A041

# Chromatographic separation of full and empty Adeno-Associated Virus (AAV) particles on CIM® monoliths

Adeno-Associated Virus (AAV)-based vectors of various serotypes are considered to have high potential in human gene therapy and genetic vaccination applications. During manufacturing of AAV vectors undesired, incomplete particles are co-produced. They lack recombinant viral genomes and consist of empty capsid proteins only. Empty capsids increase the required dose of AAV virus for medical applications and are thought to cause immunological reactions against the vector capsid, leading to unwanted side effects. Removal of empty capsids during manufacturing, as well as the ability to quantify the amount of empty AAV particle content in a formulation is hence a critical requirement for any AAV production process.

Current methods for preparative separation of empty capsids (CsCl or iodixanol gradients) are challenging to scale-up and are not suitable for large-scale production. Furthermore, analytical methods for detection of empty capsids and determination of full to empty particle ratio (electron microscope (EM) assay, total particle assay [ELISA] combined with genome copy titration [qPCR]) are time- and labour consuming, influenced by operator technique or do not provide readily available reagents for different serotypes of AAV.

A new approach for separation of full and empty AAV8 particles by exploiting minor charge differences is presented in this application note. By using linear gradient elution on a CIM QA Disk Monolithic Column, a simple, rapid and reproducible assay for analysis of AAV particles is introduced. The method was successfully applied to AAV8 particles prepared by two different manufacturing processes.

## MATERIAL AND METHODS

Virus preparations of purified full and empty AAV8 vector particles were prepared by CsCl gradient centrifugation. An alternative preparation of AAV8 was performed by tangential flow filtration (TFF) followed by an iodixanol gradient.

| | |
|---|---|
| Column: | CIM® QA Disk Monolithic column, bed volume of 0,34 mL |
| Mobile phases: | Buffer A: 20 mM Bis-Tris propane (BTP), pH 9, 50 mM NaCl<br>Buffer B: 20 mM BTP, pH 9, 1 M NaCl |
| Flow rate: | 3 mL/min |
| Gradient elution method: | Wash after load: 10 columns volumes (CV) buffer A<br>Linear gradient: 80-115 mM NaCl<br>High salt wash: 1M NaCl in 20 mM BTP, pH 9 for 10 CV |
| Sample: | An equivalent to $1x10^{12}$ or $5x10^{12}$ genome copies (GC) of full AAV8 vector preparations<br>75 µL of empty AAV8 vector preparations (CsCl gradient)<br>Diluted in 10 mL buffer A |
| Sample loop: | 10 mL |
| Detection: | UV detection, 280 nm |


Publication #: A041-AAV-1505

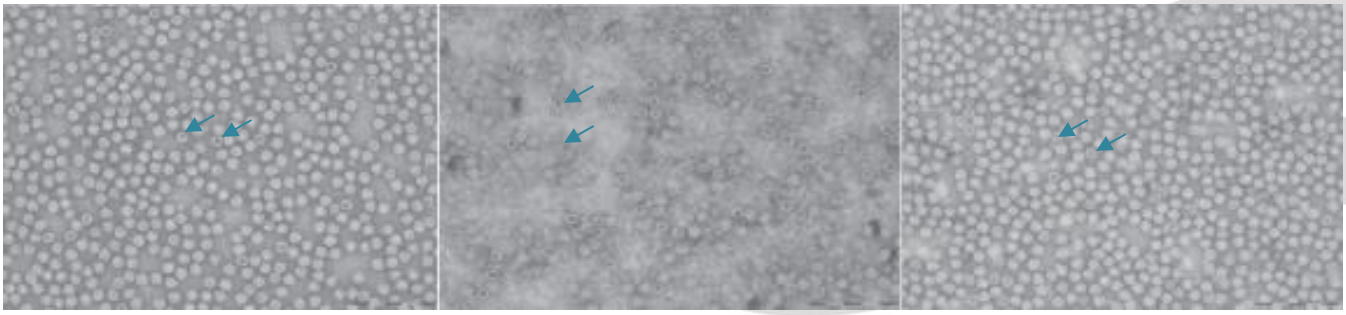
## RESULTS

### *Electron microscopy*

The presence of full and empty particles in AAV8 preparations preparations was analyzed by electron microscopy. Empty particles are indicated by arrows (Figure 1).

***> Figure 1***

CsCl vector fraction, full particles | CsCl vector fraction, empty particles | Iodixanol vector fraction, full particles



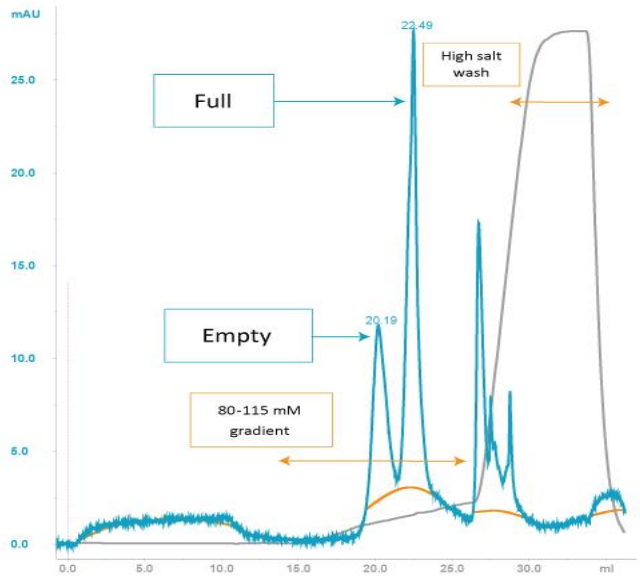
6,28 % empty particles | Empty particles | 4,4 % empty particles

### *Chromatographic separation of full and empty AAV particles*

Initial binding-elution experiments with CsCl gradient preparations of full and empty AAV8 vector particles were performed. Different retention volumes at linear salt gradients indicated that chromatographic separation of full and empty vector particles is possible (data not shown).

Based on these results a mixture of full and empty particles was applied to a CIM QA Disk Monolithic Column (Figure 2). An arbitrary volume of 75 µL of an empty capsid preparation was mixed with $1x10^{12}$ GC of a preparation containing 93.7% full AAV vector particles (as measured by EM) (Figure 2).

***> Figure 2***




| Peak | Retention [mL] | Area [mAU*mL] | Height [mAU] |
|---|---|---|---|
| 1 | 20,19 | 9,2455 | 9,387 |
| 2 | 22,49 | 17,6555 | 24,557 |
| | | | |
| Empty: Full ratio | 0,52:1 | | |
| % Empty | 34 | | |

Clearly distinguishable peaks representing empty and full capsids were detected. The later elution peak contained >99% of the loaded full particles (determined by qPCR). The calculated relative amount (according to measured peak areas) of empty capsids was 34% (Figure 2).

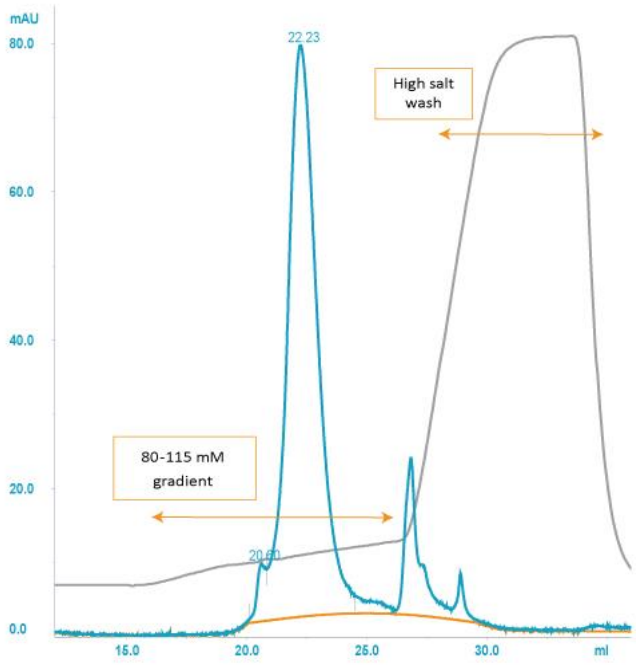

Publication #: A041-AAV-1505

Chromatographic separation of AAV8 capsids was then used for analysis of full to empty particle ratio in different AAV preparations. Based on chromatographic peak integration the quantification of an AAV preparation by CsCl gradient (93.7% full as measured by EM) resulted in an empty-to-full ratio of 0.016 (1.6% empty particles), compared to 0.067 (6.28% empty particles) as determined by EM analysis (data not shown).

### *Chromatographic separation and quantification of AAV particles produced by alternative process*

The applicability of CIM QA-based separation and quantification was next assessed for an alternative AAV preparation (prepared by TFF followed by iodixanol gradient). AAV preparation that contained an equivalent of $5x10^{12}$ GC was loaded on CIM QA disk and separated with linear gradient elution as depicted on the chromatogram (Figure 3). Five times more AAV particles were loaded compared to AAV prepared by CsCl (Figure 2), which resulted in broadening of the elution peak. Intergration of peak areas for a minor peak eluting at 20.0 min and major peak eluting at 22.2 min (Figure 3) suggested an empty-to-full ratio of 0.026 (2.5% empty particles), in good agreement with the ratio obtained by EM (empty-to-full ratio of 0.05; 4.5% empty particles).

***> Figure 3***



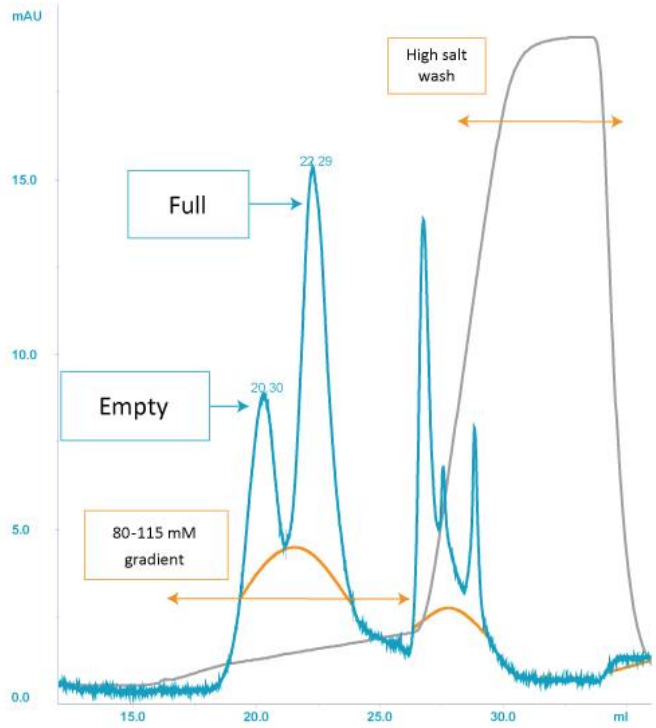

| Peak | Retention [mL] | Area [mAU*mL] | Height [mAU] |
|---|---|---|---|
| 1 | 20,6 | 2,9021 | 7,421 |
| 2 | 22,23 | 109,9566 | 76,568 |
| | | | |
| Empty: Full ratio | 0,026:1 | | |
| % Empty | 2,5 | | |

To demonstrate the applicability of the method to different AAV production processes, AAV sample was spiked with an empty particle preparation an injected on the CIM QA disk using the same gradient conditions (Figure 4).


Publication #: A041-AAV-1505

*Separation and quantification of AAV particles (TFF and iodixanol gradient), spiked with empty capsids AAV*

***> Figure 4***




| Peak | Retention [mL] | Area [mAU*mL] | Height [mAU] |
|---|---|---|---|
| 1 | 20,3 | 4,9253 | 4,887 |
| 2 | 22,29 | 12,9095 | 11,03 |
| | | | |
| Empty: Full ratio | 0,382:1 | | |
| % Empty | 27,6 | | |

## CONCLUSIONS:

A rapid and reproducible method for separation and quantification of empty and full particles AAV8 vector by linear gradient elution on CIM QA disk was developed. The protocol was succesfully employed for two different preparations of AAV and enabled quantification of empty capsid content that was in line with alternative methods. The advantages of the chromatographic method are simplicity, speed, independence from operator technique and need for specialized reagents. Instead, it offers quantitative analysis of particle contect in a single assay, with a run time of 30 min.

More details can be found in the following article:

1. M. Lock, M. R. Alvira, J.M. Wilson: Analysis of Particle Content of Recombinant Adeno-Associated Virus Serotype 8 Vectors by Ion-Exchange Chromatography. Human Gene Therapy Methods: Part B 23:56-64 (2012)

## For any additional information please contact us:

Tel.: +386 5 9699 500 | sales@biaseparations.com | www.biaseparations.com





Publication #: A041-AAV-1505