# From Laboratory to Manufacturing; Methods and Challenges in Exosome Purification

Mitch Shambrook[1], Ella L. Johnston[1],Johannes F. Weber[1], Smriti M. Krishna[1], Craig Newton[1], Ian Nisbet[1], Xenia Sango[1], Louise White[1,2], David N. Haylock[1]

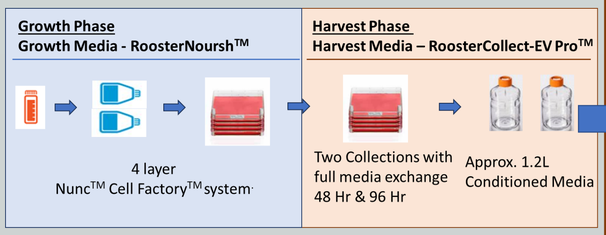
[1]VivaZome Therapeutics Pty Ltd, Victoria, Australia [2]SeerPharma Pty Ltd, Victoria, Australia

## Introduction

Extracellular Vesicles (EVs) have significant diagnostic and therapeutic potential, however the production of functional EVs at scales appropriate to clinical practice remains a difficult prospect. To address this, the identification of research scale isolation technologies that are suitable for translation to large scale expansion is critical. With the increased interest in EV therapeutics there has been a rise in the multitude and variety of research scale technologies available. Many provide advantages as well as challenges in the efficient separation of functional EV populations amongst a complex heterogenous mixture of secreted cellular components. Importantly, it is essential that these technologies and procedures not only efficiently isolate EVs at a level that is cost and time effective, but that they maintain their integrity and functional capacity via their therapeutic cargo. Tangential Flow Filtration (TFF), Size Exclusion Chromatography (SEC), and Ultrafiltration represent widely established research scale methods suitable for large-scale production of purified EV preparations (PEPs). Here we outline a research scale isolation process utilising these techniques which provides EVs at concentrations appropriate for functional assessment and for future upscaling.

## Current Research-scale Downstream Process

### 1. Cell Factory System




- Human Bone Marrow MSCs or proprietary adherent cells are expanded from cell banks into T175 flasks before being seeded into a 4 layer Nunc™ Cell Factory™ and monitored for 3-4 days.
- Media is switched from growth to harvest at 80% confluency (Figure 1).
- Conditioned Media (CM) undergoes a clarification spin of 2,000g for 10 minutes.
- Culture produces CM with particle concentrations approximately 1.0E+09 particles/mL (NTA; Figure 1).

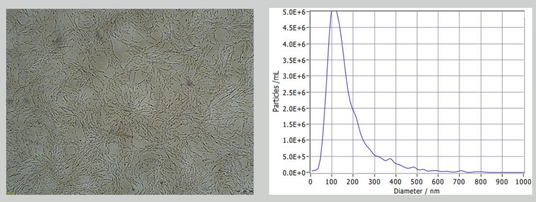



Figure 1. Adherent proprietary cells cultured in RoosterCollect-EV Pro™ and size v particles/mL of harvested CM.

### 2. Tangential Flow Filtration

- Vivaflow® 50R TFF cassette with a 10kDa cutoff is utilised to concentrate Approx. 500mL CM to 10mL (50x).
- Conditioned media subjected to TFF, which retains >90% particles. (Figure 2).
- CM maintained at 4ºC via a chilled bead bath during TFF.

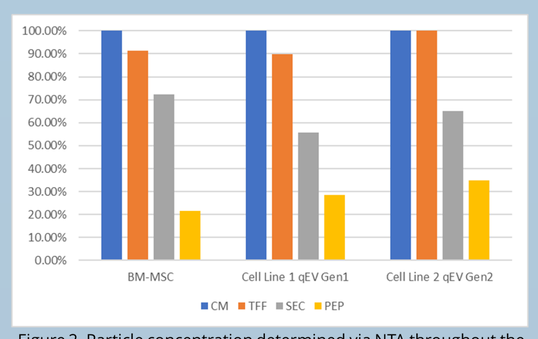



Figure 2. Particle concentration determined via NTA throughout the isolation process indicates a gradual reduction in particle recovery.

### 3. Size Exclusion Chromatography

- qEV10/35 nm Gen 2 columns are used along with the Automatic Fraction Collector (AFC) after TFF.
- Provides standardised isolation with 15 minutes sample fractionation time.
- Consistent performance in separating particles from protein (Figure 3).
- Variation in particle recovery, which could be a real effect of non-specific column binding, or reflection of inefficient EV quantification method.

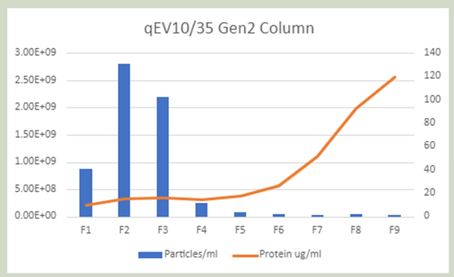



Figure 3. Separation of Particles from proteins via SEC utilising qEV10-35 Gen2 Columns.

### 4. Concentration (Ultrafiltration)

- Amicon® Ultra spin columns are then utilised to concentrate EV-containing SEC fractions to their final volume (Figure 4).

| | Vol (ml) | Particles/mL | Total Particles | Recovery % |
|---|---|---|---|---|
| Input per column | 10 | 5.40E+08 | 5.40E+09 | |
| Sartorius 30K | 0.39 | 4.20E+09 | 1.64E+09 | 30 |
| Sartorius 10K | 0.41 | 4.20E+09 | 1.72E+09 | 32 |
| Amicon 10K | 0.5 | 6.30E+09 | 3.15E+09 | 58 |
| Amicon 100K | 0.15 | 6.60E+09 | 9.90E+08 | 18 |

Figure 4. Ultrafiltration Column performance using TFF concentrated and qEV isolated separated CM as input.

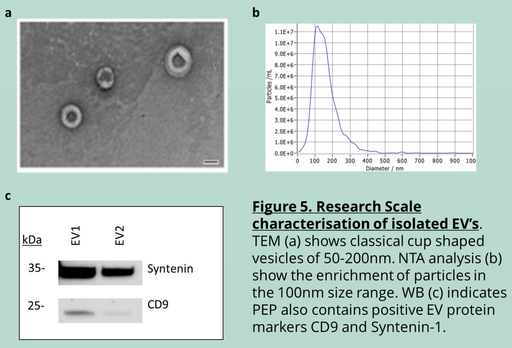



**Figure 5. Research Scale characterisation of isolated EV's**. TEM (a) shows classical cup shaped vesicles of 50-200nm. NTA analysis (b) show the enrichment of particles in the 100nm size range. WB (c) indicates PEP also contains positive EV protein markers CD9 and Syntenin-1.

## Upscaled Downstream Process


A variety of bioreactor technologies are available for culture of adherent cells to generate larger volumes of CM.

- Nunc™ Cell Factory™ systems, both 4 and 10 layer.
- The iCELLis nano produces CM with high particles/mL (1-4 x $10^{12}$/mL).
- Adherent cells can be cultured on microcarriers, thus facilitating use of stirred tank bioreactors and novel mixing systems such as the PBS vertical bioreactor.

AKTA Flux s Semiautomated TFF with Data logging.

- Can be used along with a variety of TFF filtration units including the MidGee Hoop hollow fibre 750kDa.
- Recovers >90% particles.
- 10x concentration can take >5 hours, additional testing of alternative hollow fibre units may reveal improved concentration times.

- MidGee Hoop hollow fibre 750kDa 73cm2 membrane area

SEC systems show good separation of particles and protein (Figure 6).

- Highscale SEC system utilising AKTA pure systems 25 and 150.
- Investigating appropriate resins for large scale SEC, including CL-2B, Sephacryl S-500, and other 'High Flow' resin in development.

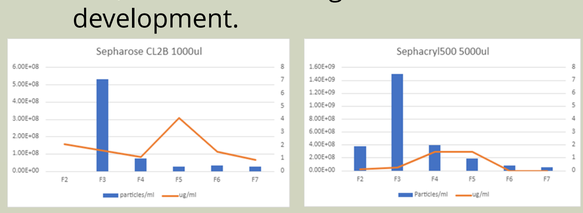



Figure 6. Distribution of EVs (particles) and protein in SEC fractions.

- With upscaled downstream volumes the Amicon ultrafiltration step can be replaced with a larger volume TFF concentration step.

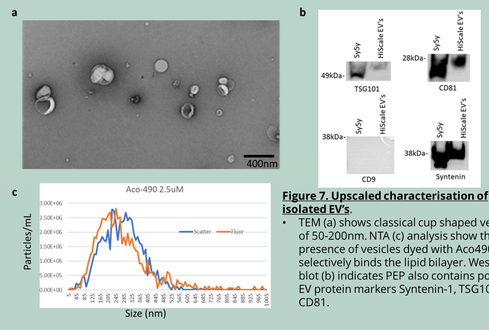



**Figure 7. Upscaled characterisation of isolated EV's**.

- TEM (a) shows classical cup shaped vesicles of 50-200nm. NTA (c) analysis show the presence of vesicles dyed with Aco490 which selectively binds the lipid bilayer. Western blot (b) indicates PEP also contains positive EV protein markers Syntenin-1, TSG101 & CD81.

## Quality Management

- Maintaining accurate and detailed record keeping of batch production is essential as this highlights production trends which serve to inform the Critical Process Parameters (CPP) of the process.
- Identifying these CCPs at research scale will inform the development of upscaled systems and ensure a faster and more informed transition to a reproducible process with quantifiable Critical Quality Attributes.

## Conclusion

- The research scale isolation process produces EVs which meet classical characterisation; they are of the correct size range, shape and express EV associated protein markers. The process is currently 4-5 hours which means the CM can be collected and processed on the same day.
- While particle loss occurs through the process, the current NTA methods for assessing recovery requires improvement to measure EV yield more accurately. Incorporating bi lipid specific dyes may help improve this assessment.
- We are currently developing an immunolabeling panel for phenotypic flow cytometric analysis of EVs and donor cells utilising the A1 and A3 symphony systems (BD Biosciences).