WORKING PROTOCOL FOR DOWNSTREAM ANALYSIS OF EVs ISOLATED USING qEV

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1 / INTRODUCTION

Multiple studies show that concentration and composition of EVs vary with pathology[1]. However, owing to the complex nature of EVs, robust and validated techniques like Size Exclusion Chromatography (SEC) can produce EV volumes for many downstream assays. Izon's qEV SEC columns consistently yield EVs with greater sensitivity and precision[2]. The optimal output volume dilution in a qEV column ranges from 2-4 times and, hence may require a post-column concentration for further downstream analysis.

Nanotrap® particles are hydrogel particles made of cross-linked N-isopropylacrylamide (NIPAm) polymers functionalized with chemical affinity baits. Nanotrap® Extracellular Vesicle Particles are designed to capture and concentrate extracellular vesicles prior to downstream analysis.[2-4]

2 / CONSIDERATIONS AND RECOMMENDATIONS

All qEV columns are available in one of two isolation ranges, the qEV / 35nm series and the qEV / 70nm series. For optimal recovery of particles between 35 and 350 nm a qEV / 35nm series column is recommended. For optimal recovery of particles between 70 and 1000 nm a qEV / 70nm series column is recommended.

Size Exclusion Chromatography

- The choice of separation and concentration method must be informed by factors that may vary between studies.
- SEC performance is determined by the column height, ratio of sample volume to collected volume, and the pore size of SEC media. Combining multiple fractions containing EVs increases the recovery but reduces the purity.
- A second SEC using a new column and starting with the EV fractions from the first SEC will further reduce the contamination with soluble components below the size cutoff. However, it may increase dilution.

Biological Fluid

- Concentration is a means to increase the number of EVs per unit volume, with or without separation. The term “enrichment” can refer to increasing concentration, i.e. EV counts relative to volume, or to increasing EV counts/ marker relative to another component.
- EVs can be obtained from a variety of biological fluids namely, plasma, serum, urine, cerebrospinal fluid (CSF), broncho-alveolar lavage fluid (BALF) and cell culture media. The output volume from qEV columns might require a concentration step depending on the downstream application.

- In case of dilute biological matrices, such as urine samples, cell culture supernatants and frozen human CSF samples, additional concentration steps before and/or after EV isolation may be required.

Table 1 Different qEV columns available for use

qEV COLUMN	INPUT qEV VOLUME	OUTPUT VOLUME
qEVsingle	150 µL	600 µL
qEVoriginal	0.5 mL	1.5 mL
qEV2	2 mL	8 mL
qEV10	10 mL	20 mL
qEV100	100 mL	200 mL

Table 2 Nanotrap® particles available for use

PARTICLE TYPE	FORMAT
Nanotrap® Extracellular Vesicle Particles	Non-magnetic Nanotrap® particles

3 / METHODS

A / Isolation of EVs from plasma and cell culture supernatant:

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. Collect sample and centrifuge to remove debris. **For detailed steps, refer to Izon's technical notes.**
4. Affix an appropriately sized qEV for the sample volume to an AFC or qEV rack and load the sample.

   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.
5. Begin collecting the void volume and EV volume.

   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.
6. After completing collection of the EV volume, flush the column with at least 1.5 column volumes of buffer before loading another sample or storing the column for future use.
7. EVs are ready for downstream applications. Izon recommends performing TRPS analysis for a standardised EV characterisation and quantification (size, concentration and charge).

B / EV concentration using Nanotrap® particles after qEV separation/purification

1. Pool purified collection volumes of interest.
2. The volume of Nanotrap® Extracellular Vesicle Particles required to concentrate EV-containing samples will depend on the volume of sample (See Table 3). Add the appropriate volume to the pooled purified collection volume.

qEV COLUMN USED FOR PURIFICATION	PURIFIED COLLECTION VOLUME (PCV) (mL)	VOLUME OF NANOTRAP® EXTRACELLULAR VESICLE PARTICLES (µL)
qEVsingle	0.60-1.0	50.0
qEVoriginal	1.5-3.0	100.0
qEV2	6.0-8.0	150.0
qEV10	10.0-20.0	200.0

Table 3: Volume of Nanotrap® particles added to specific volumes of EV-containing samples.

3. Incubate the mixture with rotation for one hour at room temperature.
4. Centrifuge the sample at 16,800 RCF for 10 minutes to pellet the Nanotrap® particles.
5. Remove the supernatant being careful not to disturb the pellet.
6. Pellet is ready for downstream processing.

C / Downstream protein analysis using Western blot of Nanotrap® particles concentrated with plasma EVs[3,4]

1. Add minimal volume (≥50 μL) of Laemmli Buffer with 10% Beta-mercaptoethanol to the Nanotrap® particle pellet to fully suspend while keeping in mind the higher the volume added to the pellet the less concentration of protein that will occur.
2. For Nanotrap® particle sample:

   a. Heat at 95°C for 3 minutes.

   b. Vortex strongly for 5 seconds.

   c. Repeat Steps 1a and 1b.
3. Heat all samples at 95°C for 3 minutes.
4. Vortex Nanotrap® particle samples for 5 seconds.
5. Centrifuge Nanotrap® particle samples at 16,800 RCF for 5 seconds to pellet NT. Avoid loading Nanotrap® particles onto gel.
6. Load each sample onto the gel.
7. Run gel at recommended time and voltage per manufacturer's instructions.
8. Remove gel from gel box, per manufacturer's instructions, and proceed with your Western Blot.

D / Downstream protein analysis using mass spectroscopy of Nanotrap® particles concentrated EVs[3,4]

1. Re-suspend Nanotrap® particles in 20 µL of 8 M urea and add 1 µL of 1 M DTT.
2. Heat sample at 50°C for 6 minutes.
3. Alkylate samples with 6 µL of 500 mM iodoacetamide in 500 mM ammonium bicarbonate and incubate for 10 minutes in dark at room temperature.
4. Dilute sample with solution of equal parts water (27 µL) and 500 mM ammonium bicarbonate (27 µL).
5. Digest samples with trypsin (1 µL) for 4 hours at 37°C.
6. Centrifuge samples at 15,000 RCF at room temperature to remove the Nanotrap® particles. Collect the supernatant and freeze overnight at - 20°C to deactivate trypsin.
7. Perform ZipTip peptide drying.

   a. Prepare ZipTip with 20 µL of Buffer B (0.1% trifluoroacetic acid + 80% acetonitrile) 3 times.

   b. Wash ZipTip with 20 µL of Buffer A (0.1% trifluoroacetic acid) 3 times.

   c. Pipette sample through ZipTip, discarding the flow-through.

   d. Wash ZipTip with 20 µL of Buffer A (0.1% trifluoroacetic acid) 3 times.

   e. Elute ZipTip with 20 µL of Buffer B (0.1% trifluoroacetic acid + 80% acetonitrile) 3 times, collecting in a clean tube.
8. Dry sample using a SpeedVac for 15 minutes.
9. Re-suspend sample in 10 µL of 0.1% trifluoroacetic acid and load on mass spectrometer.

E / RNA extraction using Izon's Exosomal RNA Isolation Kit

RNA Isolation from Exosomes Purified and Concentrated Using qEVoriginal and Nanotrap® Particles. **For further detailed notes, please reference working protocol insert in Izon's Exosomal RNA Isolation Kit.**

Notes Prior to Use

- All centrifugation steps are performed at room temperature.
- Ensure that centrifuge tubes used can withstand the centrifugal forces required.
- The provided spin columns are optimized to be used with a benchtop centrifuges and not to be used on a vacuum apparatus
- Most standard benchtop microcentrifuges will accommodate the provided mini Spin Columns.
- Centrifuging the mini spin columns at a speed higher than recommended may affect RNA yield.
- Centrifuging the mini spin columns at a speed lower than recommended will not affect RNA yield. However, centrifugation at a lower speed may require longer time for the solutions to pass through the spin column
- Ensure that all solutions are at room temperature prior to use.
- It is highly recommended to warm up **Lysis Buffer A** at 60oC for 20 minutes and mix well until the solutions become clear again if precipitates are present.
- Prepare a working concentration of the **Wash Solution A** by adding **90 mL** of 96 - 100% ethanol (provided by the user) to the supplied bottle containing **38 mL** of concentrated Wash Solution A. This will give a final volume of **128 mL**. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- **If any of the solutions do not go through the Spin Columns within the specified centrifugation time, spin for an additional 1-2 minutes until the solution completely passes through the Column. Do NOT exceed the centrifugation speed as this may affect RNA yield.**

1. Add 600 µL of PBS buffer to the centrifuged pellet containing the Nanotrap® particles and captured EVs. Add 900 µL of Lysis Buffer A and 125 µL of Lysis Additive B.
2. Mix well by vortexing for 10 seconds then incubate at room temperature for 20 minutes.
3. Separate the depleted Nanotrap® particles from your extracted sample by centrifuging the particle-sample suspension at 16,800 RCF for 10 minutes at room temperature. Without disturbing the Nanotrap® particle pellet, transfer the extracted sample supernatant into a clean tube.
4. After incubation and Nanotrap® particle removal, add 1.5 mL of 96-100% Ethanol to the mixture from Step 3 and mix well by vortexing for 10 seconds.
5. Transfer 750 µL of the mixture from Step 4 into a Mini Spin column. Centrifuge for 1 minute at 3,300 RCF (~6,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.
6. Repeat Step 5 two more times to transfer the remaining mixture from Step 4 into the Mini Spin Column.
7. Apply 600 µL of Wash Solution A to the column and centrifuge for 30 seconds at 3,300 RCF (~6,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.
8. Repeat Step 7 one more time, for a total of two washes
9. Spin the column, empty, for 1 minute at 13,000 RCF (~14,000 RPM). Discard the collection tube.
10. Transfer the spin column to a fresh 1.7 mL Elution tube. Apply 50 µL of Elution Solution A to the column and centrifuge for 1 minute at 400 RCF, followed by 2 minutes at 5,800 RCF.
11. For maximum recovery, transfer the eluted buffer back to the column and let stand at room temperature for 2 minutes. Centrifuge for 1 minute at 400 RCF (~2,000 RPM), followed by 2 minutes at 5,800 RCF (~8,000 RPM).
12. Exosomal RNA is now ready for downstream applications.

4 / REFERENCES

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