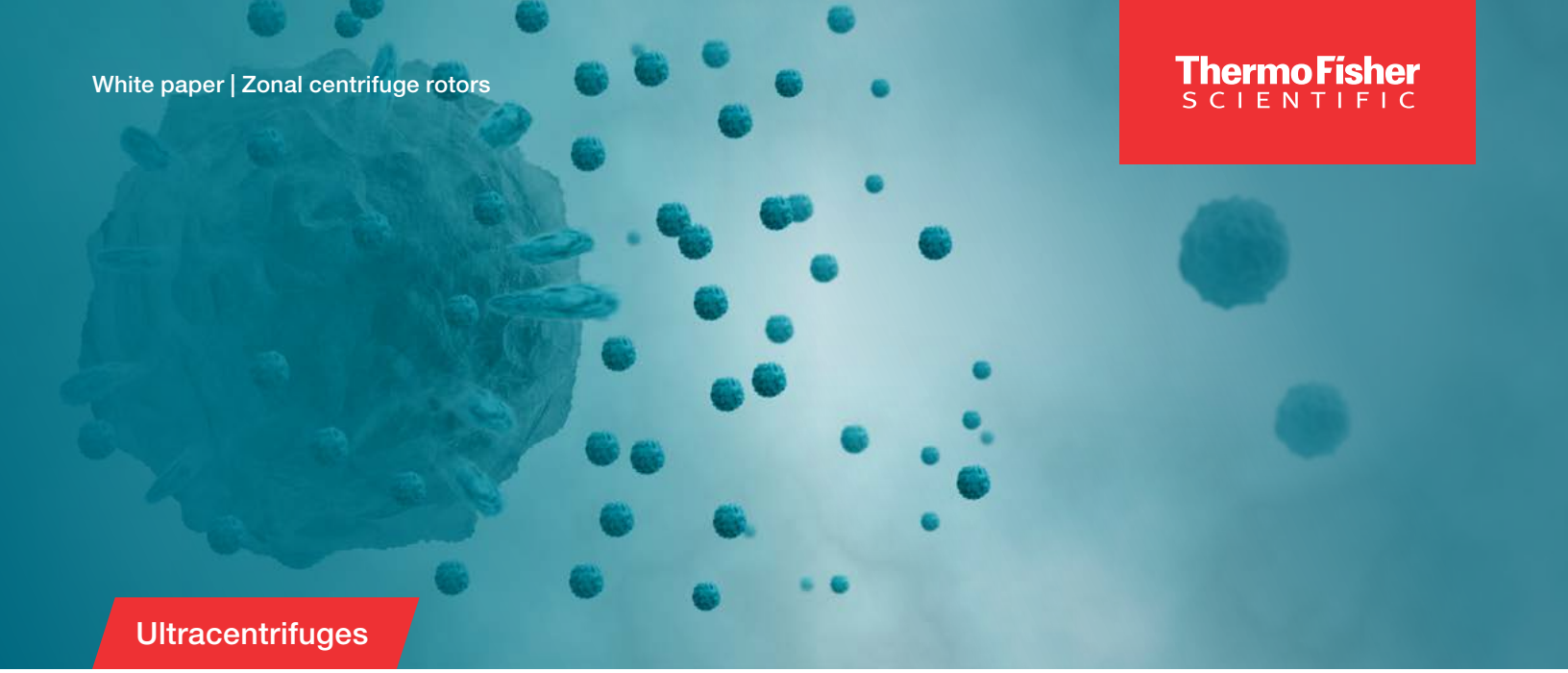

Ultracentrifuges

High yield, high purity sEV purification scale-up method by zonal density gradient ultracentrifugation

Authors

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Keywords

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Introduction

Extracellular Vesicles (EVs) are increasingly seen as foundational transporters in the human body's communication machinery. They can transport various types of biomacromolecules – for instance, protein and nucleic acid-based therapeutics – but can also be loaded with different drug cargos. Crucially, EVs have also been found to improve the uptake of molecules across natural barriers such as the blood-brain barrier. Hence, there is an increasing interest in combining the highly versatile delivery capabilities of EVs with targeting technology and sophisticated biomolecular engineering approaches to develop an entirely novel class of biotherapeutics.

For therapeutics needs, EVs are often isolated from cell culture supernatant. To remove impurities, a mix of Downstream Processes that rely on the properties of EVs can be used but there is a balance to be found between purity and yield of final material. It is desirable to have an approach that is transferable to multiple EV products.

During manufacturing runs for higher order species and patients, purification processes are usually very specific to an entity of interest where defined molecular signatures such as charge, size, mass, density, or affinity to a specific ligand, are utilized in order to separate the product of interest from cellular and proteinaceous contaminants. While these methods are great at providing a product of high purity, they need to be optimized for each novel type of engineered EVs as they will carry slightly different molecular signatures.

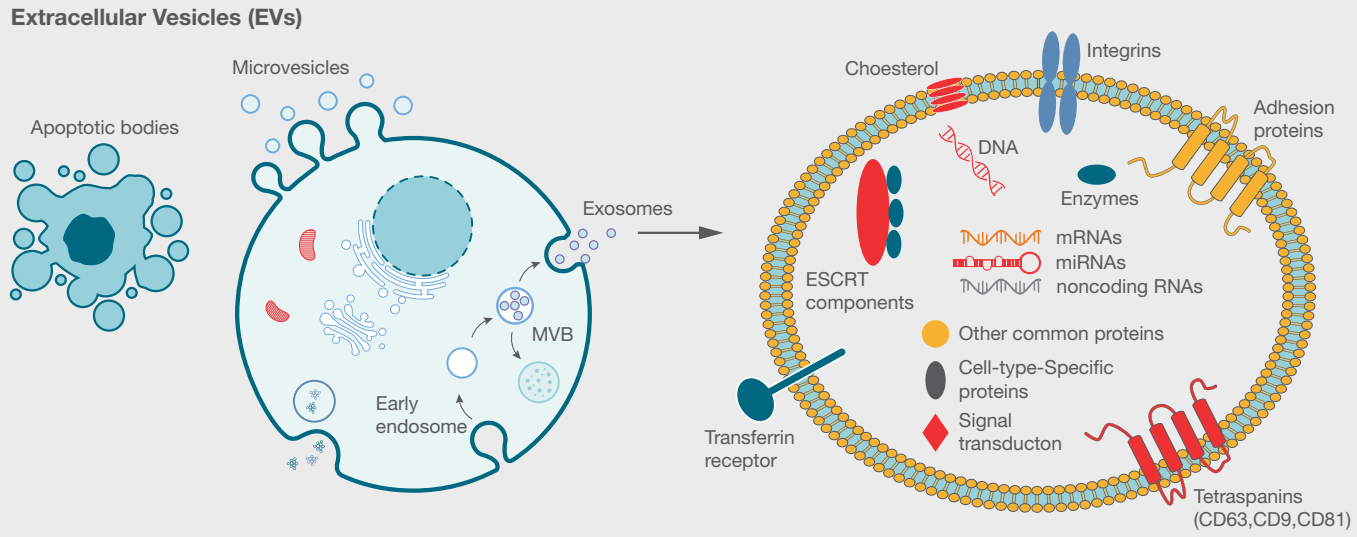


Figure 1. Extracellular vesicles

Early research programs do not have this luxury and will therefore often opt for cruder purification methods in order to still capture the majority of the intended product even if it contains higher levels of impurities. Common methods include Size Exclusion Chromatography (SEC), ultracentrifugation or commercially available kits.

Thermo Fisher Scientific and Evox Therapeutics have collaborated to develop an iodixanol density gradient ultracentrifugation method for the isolation of EV[1] at a large scale with high purity and high recovery. This downstream purification method was successfully adapted from a small-scale 12 mL swinging bucket rotor to a large-scale bowl rotor with a more than 23-fold scaling factor for only a 1.5-fold increase in time.

Figure 2. Zonal rotor

Iodixanol density gradient ultracentrifugation method for the isolation of EV

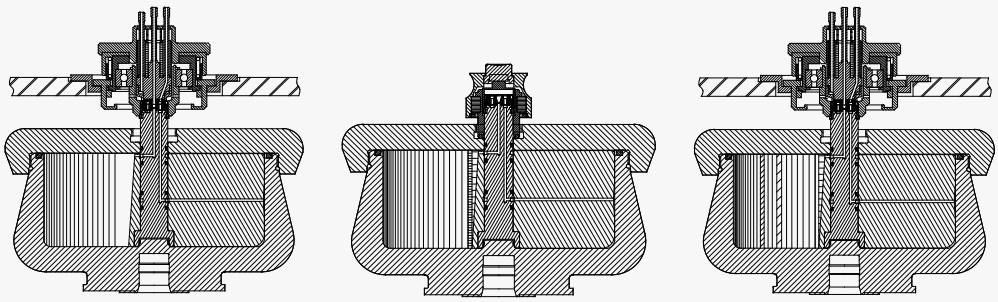
This work was carried out to isolate EVs from the equivalent of 11 L of EVs from cell culture supernatant. Post Harvest and clarification, the material was concentrated down using Tangential Flow Filtration 17-fold. The concentrated material was then combined with iodixanol for a final sample density equivalent to 6% Iodixanol.

The Ultracentrifuge was set to the Zonal Rotor setting, speed up to 3,000 rpm and primed with PBS to ensure the absence of air bubbles. The iodixanol density gradient was formed by underlaying successively 700 mL of the sample in 6% iodixanol, 100 mL of 18% iodixanol, 100 mL of 23% iodixanol, 200 mL of 30% iodixanol and finally 200 mL of 45% iodixanol from the zonal rotor outer wall.

The centrifugal separation was performed maintaining 32,000 rpm (equivalent to 102,000 x g) during 24 hours at a temperature of 4°C. The speed was then reduced down to 3,000 rpm and 60% iodixanol extrusion solution was loaded from the zonal rotor outer wall displacing the fractions out from the zonal rotor inner wall **(Figure 2)**. 36 fractions of 50 mL, from the low to the high-density order, were collected and further analyzed using Evox Therapeutics Proprietary analytics.

1. The higher density of proteins (1.35–1.41 g/cm3) enables separation of EVs (density: 1.08–1.19 g/ml) based on difference in density while maintaining EV integrity.

Zonal rotor process flow



Item \ Procedure	Loading	Centrifugal separation	Unloading
Speed	3,000 rpm	Specified speed	3,000 rpm
Procedure	Inject density gradient, sample, overlay solution	Separation	Inject high density extrusion and recover separated solution

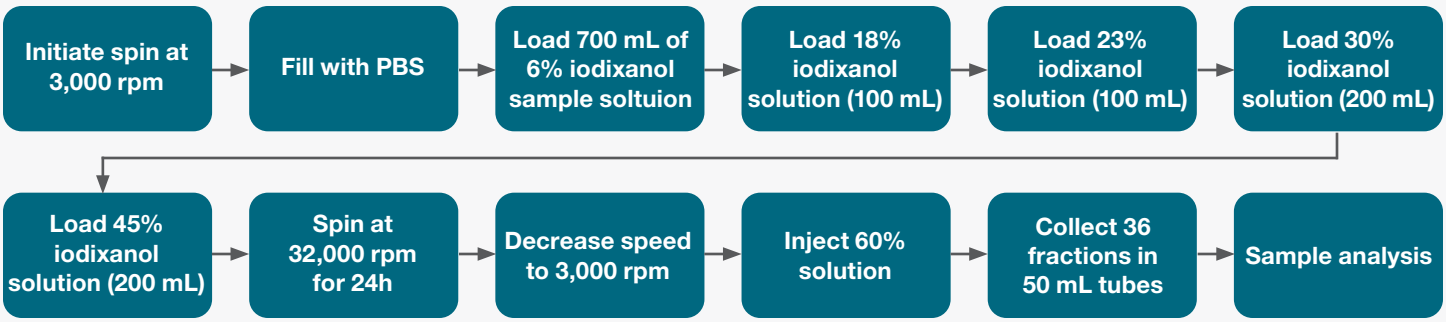


Figure 3. Schematic of zonal rotor process flow for density gradient ultracentrifugation (DGUC)

The densities of each layer loaded into the zonal rotor were measured prior to loading. At harvest, the density of each fraction was measured with the exception of the key fractions of interest that were pooled and used for further work; namely fractions 24 to 31 and fractions 33 to 34 **(Figure 4)**. These data confirm that fractions 24 to 31 have a density ranging from 1.05 g/mL to 1.15 g/mL while fractions 33 to 34 have a density ranging from 1.15 g/mL to 1.32 g/mL.

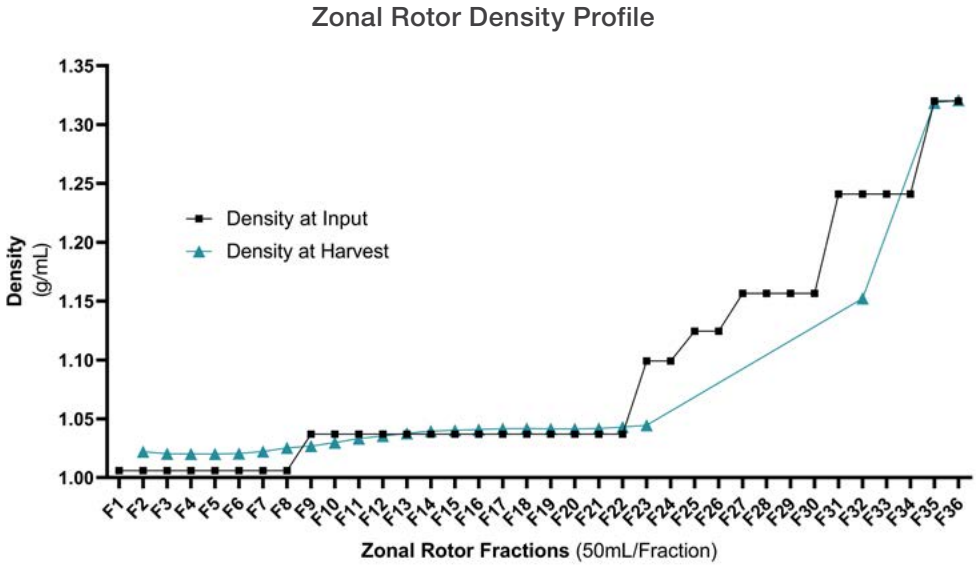


Figure 4. Density profile of fractions isolated from zonal rotor density gradient ultracentrifugation (DGUC) showing density separation between input and harvest from F23 onwards.

NTA and sandwich ELISA assay

The particle distribution across the Zonal rotor density gradient was assessed using Nanoparticle Tracking Analysis (NTA) showing that fractions 24 to 33 contained 86% of total particles recovered across the gradient **(Figure 5)** in line with that expected from the density ranges of 1.05 g/mL to 1.32 g/mL.

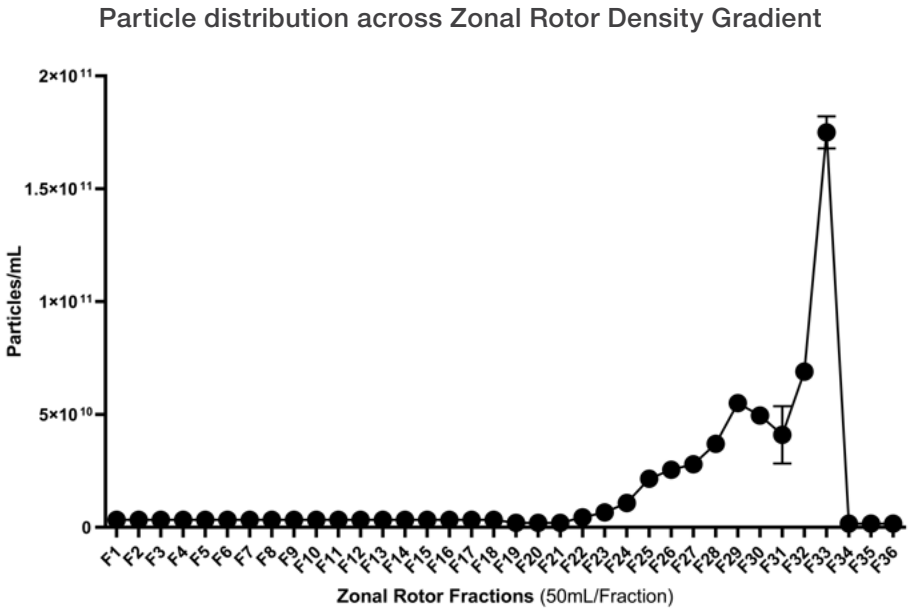


Figure 5. NTA analysis of fractions isolated from Zonal rotor density gradient of EV sample.

To further decipher the nature of these particles and to understand which fractions were enriched in the canonically described Tetraspanins CD9, CD63 and CD81 EVs (MISEV[2] 2018 guidelines), a proprietary sandwich ELISA assay that quantifies Tetraspanin positive particles was used. The concentration of Tetraspanin positive particles is interpolated from a standard curve made from a calibrator of a defined Tetraspanin positive particle concentration expressed in A.U./mL. Fractions 21 to 33 were selected for quantification on the assay as these fractions contained the majority of particles and have densities expected of sEVs **(Figure 6)**.

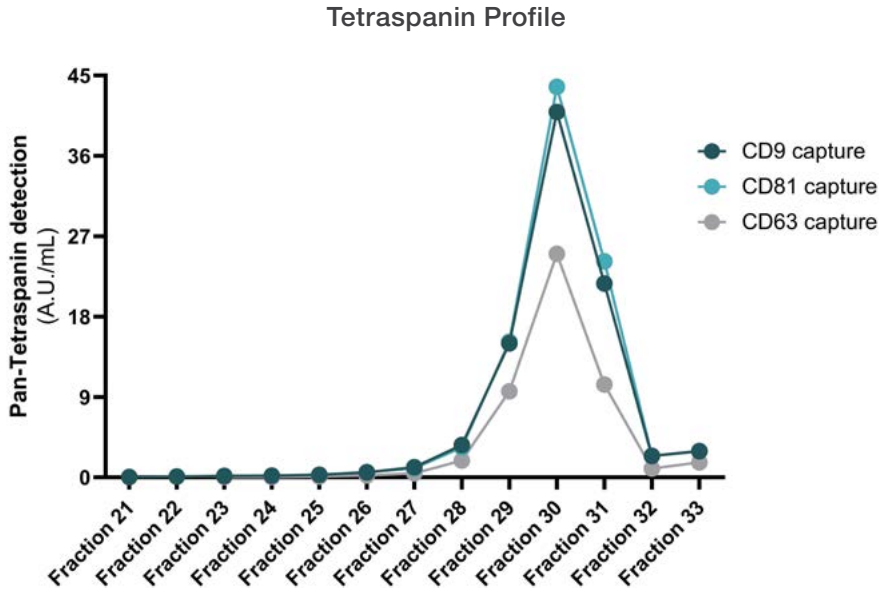


Figure 6. Tetraspanin ELISA profile demonstrates that EV particles are present in expected density of EVs

Mass balance across the density gradient was achieved within 15% of the total amount of Tetraspanin positive particles loaded into the gradient which corresponds to less than the variation of the assay. Over 90% of Tetraspanin positive particles were found between fractions 28 and 31 corresponding to a density of less than 1.15 mg/mL with the peak being found in fraction 30 **(Table 1)**.

Table 1.

	CD9 Capture-Pan Tetraspanin detection	CD81 capture-Pan Tetraspanin detection	CD63 capture-Pan Tetraspanin detection
Mass Balance achieved within 15%*	Yes	Yes	Yes
Percentage of Tetraspanin positive particles in Fraction 28-31	91%	92%	93%

* This assay has precision of 20%

2. Minimal information for studies of extracellular vesicles

Western blot

To understand whether these fractions were successfully depleted from cellular and proteinaceous contaminants, an SDS-PAGE was carried out with volumetric loading of fractions 24 to 33 **(Figure 7)**. It confirmed that most proteinaceous contaminants were located in fractions 32 and 33.

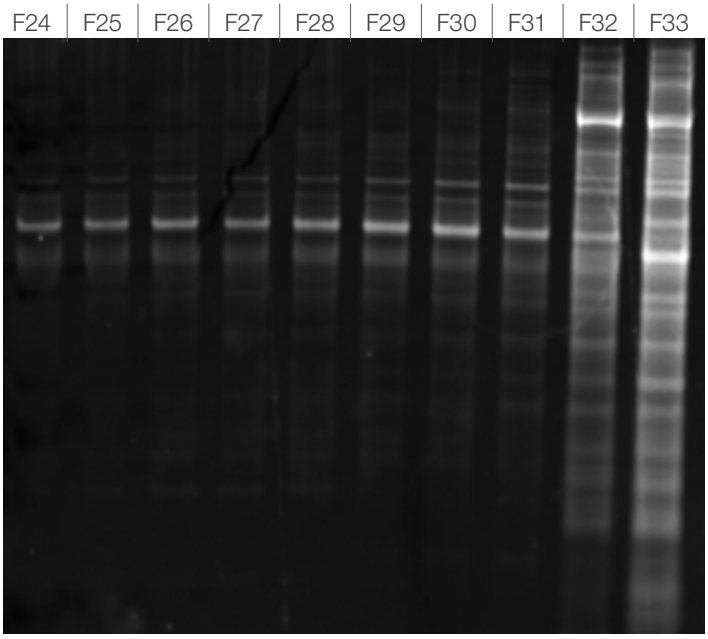


Figure 7. SDS-PAGE analysis of fractions 24 to 33 isolated from zonal rotor density gradient ultracentrifugation (DGUC) showing separation of proteinaceous contaminants in fractions 32 and 33.

To confirm the protein composition of fractions 28 to 31 as being EVs in accordance with the MISEV 2018 guidelines, these fractions were run on Jess, an automated western blot platform, loading volumetrically and probing against Syntenin (left panel, 6). Fraction 29-31 had the highest signal while fractions 32 and 33 were depleted of this signal. There was some non-specific binding in fraction 33 but this is likely to be due to a significantly higher concentration of protein contaminants. This contamination is demonstrated in right panel of 6 where fractions were probed against a Calnexin primary antibody. Further, these results align with the Tetraspanin data **(Figure 6)**.

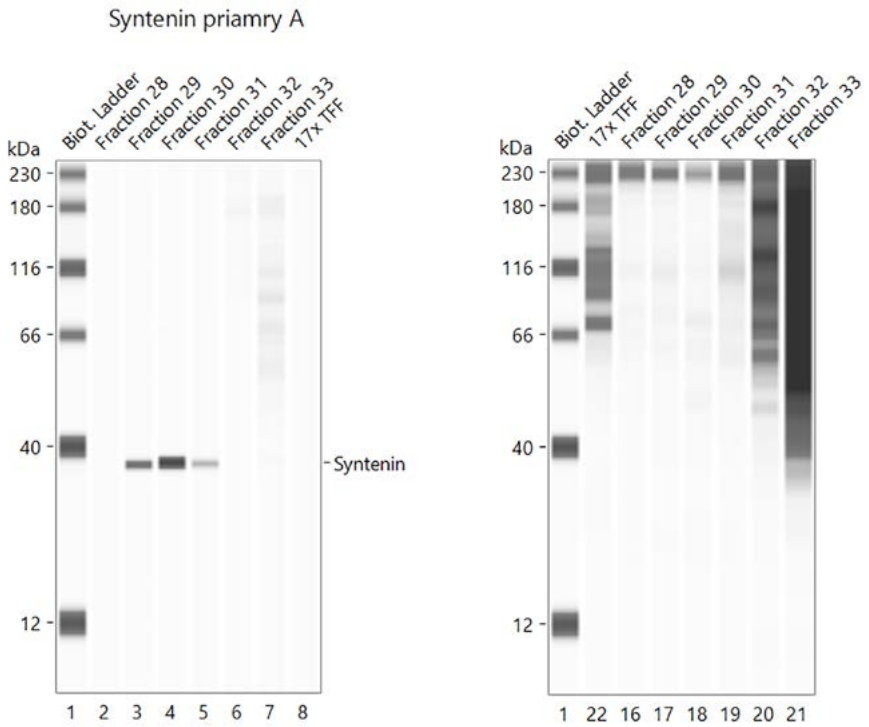


Figure 8. Western blot analysis showing presence of EV markers Sytenin (left) in fractions 29,30 and 31, which is being separated by zonal rotor from contaminating proteins like Calnexin (right).

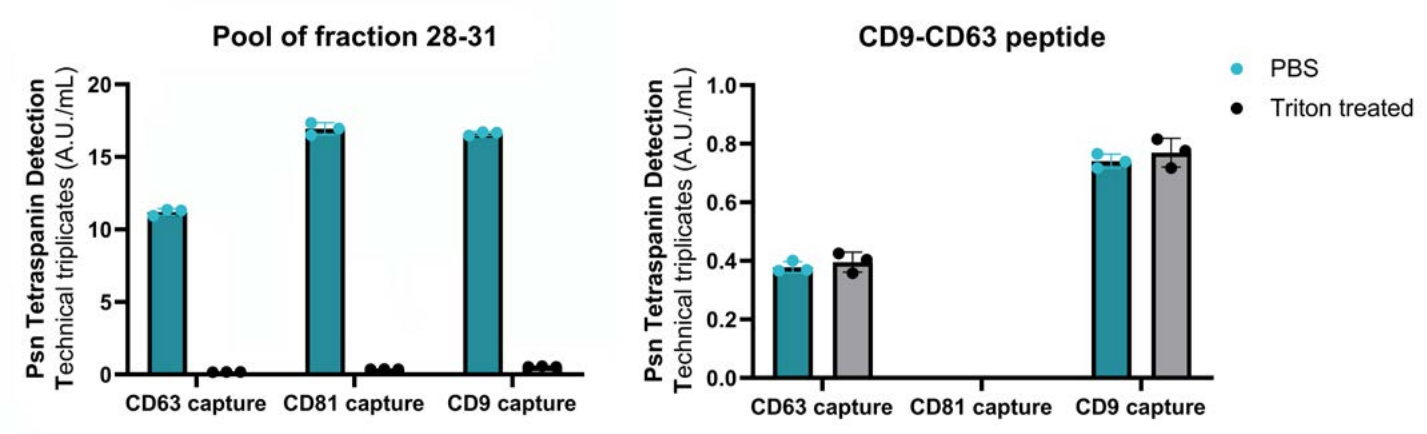


Figure 9. Tetraspanin ELISA confirms the presence of EVs in fractions 28-31 as demonstrated by loss of CD63, CD9 and CD81 signal upon triton treatment (left). Peptides derived from CD9 and CD63 were unaffected by triton treatment (right).

Tetraspanin ELISA

One of the potential concerns with the utilization of ultracentrifugation is that the high forces used can lyse or disrupt the EV membrane. To ensure that the sEVs isolated in fractions 28-31 were intact, the pool was measured in a proprietary Tetraspanin particle sandwich ELISA, diluted either in PBS or in 1% Triton X-100. The significant drop in Tetraspanin positive particles in the presence of Triton, suggests that the assay is indicative of sEV membrane integrity and therefore that the concentrations obtained in the assay are that of intact EVs. This is further confirmed by the control in the form of a peptide containing both a CD9 and CD63 epitope which is detected equally in both diluent displaying that these results were not due to matrix interference **(Figure 9)**.

Conclusion

Together, these data confirm that the zonal rotor successfully concentrated over 90% of sEVs inputted into the gradient into Fractions 28-31 and that these particles were not negatively impacted by the process of ultracentrifugation. These fractions contained the hallmarks of sEVs according to the MISEV guidelines and were depleted in cellular contaminants.

This method can further scale up using the **Thermo Scientific™ Sorvall™ CC40NX Continuous-Flow Centrifuge** allowing a total rotor volume of 8.0 L at up to 118.000 x g force in full accordance to GMP guidelines and regulation offering a manufacturable and scalable agnostic platform to go to clinic.

Ordering information

Description	Cat. No.
Thermo Scientific™ Sorvall™ WX 80+ Ultracentrifuge	75000080
Thermo Scientific™ TZ-32 Zonal Rotor	75000008
Seal assembly for TZ-32 rotor	75000013

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