# Integrated Platform for EV Analysis: SEC (qEV) + TRPS (qNano)

## Introduction

This technical note details the combination of Size Exclusion Chromatography (SEC) separation and Tunable Resistive Pulse Sensing (TRPS) analysis of extracellular vesicles (EVs). This integrated platform for the first time offers EV researchers a simple, accurate and certain solution for the isolation and characterisation of EVs. EVs, membranous vehicles with a range of important biological functions, are the subject of intense research. Medical diagnostics has a compelling need for the detailed data that EV biomarkers appear to offer. EVs are also being developed as therapeutics. Standardised measurement information is a fundamental requirement for the adoption of EVs as biomarkers and for EVs as therapeutics. The breakthrough created by this platform will enable the EV field to progress and achieve its potential.


## Context

Ultra-centrifugation and chemical precipitation methods are both unsuitable as separation methods for several reasons including inconsistency, contamination and aggregation.


The small physical size and heterogeneity of EVs has also exposed the limitations of most analytical technologies including confocal microscopy, flow cytometry, dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and Electron-microscopy (EM).

EM can resolve EVs but is non-quantitative impractical for routine screening and the vacuum alters the particles.

## SEC + TRPS

SEC as a separation system preserves the structural integrity of the EVs[1,2,3] and provides a clean population of EVs, which makes it possible to measure them consistently. It is simple, effective to use and is the only way to provide a standard representative set of EVs. SEC can be accessed via the industry standard qEV columns, aimed at 500µL samples or the qEVsingle columns for 100µL samples.

TRPS is a powerful, calibrated and certain approach to nano-particle measurement with individual size resolution of around 1 nm and minimum particle size down to~40 nm. TRPS provides particle-by-particle size distribution, accurate number concentration by size fraction, and individual particle zeta potential. These are achieved by analyzing individual particles as they translocate through a single nanopore [4, 5, 6]. Each particle transiting the pore triggers a resistive pulse (blockade event), sometimes referred to as the Coulter principle. The size/volume of a particle is measured by the height of each event, particle charge (zeta potential) is derived by analysing the shape and width of the pulse and the particle concentration is reflected in frequency of the events relative to a known calibration standard.

The qEV protocol takes 15 minutes, TRPS analysis of a post-qEV sample typically takes 20 minutes including calibration. Data analysis and the PDF report generation is rapid. Consequently the whole procedure (purification + analysis) can easily be completed in less than an hour (***Figure 1***). Workflow for multiple samples can be managed to reduce the time per measurement further.

Due to the heterogeneity of EVs and the need for standardised data, it has become very obvious that the particle concentration must be specified within a given size range (e.g. $C_{50\text{-}250nm}$), which TRPS does. A single number concentration is by itself meaningless. That applies regardless of measurement method.

EVs are present in complex biological fluids such as serum, plasma, cerebral spinal fluid (CSF), saliva, urine and cell culture media. Contaminating proteins and other non-vesicular component (e.g. lipids) can interfere with all methods of measurement and will provide false data if included in biological assays. Consequently these must be removed to achieve consistency and accuracy of the EV specific measurement and analysis.

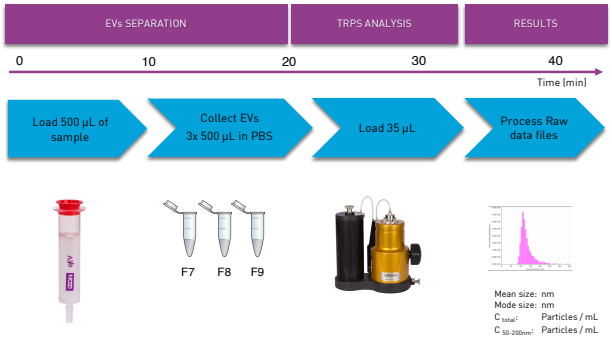


***Figure 1**: Standardized protocol for EVs separation to analysis. General Workflow for EVs isolation by SEC and analysis by TRPS. The overall procedure is usually accomplished within 30-40 min.*

SEC columns have been shown to have the highest purity of EVs with respect to free proteins[1]. They are now considered by many researchers to be the only viable method of separation for clinical use.

This technical note outlines a single workflow for EVs isolation from cerebral spinal fluid and plasma using SEC based qEV columns and analysis by TRPS. The whole procedure can be easily applied to other biological media.

## Method & Results

### Sample preparation

Extracellular vesicles (EVs) were prepared from ovine cerebral spinal fluid[i] and human plasma. All samples were cleared of residual cells and debris by three sequential 10-minute centrifugation steps (CSF 1500 g, 3000 g, 10,000 g; plasma 1500 g, 3000 g, 3000 g, in each case the supernatant is retained). Pre-equilibrated standard qEV columns were loaded with 500 µL of sample and 500 µL fractions were collected. The EVs typically elute in fractions 7-9 (see [2] and ***Figure 2***)

[i]Sample obtained from inside the skull post mortem

## TRPS Setup

All measurements were conducted using a qNano (Izon Science Ltd., NZ) and Izon software version 3.1. The lower fluid cell contained the electrolyte (75 µL) and the upper fluid cell contained 35 µL of sample.

Prior to TRPS analysis, all samples were purified by qEV columns as detailed above. Between each sample run, the system was washed using PBST (35 µL) into the upper fluid cell several times to ensure there was no residual particle carry over between samples.

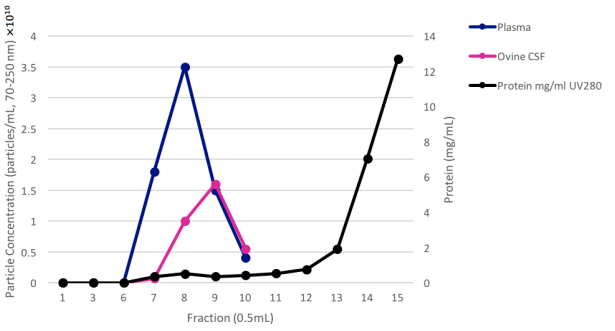


***Figure 2**: Typical qEV separation profile for plasma and CSF (500 µL).*

A detailed description of such a tunable resistive pulse sensing device is described elsewhere[5, 6]. The pore (NP150) was calibrated using 200 nm (mean 210 nm) CPC polystyrene particles of known concentration (5.0 x $10^9$/mL) at 3 pressures (10, 8, 5 cm$H_2O$). The samples were then analyzed at the same voltage and pressures using a consistent baseline current (e.g. 135 nA ± 2-5% max.) to ensure valid comparative data sets between runs.

TRPS analysis requires the use of Izon reagents, which includes a coating solution for pore pre-treatment, minimising particle binding to the pore through non-specific interactions.

Freshly prepared reagents were filtered (0.22 µm) on the day of analysis to avoid contamination/ particulates that can occur over time and interfere with TRPS analysis. For TRPS analysis of the qEV fractions, an initial dilution of 1:5 or 1:10 in electrolyte was used. This dilution was then optimised to achieve a translocation rate at the highest operating pressure of approximately 200 to 1600 particles per minute. If the EV concentration is low TRPS analysis takes longer so the sample may be concentrated using the Merck Millipore Microcon-30 spin filters[7] or an equivalent.

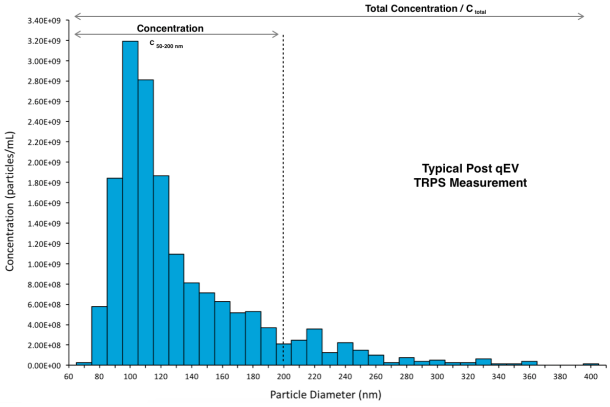


***Figure 3**: A TRPS measurement on a post-qEV sample*

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## TRPS analysis of CSF and plasma samples

The qEV fractions of the CSF and plasma samples were diluted in electrolyte and analyzed by TRPS as outlined above. The EVs from plasma and CSF eluted in qEV fractions 7-9 with the peak fraction for plasma contained in F8 and CSF F9 (***Figure 2***). Some EVs eluted in F10 but this fraction was typically more contaminated with protein and usually discarded. A typical particle size histogram and concentration data is shown in ***Figure 3***. In some instances, where biological samples contain a low level of EVs, additional concentration steps using a spin filter may be required before or after the qEV purification. This is frequently the case with urine samples, cell culture supernatants and frozen human CSF samples.

## Conclusion

This technical note details the methodology for isolation and TRPS analysis of EVs. Biofluids contain EVs and these vesicles can be now readily isolated by standardised qEV size exclusion chromatography and analyzed by TRPS to provide certainty and repeatability. The procedure is simple, gentle and can be completed within 60 minutes. We purified EVs from ovine CSF and human plasma using qEVs and performed TRPS analysis with excellent stability at 1:10 dilution of the samples. TRPS is a powerful method for EV analysis; it is a non-averaging approach applicable for the analysis of poly-disperse size samples. TRPS particle-by-particle sensing is the only method that provides certain and precise size, concentration and zeta potential measurements. The future of medical diagnostics is expected to include EV profiling. Understanding the biophysical diversity in EV populations in a standardised format is paramount for linking the impact of EVs properties to their biological role and function. SEC + TRPS is the only way that this can be achieved for clinical use.

## References

**[1]** Anita N. Boing; et al. Single-step isolation of extracellular vesicles by size-exclusion chromatography. Journal of Extracellular Vesicles 2014, 3: 23430

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**[4]** Emma L. C. Blundell; et al. Emergence of tunable resistive pulse sensing as a biosensor. Anal Methods 2015, 7, 7055

**[5]** Geoffrey Willmott; et al. Use of tunable nanopore blockade rates to investigate colloidal dispersions. J. Phys.: Condens. Matter 2010, 22, 454116

**[6]** Robert Vogel; et al. Quantitative sizing of nano/microparticles with a tunable elastomeric pore sensor. Anal. Chem. 2011, 83, 3499−3506

**[7]** Merck Millipore - Microcon-30 - Product reference: MRCF0R030



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