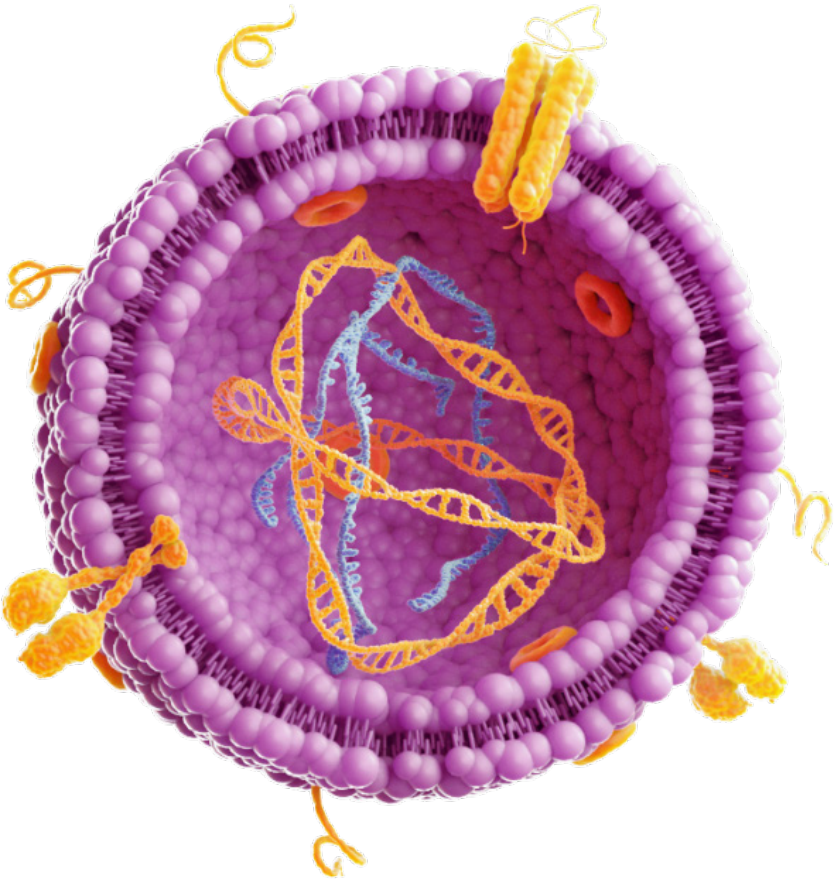
ISOLATING EVs FROM BLOOD PRODUCTS USING qEV COLUMNS



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INTRODUCTION

Extracellular vesicles (EV) are the broad classification of membrane-derived entities produced by a variety of activated and apoptotic cell types[1]. The term "EV" includes exosomes, microvesicles (sometimes called microparticles), oncosomes and other vesicles that are defined by their cellular origin, size, and surface markers[2]. They are used to transmit signals between cells, and their ability to transport molecules to specific target cell populations make them attractive tools for diagnostic and therapeutic development[3].

Although blood-derived EVs are a highly complex and heterogeneous population, comprising many EV types from multiple cellular origins, blood-derived EVs are valuable rich sources for biomarker discovery research, as many conditions/diseases can significantly impact the composition of circulating EVs.

Nevertheless, blood's pre-analytical (sample collection and processing) phase can be an important source of variability for the isolation of EVs. This is due in large part to the fact that blood cells, particularly platelets, can be activated easily and release abundant EVs during sample collection and processing[4]. Platelets can be activated by many of the molecules and conditions used in common blood processing procedures[4], so it is important that the sample collection and processing protocols used (A) prevent platelet activation, and (B) remove platelets prior to storage and analysis.

This technical bulletin provides general guidance, considerations, and recommendations on how to isolate EVs from blood products using Izon's qEV columns.

CONSIDERATIONS AND RECOMMENDATIONS

The detection and characterisation of blood-derived EV populations can be often confounded by artefacts that are generated during blood sample processing and EV isolation[5]. The collection process can alter the molecular integrity, function, and/or composition of biospecimens, so special considerations must be made when choosing the sample medium (whole blood, plasma, or serum) that will be assessed as well as the blood collection and processing method that will be used. Some of these considerations have been outlined below and are represented in the flowchart in Figure 1.

EV source

The EV source is an important consideration in the design of EV analytical studies. While some EV studies have been carried out using whole blood, it is extremely difficult to differentiate EVs from other abundant particulate matter, and this source precludes EV storage and isolation, so the number of applications is limited[5]. Plasma and serum are the predominant EV sources used.

Plasma is preferred over serum as the EV source due to factors like:

- Additional EVs are released during clot formation when preparing serum[5], which results in a sample that does not truly reflect the original EV composition.
- Platelet-derived EVs released after collection may account for a significant number of EVs present in serum[1], making it difficult to obtain a sensitive analysis of less abundant EVs.

Blood Collection

The blood collection conditions can greatly affect the quantity and characteristics of EV populations present in blood samples. Collection variables such as storage temperature, transportation state, storage time, anticoagulant, and centrifugation protocol should be kept constant for all samples, as each of these can impact the number of EVs present in processed samples[5,6].

- The choice of anticoagulant may affect downstream applications, see Table 1.
- Platelets may be activated by the physical force associated with venepuncture; the first 1-3 mL of blood collected should be discarded because of the activating effects of the tourniquet pressure and the contamination by fibroblasts[7].
- Preferably, no measurements of EVs in haemolysed samples should be done. If haemolysed samples are included, the obtained results should be interpreted with care and the degree of haemolysis should be measured[5].

Table 1: Anticoagulants Present in Blood Collection Tubes

ANTICOAGULANT	TUBE	MODE OF ACTION	EFFECTS ON EVs
Trisodium Citrate	Blue Top	Prevents blood from clotting by binding calcium	• Most commonly used anticoagulant; recommended by the International Society on Thrombosis and Haemostasis[5]
EDTA	Purple Top	Prevents clotting by binding calcium	• May activate platelets[6] • Suitable for RNA analysis[5]
Sodium Heparin	Green Top	Prevents clotting by inhibiting thrombin and thromboplastin	• Not generally recommended for EV studies • May activate platelets[6] • May block the EV uptake by other cells[7] • Interferes with PCR reaction[5]
Acid Citrate Dextrose (ACD)	Yellow Top	Prevents clotting by binding calcium	• Affects osmotic balance of erythrocytes, which may lead to changes in EV composition/release[6] • Recommended for analysis of microparticles/ microvesicles[8] • Acid citrate dextrose (ACD) and CTAD prevent platelet activation and the release of platelet EVs more efficiently than citrate[5]

Sample Processing

- After the blood has been collected, it is centrifuged to remove most circulating cells, like erythrocytes and leukocytes, as these cell types can release EVs into the blood after collection. It is important to limit the amount of time between collection and the first centrifugation step as much as possible; ideally this should be less than 30 minutes, but no more than 60 min[6].

- As platelets are smaller than other blood cells, they require specific conditions for their removal. If platelet-derived EVs are not of interest, platelet activation (release of EVs) must be carefully avoided whilst they are still present in the sample: cold can activate platelets, thus centrifuge samples at room temperatures[5]; shear can activate platelets, thus samples should be kept upright and avoid transport stress and agitation[5].

Pre-EV isolation Storage

Unless immediate plasma/serum derived EV isolation and analysis is possible, short- or long-term sample storage will be required.

- All samples should be stored at the same temperature and for the same period of time to limit effects on EV functionality.

- Storage at -80°C, has shown best results to maintain EV numbers[6,9].

- MicroEVs may increase in numbers with cold storage in Platelet Rich and Platelet Poor Plasma samples[9].

- Frozen samples should not be compared to fresh samples[6].

- Freeze thaw cycles should be kept to a minimum[5,6].

qEV Isolation

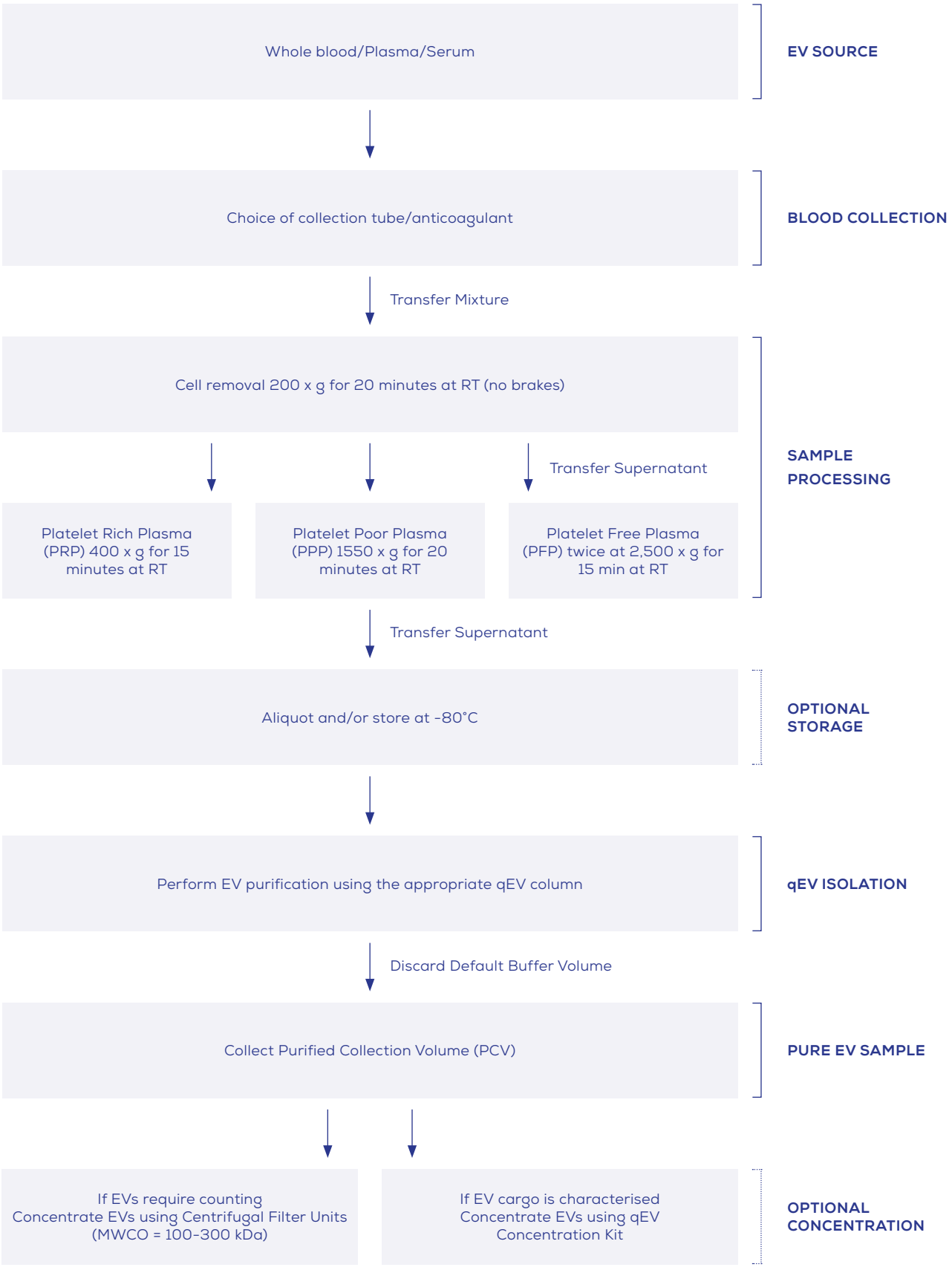
- There is a range of qEV isolation columns suited to different EV size ranges and sample volumes to match various research needs for blood-derived EVs.
- The new Gen 2 qEV columns are made with a proprietary agarose resin, which delivers a more purified extracellular vesicle (EV)-containing eluate, removing more contaminating protein than Legacy (containing previous resin) qEV columns.
- Both Legacy and Gen 2 are available now in the existing 35 nm and 70 nm series, which have optimum recovery ranges of 35-350 nm and 70-1000 nm, respectively. More information on available qEV columns normally used for blood EVs can be found in Table 2.

Table 2. Different qEV columns available to use with common volumes of plasma/ serum samples.

INPUT qEV VOLUME	qEV VOLUME	PURIFIED COLLECTION VOLUME (PCV)
150 µL	qEVsingle[a]	Legacy – 600 µL
500 µL	qEVoriginal[b]	Legacy – 1.5 mL Gen 2 – 1.6 mL
1 mL	qEV1[c]	Gen 2 – 2.8 mL
2 mL	qEV2[a]	Legacy – 6 mL

a / Legacy only, b / Gen 2 and Legacy, c / Gen 2 only

Figure 1: Flowchart summarising considerations for isolation of EVs from blood products using qEV platform.



MATERIALS

- Two evacuated blood collection tubes
- One ≥ 21-gauge needle
- Personal protective equipment
- Tourniquet
- Blood transfer device
- Puncture resistant sharps container
- Centrifuge with swing-out rotors capable of spinning at 2,500g for 15 min.
- 1000-µL pipette
- 200-µL pipette
- Fresh 1X PBS Solution
- Sterile 0.22 µm syringe filter
- Sterile syringe
- Izon qEV column

METHODS

Protocol for blood collection from an adult human subject and processing for EV isolation.

1. Collect blood by applying a light tourniquet and venepuncture the antecubital vein with a ≥ 21-gauge needle.
2. If possible, discard the first 2-3 mL of blood.
3. Collect required blood in chosen blood collection tube, considering that approximately 50% of blood volume corresponds to plasma.
4. Gently invert the tube 8-10 times to mix the blood with anticoagulant or components in the tube.
5. Allow the tubes to incubate at room temperature (20-24°C) for 10-20 min, but no more than 60 min.
6. Centrifuge tubes at 200 x g for 15-20 minutes at room temperature (RT)
    a. Make sure that the brake has been turned off.
    b. Use a centrifuge with a swing-out rotor as opposed to fixed rotors.
7. Transfer supernatant to a clean tube and centrifuge at appropriate speed and time to remove desired amount of platelets (refer to Figure 1).
8. Collect plasma/serum into a new tube using a 200 uL pipette, drawing from the top of the tube, and leave approximately 100 µL of plasma/serum at the bottom of the tube.
9. Store plasma/serum sample at -80 °C or liquid nitrogen.

EV Isolation with qEV

1. Prepare fresh 1X PBS solution and filter using a sterile 0.22 µm syringe filter

2. Equilibrate the qEV column with room-temperature PBS solution

    a. Degassed and room temperature buffers will help to avoid air bubbles forming in the gel bed.

3. Thaw plasma/serum sample and load an appropriate volume of sample onto the loading frit of the qEV column.

    a. Be sure that the volume of the sample is appropriate for the type of qEV column used (refer to Table 2 for information).

4. Begin collecting Default Buffer Volume and then Purified Collection Volume as directed in the qEV or AFC user manual.

    a. Different samples may give slightly different elution profiles and purity, hence an initial measurement of EV concentration and protein contaminants in collected volumes is recommended.

5. After all volumes have been collected, flush the column with recommended volumes of 0.5 M Sodium Hydroxide (NaOH) followed by PBS buffer, found in each specific qEV user manual.

    a. It is recommended to flush the column with buffer containing a bacteriostatic agent (e.g. 0.05% w/v sodium azide) prior to storage.

6. Store the column at 4-8°C.

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Izon Science publishes this method as a service to investigators. Detailed support for non-qEV aspects of this procedure might not be available from Izon Science.

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