads using a buffer appropriate for downstream analysis (see Note 22):

16a. For protein extraction: Add 15-20 µL RIPA buffer, mix well and incubate at 2-8^{0}C (or on ice) for 15 minutes to complete vesicle lysis. Magnetic beads can be retrieved by placing the sample on the magnet and withdrawing the supernatant. Add appropriate sample and/or loading buffers to the supernatant and proceed to gel electrophoresis.

16b. For RNA extraction: Add 1mL TRIzol reagent and vortex to homogenize sample. Magnetic beads can be retrieved by placing the sample on the magnet, if desired. Proceed with RNA extraction as per manufacturer instruction.

4. Notes

1. The list of rotors and tubes given in Table 1 is by no means exhaustive, there are numerous other rotor and tube configurations which have successfully been used for EV isolation. The Beckman Coulter website contains detailed and up-to-date information on centrifuge, rotor and tube compatibilities and it is recommended to check equipment details here before beginning any ultracentrifuge protocols.
2. The qEV column is a commercially available column developed for extracellular vesicle isolation (Izon Science, UK). Other size exclusion columns which have been used in EV experiments include the Hi-Prep Sephacryl S-400 HR column (GE Healthcare Life Sciences, MA, USA) [27] and Sepharose CL-2B (Sigma-Aldrich, MO, USA) packed in a syringe to a volume of ~10mL [8,28]. Details of the protocols for these columns can be found in the respective publications referenced above.
3. Isolation of intact exosomes have also been performed using antibodies coupled to Protein G Dynabeads® (Thermo Fisher, MA, USA). For a description of the associated protocol, please see [12].
4. There are numerous commercially available RNA extraction kits which could be used in place of a TRIzol-based method. Substitute TRIzol with the appropriate lysis buffer as per the kit instructions, and proceed as per manufacturer protocol. Eldh *et al*. [29] provide a comparison of different techniques for exosomal RNA extraction.
5. Other biofluids may require further modifications to the standard protocol. Ultracentrifugation-based protocols have previously been reported for ascites [30], human breast milk [31] and saliva [32] .
6. Performing the short (< 30 minute) centrifugation steps at 4°C is desirable but not necessary. In our experience, exosomes may be centrifuged at room temperature without substantial yield loss. However, for downstream applications which require intact functional exosomes (e.g. co-culture assays) or where RNA or proteomic analysis is to be performed, refrigeration during all centrifuge runs would be recommended.

7. Steps 1-3 of the ultracentrifugation protocol may be replaced by filtration to approximately 0.2μm. We have observed that this substitution does not lead to a substantial reduction in yield, provided that the sample volume is ≥ 2mL and the sample is not viscous.
8. Each repeat ultracentrifugation step is likely to result in some yield loss [14]. It is therefore important to consider both yield and purity requirements for isolated vesicles before deciding on the final protocol.
9. When centrifuging sample through filter in multiple aliquots, keep remaining sample on ice or at 4°C to avoid yield loss during processing.
10. The manufacturer recommendations suggest that filters should not be centrifuged more than 4-5 times as this may cause filter clogging and decrease filtration efficiency. For very large starting sample volumes, it may therefore be necessary to split the sample across multiple filter units and pool concentrates.
11. We recommend beginning with a 10 minute centrifugation speed and scaling duration up or down as necessary, as it is difficult to predict the exact behaviour of individual samples within each column. We have observed that samples with a high concentration of vesicles require a longer centrifugation times to achieve the same concentration factor as those with a lower amount of vesicles. Centrifugation times may therefore need to be adjusted for different individual samples of the same type (e.g. cell culture media from two different cell lines, urine from two different individuals). Additionally, the centrifugation time may need to be increased over subsequent centrifugation steps to produce the same concentrate volume, as filter efficiency is decreased with each centrifugation.
12. Ultrafiltration columns may also be used for buffer exchange of concentrated vesicle samples. For example, vesicles can be exchanged from phenol-red containing cell culture media to a phenol-red free buffer which will not interfere with downstream fluorescence assays. This process is similar to that described in section 3.2. The major difference is that following each centrifugation step, the sample is reconstituted in the new buffer and this is repeated until the original buffer has been removed.

13. Typically, manufacturer provided protocols for proprietary reagents will include several steps for clearing samples of contaminating debris. These are generally similar to what is described in steps 2-4 of the ultracentrifugation protocol, and either method may be used.
14. Extending the incubation time from that recommended by the manufacturer may slightly improve yields. Alternately, we have found that it is possible to reduce overnight incubation steps to 3-4 hours with only minor yield loss, however, this is not recommended for downstream applications where maximising vesicle, protein or nucleic acid input is critical (e.g. mass spectrometry, RNA sequencing).
15. We and others [11] have noted that increasing the centrifugation speed and/or time from that recommended by the manufacturer may improve yields.

    The standard ExoQuick-TC protocol calls for a 30 minute centrifugation at 1 500 x g. Alvarez and colleagues report an adapted method using a higher ratio of reagent to sample (from 1:5 to 1:3.33), and increasing centrifugation speed to 10 000 x g, which resulted in improved vesicle yield [11]. There is also a possibility that more non-vesicular debris may be co-precipitated during a longer or faster centrifugation step, and this should be considered for applications where sample purity is critical.
16. When using the column for the first time or after storage, be aware that the initial 5-6 mL to elute will contain 0.05% sodium azide and must be disposed of in accordance with local regulations.
17. The exact time for the vesicles to elute may vary. According to the manufacturer, peak elution is typically at 4 mL, but can occur anywhere between 3 and 4.5 mL.
18. The volumes given are for 13 -15 mL ultracentrifuge tubes. These volumes may be scaled up or down accordingly for use in different sized tubes.
19. Iodixanol has a molar extinction coefficient of 320 L g^{-1} cm^{-1} at this wavelength [33].
20. The protocol described here to conjugate 4µg biotinylated antibody to 1 mL (1 x 10^7) beads, as per manufacturer's recommendation. These volumes may be adjusted accordingly, however, it is recommended to keep the concentration of beads constant at 1 x 10^7 per mL to maximise the efficiency of the coupling reaction.

21. Other streptavidin coated Dynabeads may be used in place of the Exosome-Streptavidin Isolation reagent. The stock concentration of beads and their antibody binding capacity varies between individual bead types. The input of beads and antibody to the reaction will need to be scaled appropriately to account for this. We recommend starting at a bead concentration of 1 mg/mL during the reaction and adjusting as necessary.
22. The amount of material recovered after immunoaffinity isolation depends on a number of factors, including efficiency of antibody coupling to beads, composition of input sample and the method of elution. As such, achieving sufficient yields for downstream protein and RNA analysis is likely to require optimisation of conditions for each individual reaction.

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Figures and Tables

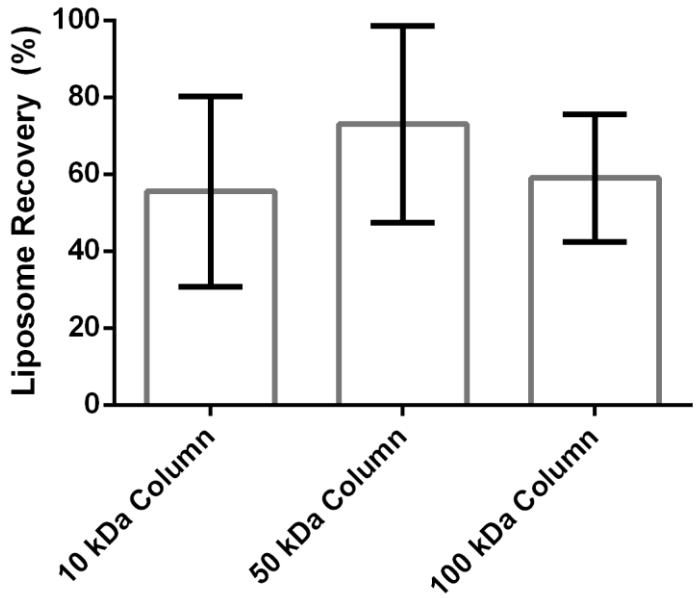

Figure 1: recovery (%) ± SD of liposomes (55:45 mol/mol 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC): cholesterol, mean size= 100 nm, *FormuMax, CA, USA*) spiked into cell culture media (RPMI1640, *Sigma Aldrich, MO, USA*) and concentrated using 10 kDa, 50 kDa or 100 kDa molecular weight cutoff (MWCO) Amicon Ultra-2 filtration columns. Recovered particle concentrations measured by tunable resistive pulse sensing (TRPS) and compared to input concentrations.

Table 1: overview of EV purification techniques, including the mechanism of separation of vesicles from other sample components, type and volume of input, approximate processing time, required equipment, sample purity and other considerations.

Method	**Mechanism of Separation**	**Input Type**	**Processing Time**	**Required Equipment**	**Purity of Isolates**	**Other Considerations**
Ultracentrifugation	Sedimentation Rate	Raw sample, any volume	3-6 hrs	Ultracentrifuge and compatible tubes	Low	May induce vesicle disruption [27]
Ultrafiltration	Size	Raw sample, any volume	1-3 hrs	Benchtop centrifuge	Low	Retains EVs in original fluid, may need to perform buffer exchange
Polymer-based Reagents	Sedimentation Rate	Raw sample, any volume	2-18 hrs	Benchtop centrifuge	Low	Not recommended for use with mass spectrometry
Size-Exclusion Chromatography	Size	Concentrated EVs, ~500µL volume	1-2 hrs	-	High	May separate vesicles from high density lipoprotein (HDL) particles [20]
Density Gradient Separation	Density	Concentrated EVs, ≤ 1 mL volume	20-24 hrs	Ultracentrifuge and compatible tubes	High	Co-isolates vesicles and HDL particles [34]
Immunoaffinity Isolation	Surface Marker Expression	Concentrated EVs, ~100 µL volume	18-20 hrs	Magnet separator, shaker	Very high	Allows targeting of specific subpopulations based on surface marker expression [12] but recovery may be low

Table 2: list of ultracentrifuge rotors and compatible tubes appropriate for use in extracellular vesicle isolation.

Centrifuge Type	**Rotor**	**Rotor Type**	**Rotor Max Capacity**	**Example Compatible Tube/Bottle**
Benchtop	TLA120.2	Fixed Angle	10 x 2 mL	Thickwall Polycarbonate tube 1 mL (11 x 34 mm)
Benchtop	TLA100.3	Fixed Angle	6 x 3.5 mL	Thickwall Polycarbonate tube 3.5 mL (13 x 57 mm)
Floor	Type 50.2 Ti	Fixed Angle	12 x 39 mL	Thickwall Polycarbonate bottle 26.3 mL (25 x 89 mm)
Floor	Type 70 Ti	Fixed Angle	8 x 39 mL	Thickwall Polycarbonate tube + cap (optional) 32 mL (25 x 89 mm)
Floor	SW32	Swinging Bucket	6 x 38.5 mL	Thinwall Polypropylene tube 38.5 mL (25 x 89 mm)
Floor	SW40	Swinging Bucket	6 x 14 mL	Thinwall Polypropylene tube 14 mL (14 x 95 mm)
Floor	SW41	Swinging Bucket	6 x 13.2 mL	Thinwall Polypropylene tube 13.2 mL (14 x 89 mm)

Table 3: recommended maximum centrifugation speed for Amicon Ultra 0.5, 2, 4 and 15 mL columns in swinging bucket and fixed angle rotors respectively. ^ For 4 mL columns in a fixed angle rotor, filtrate volume should not exceed 3.5 mL. ^^For 15 mL columns in a fixed angle rotor, filtrate volume should not exceed 12 mL.

Amicon Ultra Column Capacity	**0.5 mL**	**2 mL**	**4 mL^**	**15 mL^^**
Swinging Bucket	N/A	4 000 x g	4 000 x g	4 000 x g
Fixed Angle	14 000 x g	7 500 x g	7 500 x g	5 000 x g

Table 4: recommended maximum centrifugation speed for Vivaspin Centrifugal Concentrator 0.5, 2, 4, 6, and 20 mL capacity columns in swinging bucket and fixed angle rotors respectively. * indicates the adjusted speed for ≥ 100 kDa PES membranes. ł indicates the adjusted speed for CTA and HY membranes of all MWCO. ˆ For 20 mL columns in a fixed angle rotor, filtrate volume should not exceed 14 mL.

Vivaspin Centrifugal Concentrator Capacity	**0.5 mL**	**2 mL**	**4 mL**	**6 mL**	**20 mL ^**
Swinging Bucket	N/A	12 000 x g *9 000 x g ł 8 000 x g	10 000 x g *7 000 x g	10 000 x g *6 000 x g	8 000 x g * 6 000 x g
Fixed Angle	15 000 x g	4 000 x g	4 000 x g	4 000 x g	5 000 x g *3 000 x g

Table 5: reagent: sample ratio, incubation time and centrifugation conditions for Invitrogen Total Exosome Isolation kit, ExoQuick Exosome Precipitation Solution and ExoSpin Exosome Purification kit respectively.

Reagent	**Compatible Sample Type/s**	**Reagent: Sample Ratio**	**Incubation Time (see Note 14)**	**Centrifugation Speed**	**Centrifugation Time**
Invitrogen Total Exosome Isolation Kit (for cell culture media)	Cell culture media	1:2	Overnight	10 000 x g (see Note 13)	1 hour
Invitrogen Total Exosome Isolation Kit (for serum)	Serum, plasma	1:5	30 minutes	10 000 x g	10 minutes
ExoQuick- TC Exosome Precipitation Solution (for cell culture media and urine)	Cell culture media, urine	1:5	Overnight (minimum 12 hours)	1 500 x g	30 minutes
ExoQuick Exosome Precipitation Solution (for biofluids)	Serum, ascites, other biofluids	1:4	30 minutes (serum) Overnight (ascites)	1 500 x g	30 minutes
ExoSpin Exosome Purification Kit (for cell culture media)	Cell culture media, urine, saliva	1:2	At least 1 hour	16 000 x g (see Note 13)	1 hour
ExoSpin Blood Exosome Purification Kit (for plasma/serum)	Plasma, serum	1:2	5 minutes – 1 hour	20 000 x g	30 minutes

Table 6: preparation of 40, 20, 10 and 5% Iodixanol solutions (12 mL each) from Optiprep (60% w/v Iodixanol) stock and Tris-Sucrose buffer.

Iodixanol Concentration (w/v %)	**Optiprep Stock (mL)**	**Tris-Sucrose Buffer (mL)**
40	8	4
20	4	8
10	2	10
5	1	11