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Application Note

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Transferring EV Purification Process From CIMmultus® EV to QA HR Column

Katja Vrabec, Andrej Raspor, Marko Narobe, Ana Železnik, Valentina Novak, Ivana Petrović Koshmak and Maja Leskovec
Sartorius BIA Separations

Abstract

CIMmultus EV, an anion exchange column intended for the purification of extracellular vesicles and CIMmultus QA HR, a novel column from the CIM® QA HR line, designed for the highest batch-to-batch and scale-to-scale reproducibility, were used for isolation of extracellular vesicles following the same protocol. Both columns share the same chemistry, a quaternary amine ligand but slightly different ligand density. We demonstrate that the purification protocol developed for discontinued CIMmultus EV is straightway transferrable to the CIMmultus QA HR column. The comparability of both column types was demonstrated on elution profiles and recovery of extracellular vesicles.

Introduction

CIMmultus columns are pre-packed chromatographic monolithic columns designed for purification needs of large biologics like viral vectors, vaccines, nucleic acids, and extracellular vesicles (EVs). Monoliths are a single-unit structures with highly interconnected channels distributed homogeneously throughout the entire bed. Mass transfer through them is exclusively convective for large biomolecules allowing for high flow rates with low shear environment. On monoliths, capacity and resolution are flowrate independent as large molecules are not hindered by their diffusion rates thus shortening the processing time.

Extracellular vesicles can be purified with CIMmultus columns from various source materials, including plasma and different cultured cell lines [1][2]. This application note describes the isolation of EVs from HEK293-CD63eGFP-derived clarified conditioned media using tangential flow filtration (TFF) combined with nuclease treatment, followed by separation on CIMmultus EV or a CIMmultus QA HR column.

CIMmultus EV is an anion exchange column that was intended specifically for the purification of extracellular vesicles [3] and was discontinued in 2024. It shared the same quaternary amine (QA) ligand as CIMmultus QA and new CIMmultus QA HR columns. CIMmultus QA HR was developed to provide the best batch-to-batch and scale-to-scale reproducibility [4]. The line was improved with an additional release test on a quality control unit [5] and improved column housing for small units. The acceptance criteria are based on the elution conductivity value of a viral vector, ensuring all CIMmultus QA HR columns allow elution of large biomolecules at specific conditions regardless of the column volume and production batch. The housing of the lower volume columns, from 1 mL to 8 mL, was adjusted to minimize the void volume, for high reproducibility between all scales. The redesign of the CIMmultus QA HR in 1 mL, 4 mL and 8 mL scales ensures that the percentage of void volume of these small CIMmultus HR columns matches the percentage of void volume of larger CIMmultus QA (HR) columns.

CIMmultus QA HR and CIMmultus EV columns are manufactured using the same production methods. Both lines share the same quality assurance regulatory support file for GMP products, allowing a smooth transition from the standard CIMmultus line to the new CIMmultus QA HR line.

Methods and Results

Conditioned Media Preparation

The HEK-293T cells used in the present study had been genetically modified to express an enhanced green fluorescent protein (eGFP) fused to the C-terminal region of human CD63. The cells were generously provided by Bernd Giebel (Institute for Transfusion Medicine at the University Hospital Essen, Germany).

HEK CD63-eGFP cells were seeded in an Ambr250 vessel on SoloHill® FACTIII microcarriers and grown for 3 days in growth media (DMEM with an addition of 10% FBS). Subsequently, the media was switched to one devoid of FBS for the production phase. The conditioned media was collected on the second day of EV production, put through a cell strainer, and then clarified through a 1.2 µm Sartopure® PP3 filter.

HEK-293T EVs tagged with CD63-eGFP were isolated from the conditioned media using tangential flow filtration (TFF) combined with nuclease treatment. The EVs were then separated using either a CIMmultus EV or a CIMmultus QA HR column. Both columns were assessed using the bind-elute procedure described below, and the reproducibility of results was evaluated when transitioning from CIMmultus EV to CIMmultus QA HR.

Sample Preparation With TFF

The procedure describes the pre-treatment of clarified conditioned media with TFF and nuclease. The buffers are provided in Table 1. The resulting TFF retentate is prepared for loading to chromatographic columns.

Table 1*: Diafiltration buffers.*

Buffer name	Buffer composition
Diafiltration buffer 1	30 mM BTP, 0.5 M NaCl, 5 mM $MgCl_2$, pH 8.0
Diafiltration buffer 2	30 mM BTP, 50 mM NaCl, pH 7.0

TFF system setup:

- TFF Module: Hollow fiber filter module or a selected alternative with the cut-off that retains your product. In this experiment, hollow fiber filter with 500 kDa cut-off was used (D02-S500-05-N, Repligen surface area: 190 cm^2).
- TFF System: Sartoflow (Sartorius), KR2i (Repligen), or modular system using 2 peristaltic pumps.
- Recommended Flux LMH for Diafiltration: 250-350 L/m2/h (or as specified for the TFF module). The described procedure was performed at the flow rate 100 mL/min.
- TMP: The transmembrane pressure (TMP) was controlled at 2.0 psi in the described procedure.

TFF procedure (described for 1000 mL of clarified conditioned media):

1. Concentrate 1000 mL of clarified conditioned media to 200 mL, achieving a 5-fold concentration.
2. First diafiltration: perform buffer exchange with 1 diavolume (200 mL) of Diafiltration buffer 1.
3. Nuclease treatment: add salt-tolerant nuclease (e.g. Saltonase, Qiagen at 50 U/mL) into the feed vessel and incubate for one hour with recirculation at 20 mL/min
4. Second diafiltration: perform buffer exchange with 5 diavolumes of Diafiltration buffer 2
5. Retentate collection: at the end of the buffer exchange, concentrate the retentate to 170 mL and empty the feed tubing into the new retentate vessel.
6. Flush the retentate tubing with 30 mL of Diafiltration buffer 2 and merged with the retentate, resulting in 200 mL of the final TFF retentate sample.

7. Filtration: remove the visible aggregates that may be formed during TFF in the retentate by centrifugation or filtration (0.45 µm – 0.8 µm) before loading to the column using syringe filters such as Minisart® Syringe Filter (Sartorius).

EV Separation on CIMmultus EV and CIMmultus QA HR

The procedure describes column preparation and chromatographic procedure for CIMmultus EV (2 µm)-1 and 4 mL, and CIMmultus QA HR (2 µm)-1 and 4 mL columns. The solutions are provided in Table 2. Note the different solutions used for neutralization, cleaning, and storage of each column line.

Table 2: *Buffers and solutions used for chromatography procedure.*

Buffer | Solution	Buffer composition for CIMmultus EV	Buffer composition for CIMmultus QA HR
Chromatography buffer A	30 mM BTP 50 mM NaCl 2% sorbitol pH 7.5	
Chromatography buffer B	30 mM BTP 2 M NaCl 2% sorbitol pH 7.5	
Neutralisation buffer	0.5 M TRIS + TRIS HCl, 1 M NaCl, pH 7.5	0.5 M Phosphate buffer pH 6.5 or 1 M Sodium acetate pH 5.5
Cleaning-in-place (CIP) buffer	1 M NaOH 2 M NaCl	0.1 M NaOH 2 M NaCl
Storage solution	20 % EtOH in dH_2O	20% EtOH in 20 mM sodium acetate pH 5.5

Chromatography setup:

The described experimental procedure was performed on Akta Pure 25M (Cytiva), equipped with a preparative light scattering detector (Sartorius BIA Separations PATfix MALS 3601 detector) that measures LS at 90° angle. The absorbance was monitored at 260 and 280 nm.

- CIMmultus EV and CIMmultus QA HR columns must be connected in the direction indicated by the flow arrow on the column housing, and the flow should not be reversed.
- These columns are delivered in their respective storage solutions. It is recommended to wash the column with deionized water, sanitize, and regenerate it before use.
- Additionally, performing a run without a sample is advised to establish a baseline for comparing experimental results. Note that certain buffer components can absorb UV light, and transitions between buffers may create refractive index artifacts, which can complicate the interpretation of experimental data.
- Before connecting the column, ensure that the chromatography system is flushed with water and that the pressure release valve is set to the maximum column pressure (1.8 MPa for 1 mL columns, and 2 MPa for 4 mL columns). Wash the column with deionized water to prevent the mixing of incompatible solutions before proceeding to equilibration.
- Flow Rate: Refer to the Instructions for Use (IFU) for the recommended flow rates for your columns. The described runs were performed at 3 CV/min.

Chromatography procedure:

1. Equilibration: Pump at least 10 CV of Chromatography buffer B and at least 10 CV of chromatography buffer A through the column. Check the output pH and conductivity are the same as buffer A before loading the sample.
2. Loading: Observe operating pressure during sample application. Reduce flow rate if necessary to maintain operating pressure within acceptable limits.
3. Wash: Wash the unbound sample components from the column by applying 15 CV of chromatography buffer A
4. Elution gradient: Perform elution in linear gradient reaching 100% of Chromatography buffer B in 15 CV. Collect the fractions with EVs.

5. CIP: Perform cleaning-in-place using at least 10 CV of the appropriate CIP solution. A reduced flow rate is suggested for column cleaning and an extended contact time. After CIP flush the column with at least 15 CV of dH2O after CIP.
6. Neutralization: Flush the column with 15 CV of Regeneration buffer, followed by 15 CV of dH2O.
7. Storage: Store the column in the respective Storage solution.

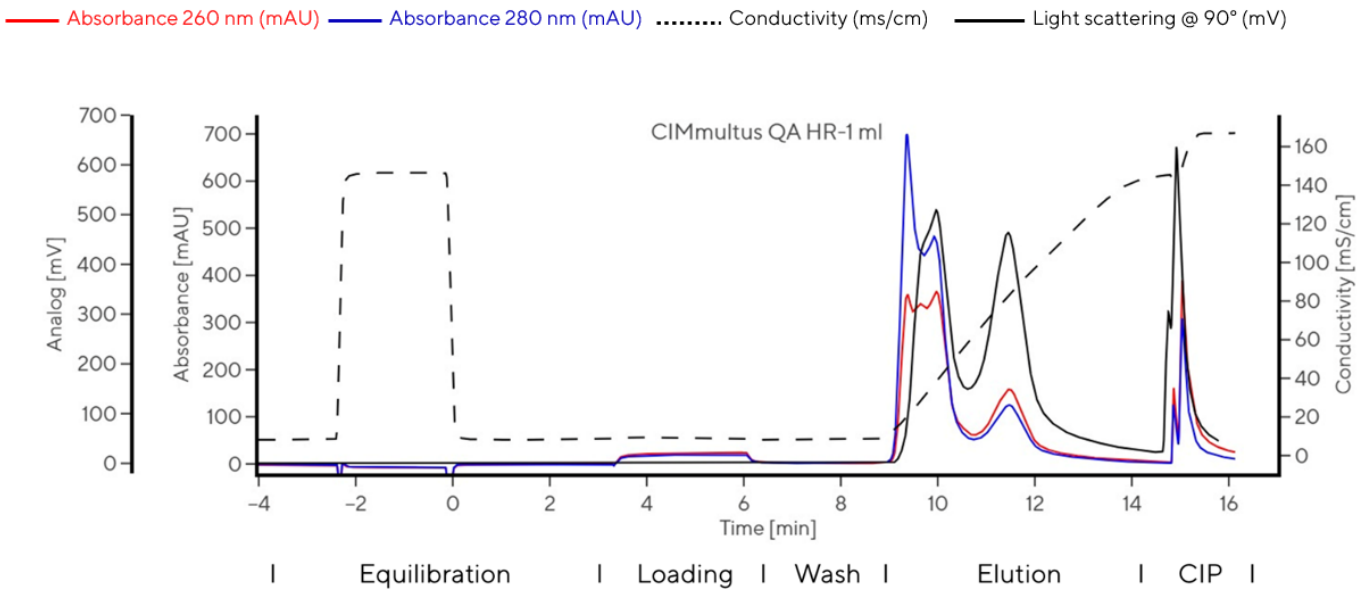

Figure 1: *Example chromatogram with equilibration using CIMmultus QA HR column.*

Comparison of EV Elution Profiles on CIMmultus EV and CIMmultus QA HR

One TFF retentate was prepared for all 4 chromatographic runs. An equal loading volume was injected to CIMmultus EV and CIMmultus QA HR 1 mL columns, and 4x larger volume was loaded on CIMmultus 4 mL columns. About 4E+11 total particles were loaded per 1 mL of monolithic support (measured by NTA).

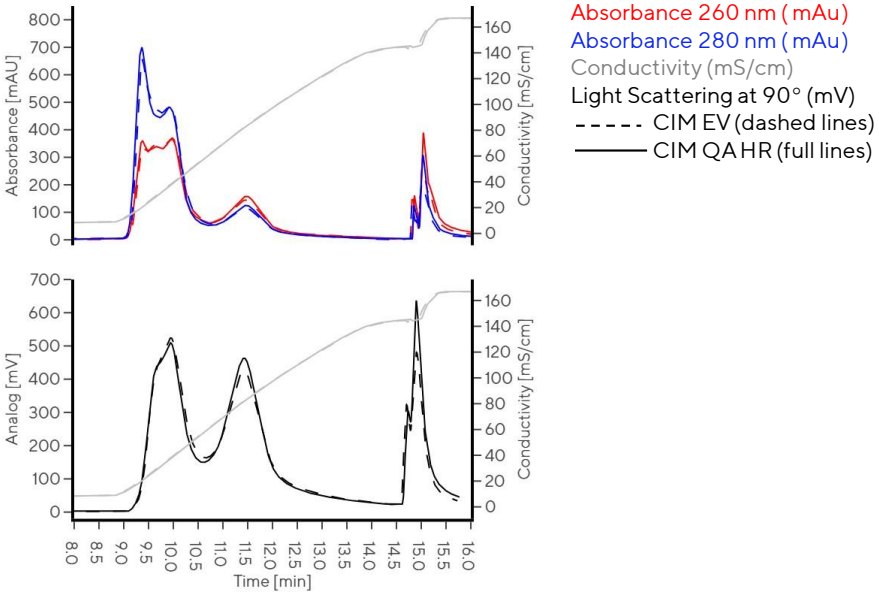

Figure 2: Overlay of the elution profile on EV and QA HR column on UV detector (Absorbance measured at 280 nm) and LS detector (Scattering measured at 90° angle). The profile from CIMmultus QA HR-1 mL is shown in full line and from CIMmultus EV-1 mL in dashed line.

EVs were separated in the linear elution gradient using the above purification protocol. UV and LS signals in the elution were reproducible between CIMmultus EV and CIMmultus QA HR, shown in Figure 2. The comparability between columns was observed on 1- and 4-mL column scale with elution profiles of all columns shown in Figure 3.

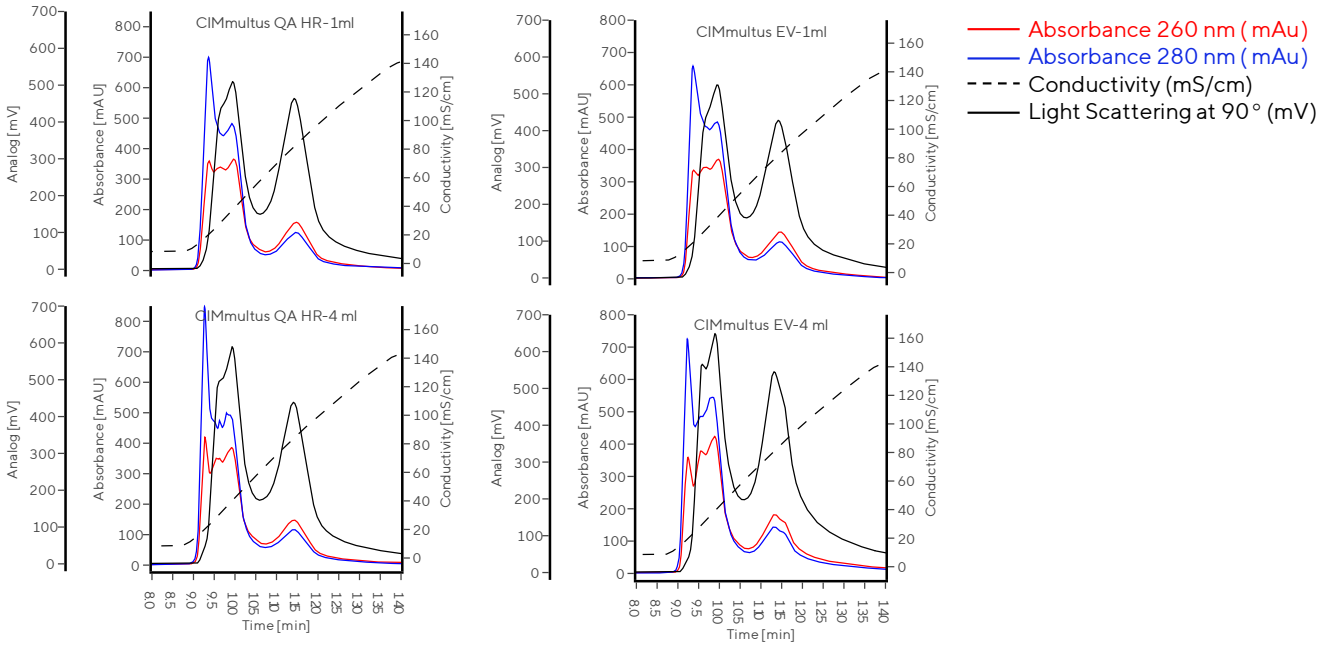

Figure 3: *Elution profiles on QA HR 1- and 4-mL column and CIMmultus EV 1- and 4-mL columns on UV detector (Absorbance measured at 260 nm, 280 nm) and LS detector (Scattering measured at 90°).*

Chromatographic fractions from CIMmultus EV and CIMmultus QA HR columns were collected and analyzed for CD63eGFP fluorescent EVs. The analysis was conducted using a multi-detector analytical chromatography platform, PATfix [6][7], coupled with a size exclusion chromatography (SEC) column. Each sample was injected into the column, where the particle-related peak was separated from smaller molecules. CD63eGFP EVs emit a fluorescence signal, which was measured in the particle-related peak (void volume of the SEC column). The fluorescence peak area was measured, accounted for dilution and sample volume, and the percentage of EVs was calculated in each fraction compared to the column load (TFF retentate). The EV recovery was plotted in Figure 4 for each column. The distribution of fluorescent particles in Peak 1 and Peak 2 was comparable across all tested columns. The recoveries of CD63eGFP EVs were calculated for all chromatographic runs (Table 3) and found to be comparable on both EV and QA HR columns.

Table 3: *Overview of recoveries of CD63eGFP particles in chromatographic fractions. The recoveries are based on PATfix SEC fluorescent peak area calculations. The relative error of the analytical method was 10%.*

			Total recovery HEK293T CD63 eGFP EVs on column	
Column	CIMmultus EV – 1 mL	CIMmultus QA HR – 1 mL	CIMmultus EV – 4 mL	CIMmultus QA HR– 4 mL
HEK CD63eGFP EV percentage in elution fractions	96%	96%	99%	96%

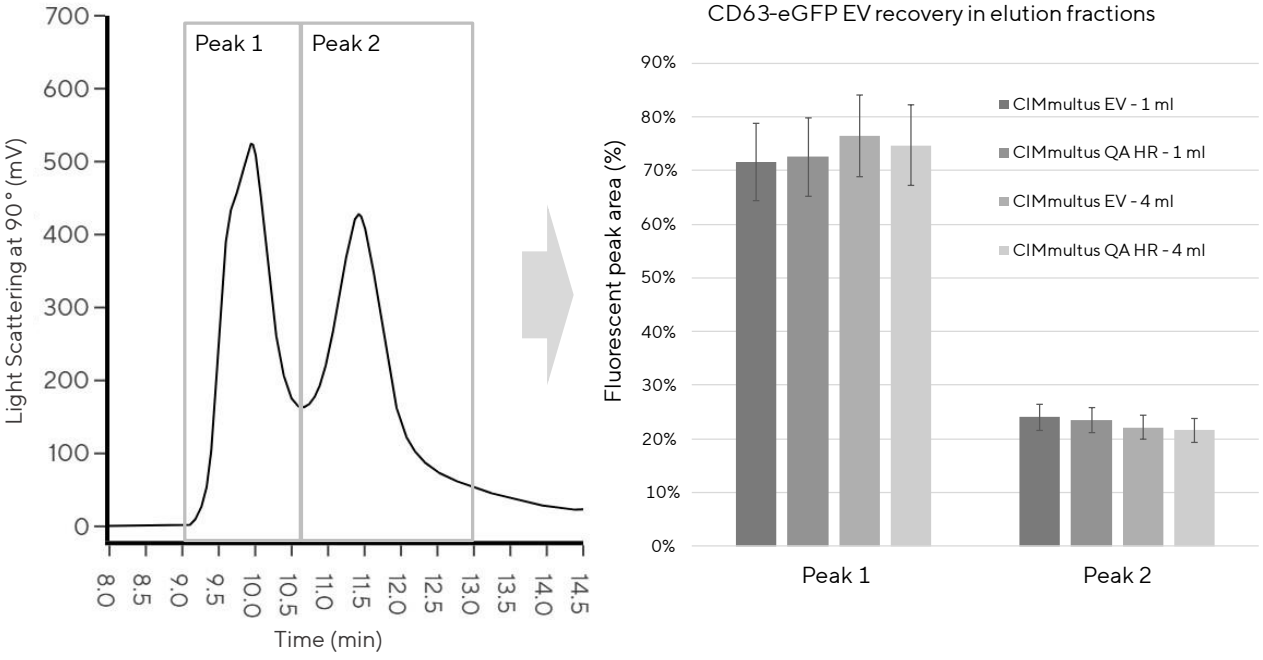

Figure 4: *Distribution of HEK293T CD63-eGFP EVs in main peaks collected from CIMmultus EV and CIMmultus QA HR, 1 mL and 4 mL columns. The collection of peaks was based on Light Scattering signals in preparative runs (left). The fractions were analyzed on the PATfix system, coupled with a SEC column. The percentage of fluorescent EVs in collected fractions is plotted on the graph (right). Comparable recoveries were measured in fractions from CIMmultus EV and QA HR columns.*

Conclusion

HEK-derived EVs were isolated from clarified conditioned media using TFF with nuclease, and anion exchange chromatography. The same procedure was performed on CIMmultus EV and CIMmultus QA HR columns, on 1- and 4-mL scales. The elution profiles on both columns are comparable and the recovery of EVs is reproducible within the margin of error of the analytical method. The protocol developed for CIMmultus EV column can be applied to CIMmultus QA HR columns with reproducible results.

Acknowledgment: We would like to thank Bernd Giebel (University Hospital Essen, Institute of Transfusion Medicine) and his team for kindly providing the HEK293 CD63-eGFP cell line used in experiments.

Ordering Information

Cat. No.	Product Name
BIA-311.5213-2	CIMmultus® QA HR - 1 mL (2 µm)
BIA-414.5213-2	CIMmultus® QA HR - 4 mL (2 µm)
BIA-411.5213-2	CIMmultus® QA HR – 8 mL (2 µm)
BIA-904.5213-2	CIMmultus® QA HR – 4 mL (2 µm) - cGMP
BIA-901.5213-2	CIMmultus® QA HR – 8 mL (2 µm) - cGMP
BIA-914.5213-2	CIMmultus® QA HR – 40 mL (2 µm) - cGMP
BIA-911.5213-2	CIMmultus® QA HR – 80 mL (2 µm) - cGMP
BIA-924.5213-2	CIMmultus® QA HR – 400 mL (2 µm) - cGMP
BIA-921.5213-2	CIMmultus® QA HR – 800 mL (2 µm) - cGMP
BIA-934.5213-2	CIMmultus® QA HR – 4000 mL (2 µm) - cGMP
BIA-931.5213-2	CIMmultus® QA HR – 8000 mL (2 µm) - cGMP

References

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Germany
Sartorius Stedim Biotech GmbH
August-Spindler-Strasse 11
37079 Goettingen
Phone +49 551 308 0

Slovenia
Sartorius BIA Separations d.o.o.
Mirce 21
5270 Ajdovščina
Phone +386 59 699 500

USA
Sartorius Stedim North America Inc.
565 Johnson Avenue
Bohemia, NY 11716
Toll-Free +1 800 368 7178

For more information, visit
www.sartorius.com
www. biaseparations.com

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