

## Optimization of Upstream Extracellular Vesicle Processes Using PATfix® Analytical Tools

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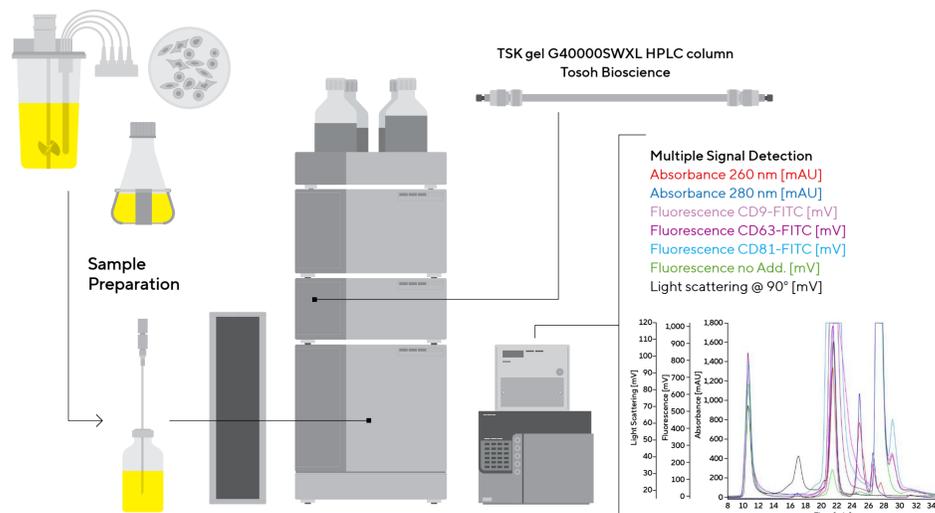
### Introduction

Extracellular vesicles (EV) are lipid-bound products secreted by cells. Among them, exosomes have great potential for clinical applications. Animal- and human-derived components used in cell culture, such as fetal bovine serum (FBS), naturally contain exosomes that can contaminate the desired product. To facilitate a more accurate analysis of exosomes derived from specific cells of interest, multiple manufacturers have developed exosome-depleted FBS (EV (-) FBS) via different approaches. In this work, we evaluated commercially available EV (-) FBS supplements for residual exosome content and tested their performance in an upstream exosome production process. The analysis was performed on a PATfix® system using PATfix® size exclusion chromatography (SEC) as an analytical method.

### 1. Exosome Markers Using PATfix®

Multiple-detector PATfix® technology enables the simultaneous detection of absorbance (monitored at 260 nm and 280 nm), fluorescence (Ex: 488 nm and Em: 520 nm), and light scattering emitted/scattered from the sample (monitored at 90° angle). In this case, the fluorescence detector was used to detect membrane bound exosome markers. Samples were spiked with antibodies, conjugated to a fluorescent probe, and incubated until they bound to exosome antigens of interest. Excess antibody is separated from the exosomes by SEC, while exosomes are too large to enter the pores of the chromatography media and are eluted in the void volume. The peaks of fluorescent-labeled exosomes are analyzed using PATfix® software (Figure 1).

Figure 1: A Flow Chart of the SEC PATfix® Analytical Method

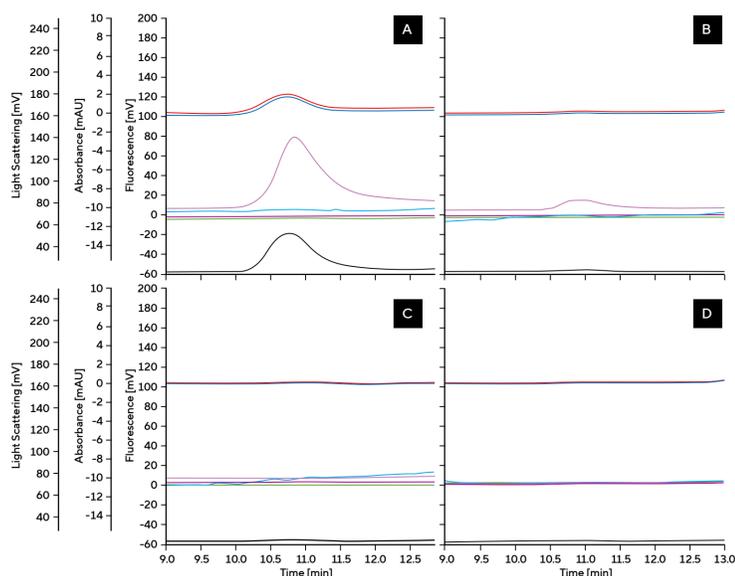


Note. Samples were mixed with a specific antibody (FITC anti-human CD63, FITC anti human CD9) and incubated in the autosampler of the PATfix® system until the response was stable. The separation is performed on SEC TSK gel G4000SWXL column (Tosoh Bioscience) at a flow rate of 0.5 mL/min. Control samples are prepared without any antibodies. An example of signal recording is shown on the right, each signal labelled with a different color noted in the legend above.

### 2. Screening Input Materials for Exosome Production

Various FBS products were tested for exosome markers (Figure 2). Regular FBS is known to contain serum derived exosomes and was used for positive control. Two exosome depleted FBS alternatives intended for exosome production were confirmed to be free of chosen exosome markers (Figure 2C and D). One product contained traces of CD9 positive vesicles. (Figure 2B)

Figure 2: Zoomed in Chromatograms of Different Fbs Supplements Incubated With Fluorescent-Labeled Antibodies Against Exosome Markers



Note. (A) EV(+) FBS - positive control, (B) Exosome depleted FBS (producer 1), (C) Exosome-depleted FBS (producer 2), (D) Exosome-depleted FBS (producer 3)

### 3. Screening of Upstream Conditions for Exosome Production

An adherent HEK293 cell line expressing green fluorescent protein tagged tetraspanin CD63 (HEK293 CD63 eGFP) was used in this screening. Cells were cultivated in DMEM (high glucose, with GlutaMAX™, without phenol red) supplemented with 10% FBS. At the first passage, cells were split and grown for three subsequent passages in the presence of different FBS supplements (Table 1).

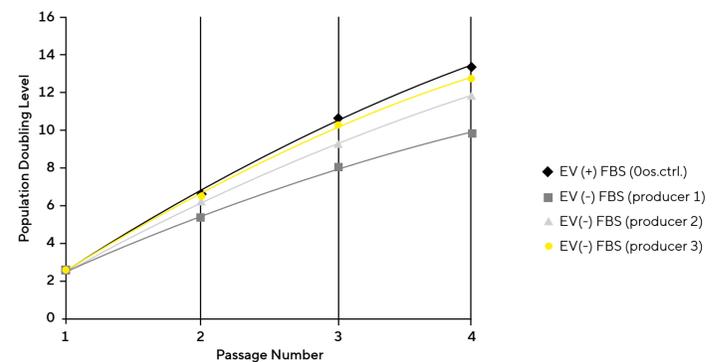
Cell culture data for different FBS supplements (Table 1, Figure 3) shows that growth is reduced in all EV (-) FBS when compared to EV (+) FBS. Viability in all samples was above 90%. The observed differences between EV (-) FBS are a result of the distinct approaches to removing exosomes during production.

During production, CD63 eGFP is integrated into exosomes, which allows detection of these vesicles with the fluorescent monitor on the PATfix® system. Conditioned media samples collected from the third passage and analyzed via PATfix® SEC are shown in Figure 4. The highest CD63 eGFP response was measured with EV (-) FBS from producer 3, where we also observed the best cell growth, compared to other EV (-) FBS

Table 1: Cell Density and Viability of HEK293 CD63-egfp in Passage at Which Conditioned Media Was Collected for Analysis. Samples Were Collected From the Third Passage After 72H in Culture.

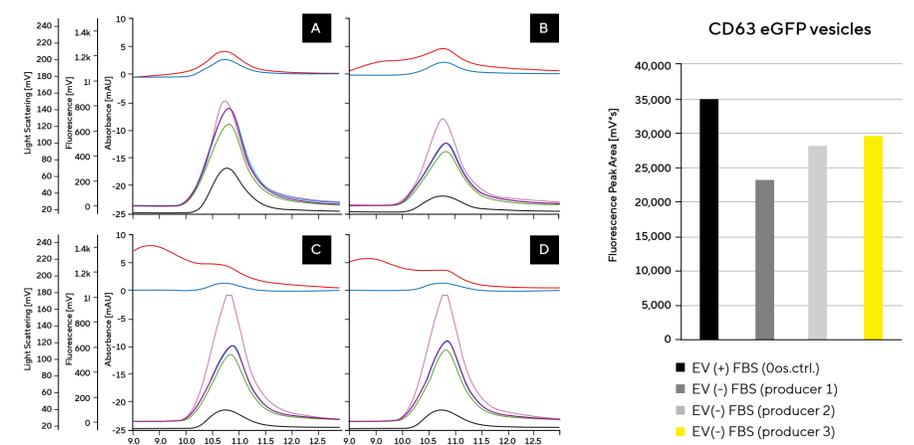
	EV (+) FBS (positive.ctrl)	EV (-) FBS producer 1 EV	EV (-) FBS producer 2	EV (-) FBS producer 3
Cell density [cells/cm <sup>2</sup> ]	2.32E+05	9.45E+04	1.16E+05	2.00E+05
Viability (% viable cells)	98.3	91.0	98.6	97.8

Figure 3: Effect of Different EV (-) Fbs Supplements on HEK293 CD63-eGFP Cell Growth Compared to EV (+) FBS (In Black)



Note. All EV (-) FBS supplements reduced cell growth to some extent.

Figure 4: Zoom in chromatograms of supernatants from adherent HEK293 cells expressing CD63-eGFP cultivated in different media



Note. (A) HEK293 CD63-eGFP in EV (+) FBS, (B) HEK293 CD63-eGFP in EV (-) FBS (producer 1), (C) HEK293 CD63 eGFP in EV (-) FBS (producer 2), (D) HEK293 CD63-eGFP in EV (-) FBS (producer 3), (E) Comparison of intrinsic CD63 eGFP fluorescence signal peak area (mV\*s) in upstream

### 4. Conclusion

The data presented here demonstrate that:

- PATfix® SEC analytics is a powerful analytical tool for screening both raw materials and exosome harvests and can complement common process development approaches.
- Exosomes labeled with fluorescent antibodies, or eGFP labeled exosomes can be tracked using a fluorescence detector on the PATfix® system.
- Among EV depleted FBS, the one from producer 3 performs best in the upstream process.

### Acknowledgements

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