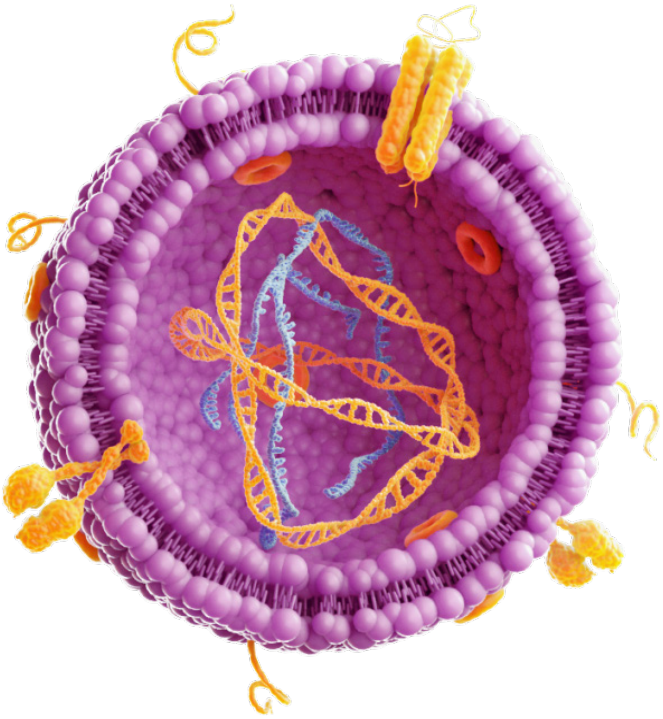
ISOLATING EVs FROM BLOOD PRODUCTS USING qEV



TECHNICAL NOTE

T006-01E

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CONTENTS

INTRODUCTION

Extracellular vesicles (EV) are the broad classification of membrane-derived entities produced by a variety of activated and apoptotic cell types[4]. The term "EV" includes exosomes, microvesicles (sometimes called microparticles), oncosomes and other vesicles that are defined by their cellular origin, size, and surface markers[6]. They are used to transmit signals between cells[5], and their ability to transport molecules to specific target cell populations make them attractive for diagnostic and therapeutic development.

Blood represents the major biofluid for isolation of EVs, and the pre-analytical (sample collection) phase can be an important source of variability. This is due in large part to the fact that blood cells, particularly platelets, can be activated easily and release EVs during sample collection and processing[5]. Platelets can be activated by many of the molecules and conditions used in common blood processing procedures, so it is important that the sample collection and processing protocols used (A) prevent the activation of platelets, 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 characterization of EV populations is often confounded by artefacts that are generated during 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.

Sample Medium

The type of sample medium is an important consideration in the design of EV analytical studies. While some EV studies have been carried out using whole blood, this medium precludes EV storage and isolation, so the number of applications is limited[5]; plasma and serum are the predominant sample types used.

Plasma is the preferred over serum as a source of EVs due to a variety of factors:

- Additional EVs are released during the clot formation when preparing serum[5].
- Serum will contain a higher number of vesicles than plasma[1].
- Platelet-derived EVs that are released after collection may account for a significant number of the EVs present in serum[1].

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[2,5].

- Blood should be collected from fasting patients, preferably in the morning[4].
- 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[1].
- Preferably, no measurements of EVs in haemolyzed samples should be done. If haemolyzed 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[2] • 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[2] • May block the EV uptake by other cells1 • 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[2] • Recommended for analysis of MP/MVs[3] • Acid citrate dextrose (ACD) and CTAD prevent platelet activation and the release of platelet EVs more efficiently than citrate[5]

Blood Processing Considerations:

After the blood has been collected, it is centrifuged to remove platelets, 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[2]. The processing steps that are used may depend on the specific scientific question being asked, however general guidelines have been outlined in Table 2.

Table 2: Effects of Blood Processing Steps on EV Levels

Anticoagulant	Mode of Action
Incubation prior to centrifugation	• The anticoagulants present in blood collection tubes may impact the efficiency of the initial centrifugation step[2]. • It is generally recommended that blood is centrifuged within 1 hour of collection[1,3].
Transport Stress/agitation	• It is recommended to transport samples in a frozen state[2] • If samples cannot be transported frozen, then they should be kept upright[5].
Storage Temperature and Time	• All samples should be stored at the same temperature and for the same period of time to limit effects on EV functionality. • Frozen samples should not be compared to fresh samples[2].
Freeze-Thaw Cycles	• Freeze thaw cycles should be kept to a minimum[2,5].
Centrifugation Protocol	• Centrifuge blood at room temperature[5]. • Remove platelets by using 2 subsequent centrifugations steps of 2500g for 15 minutes as recommended by the International Society on Thrombosis and Haemostasis[5].

MATERIALS

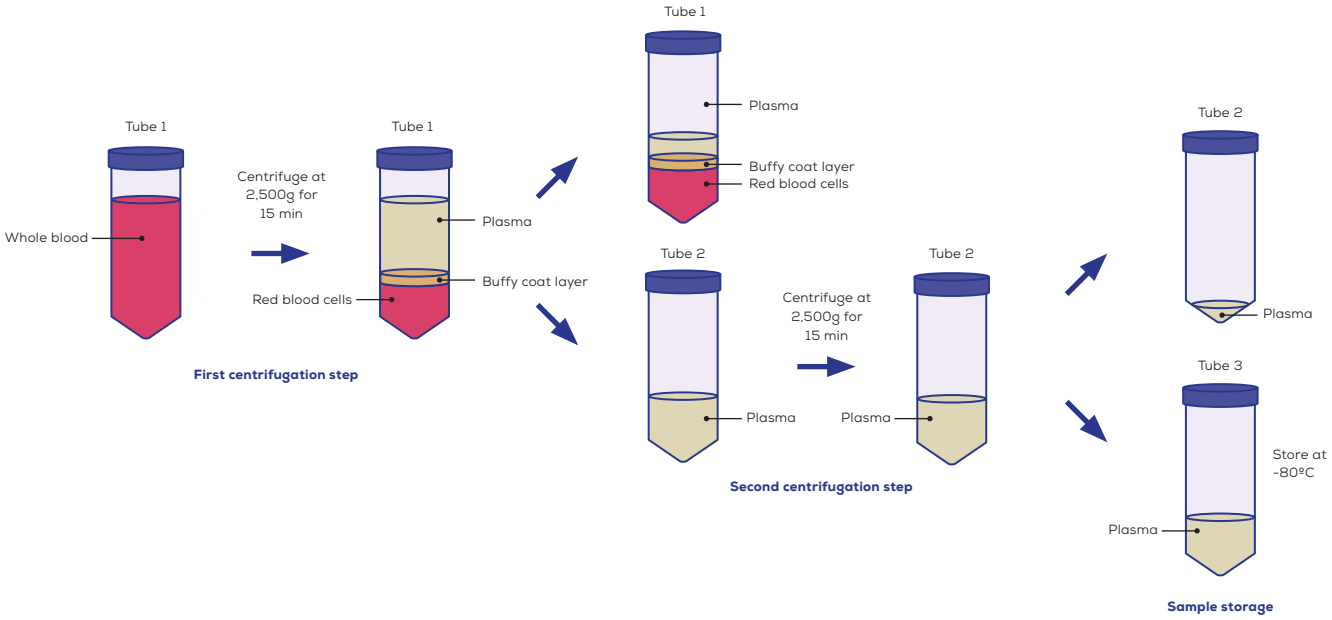
- Two evacuated blood collection tubes
- One ≥ 21-gauge needle
- Personal protective equipment
- Tourniquet
- Blood transfer device
- Puncture resistant sharps container
- Centrifuge with swing-out rotors capable of spinning at 2,500g for 15 min.
- 1000-µL pipette
- 200-µL pipette
- Fresh 1X PBS Solution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

METHODS

Blood Collection and Processing

1. Collect blood from fasting subjects, preferably in the morning (08.00 to 11.00).
2. Apply a light tourniquet and venepuncture the antecubital vein with a ≥ 21-gauge needle.
3. Discard the first 2-3 mL of blood.
4. Collect 2 tubes of blood with a minimum volume of 3.5 mL per tube.
5. Gently invert the tube 8-10 times to mix the blood with anticoagulant.
6. Allow the tubes to incubate at room temperature (20-24°C) for no more than 60 min.
7. Centrifuge tubes at 2,500g for 15 minutes at room temperature (See Figure 1 below).
    a. Make sure that the brake has been turned off.
    b. Use a centrifuge with a swing-out rotor as opposed to fixed rotors.
8. Collect plasma from the top and put into a new tube with a 1000-µL pipette, leaving at least 1 cm of plasma above the buffy coat/layer.
    a. NOTE: Be very careful not to disturb the buffy coat (the layer of white blood cells and platelets above the red blood cells). If the coat has been disturbed, begin the first centrifugation step again.
9. Centrifuge the plasma again at 2500g for 15 min at room temperature.
10. Collect plasma into a new tube using a 200-uL pipette, drawing from the top of the tube, and leave approximately 100 µL of plasma at the bottom of the tube.
11. Snap freeze plasma using liquid nitrogen and store at -80 °C.

Figure 1: Two-step centrifugation procedure



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 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; for more information, visit www.izon.com.
4. Begin collecting the void volume and sample fractions 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 fractions is recommended.
5. After sample fractions have been collected, flush the column with 1.5 column volumes of buffer before loading another sample or storing the column for future use.
    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.

REFERENCES

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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RESOURCES

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