

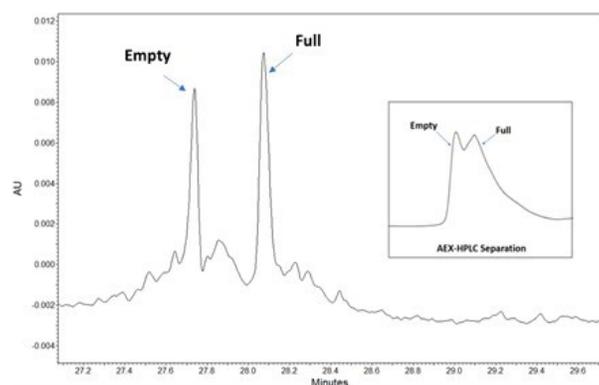
# Determination of Full, Partial and Empty Capsid Ratios for Adeno-Associated Virus (AAV) Analysis

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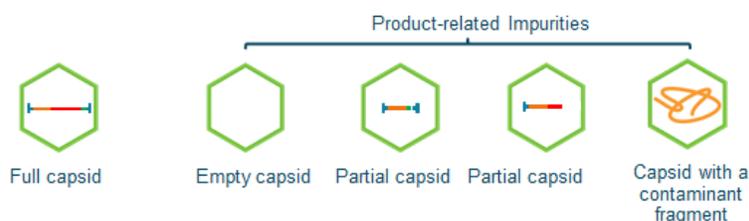
## Introduction

**Adeno-associated virus (AAV)** is one of the most commonly used delivery vehicles in gene therapy development. The rAAV is made of a shell of proteins, called capsid, encompassing a single-stranded DNA called a transgene.<sup>1</sup> During the manufacturing of AAV vectors, a high percentage of capsids might not incorporate any of the transgenes and are referred to as empty capsids. Additionally, capsids that contain fragments of the transgene are called partial capsids. These undesired product-related impurities are co-produced with the full capsids which contain the full length of the desired transgene. The presence of these impurities could affect the efficacy and safety of AAV vector products because of their risk for increasing immunogenicity of the AAV product. In addition, it can inhibit transduction of full capsids by competing for vector binding sites on cells.<sup>2</sup> The ability to determine the amount of these impurities along with the drug product is therefore a critical requirement for any AAV production process and quality control.

There are multiple technologies being used concurrently for determining the ratios of these empty or partially filled capsids along with the full AAV such as analytical ultracentrifugation (AUC), transmission electron microscopy (TEM), etc.<sup>3,4,5</sup> However, these traditional methodologies have their own set of challenges and hence drive a need for a parallel technique which is faster and easier to perform.



**Figure 2.** Separation of AAV serotype 5. Shown is an electropherogram with well resolved peaks between the empty and full capsids with 65nL of 1x10<sup>13</sup> GC/ml sample load. The inset shows chromatogram of a 30ul load of the same sample analyzed with anion exchange chromatography.



**Figure 1.** Along with the full capsid containing the transgene of interest, various product related impurities can be present such as empty capsid with the transgene missing, partial or truncated fragments of the gene or capsid with contaminant gene from the host cell

This technical note demonstrates a robust capillary isoelectric focusing (cIEF)-based method for AAV full and empty capsids analysis that can be completed in less than an hour. The results show excellent resolution between full and empty capsid as well as potential partial capsid peaks for determination of their ratios. It is also capable of analysis of different serotypes of AAV. The results from this methodology correlate well with orthogonal approaches such as anion exchange high performance liquid chromatography (AEX-HPLC), although AEX-HPLC provides less resolving power for these species and can struggle to quantify a smaller abundant peak vs. a closely eluting dominant peak.

## Key Features

- A cIEF based platform method with the capability of method optimization for optimal separation of full and empty capsids for AAV samples across multiple serotypes
- This methodology has high resolving power of separating full and empty AAVs with very small pI differences ( $\leq 0.1$  pH unit)
- Provides rapid analysis time with less than 1 hour per sample compared to traditional methods such as AUC and EM which can take days
- Good data correlation of full and empty capsid ratio with orthogonal technologies such as AEX-HPLC is obtained

## cIEF Experimental

### Instrument

All cIEF experiments were performed using a PA 800 Plus Pharmaceutical Analysis System (SCIEX, Brea, CA) equipped with a UV detector and a 280 nm filter (P/N 969136) as shown in Figure 1. Data were collected and analyzed using 32 Karat™ Software. The installed N-CHO capillary (SCIEX, P/N 477601) was 30.2 cm long (20 cm from injector to detector). The capillary temperature was maintained at 20° C in all separations. Normal polarity was used during voltage application.

### Chemicals and Materials

The cIEF gel (P/N 477497) and cIEF peptide marker kit (PN A58481) were purchased from SCIEX.

The anolyte solution (A), catholyte solution (C), chemical mobilizer solution (CM) and capillary cleaning solution (U) were prepared as follows for buffer tray set up. The symbol in parentheses were used for buffer tray configuration in Figure 8.

**Anolyte Solution (A)** Anolyte solutions of 200 mM phosphoric acid was prepared by adding 0.685 mL 85% phosphoric acid to a total volume of 50 mL with DDI water.

**Catholyte Solution (C)** Catholyte solutions of 300 mM sodium hydroxide were prepared by adding 15 mL of 1 M NaOH (Sigma 720820) to a total volume of 50 mL with DDI water.

**Chemical Mobilizer (CM)** Chemical mobilizer solutions of 350 mM acetic acid was prepared by adding 1 mL of glacial acetic acid to a total volume of 50mL with DDI water.

**Capillary Cleaning Solution (U).** Capillary cleaning solution was 4.3M urea.

The cathodic stabilizer solution, anodic stabilizer solution and 3 M urea-cIEF gel solution were prepared as follows:

**Cathodic Stabilizer Solution.** Cathodic stabilizer solution of 500 mM L-Arginine was prepared by dissolving 0.87 g of L-Arginine (98%) (Sigma P/N A5006) in 8 mL of DDI water, mixing for 15 min for complete solvation, and finally scaling up to a total of 10 mL with DDI water.

**Anodic Stabilizer Solution.** Anodic stabilizer solution of 200 mM iminodiacetic acid (IDA) was prepared by dissolving 0.27 g of iminodiacetic acid (98%) (Sigma P/N 220000) solid in 8 mL DDI water, mixing for 15 min for complete solvation, and finally increasing the total volume to 10 mL with DDI water.

**3 M Urea-cIEF Gel Solution (U-Gel).** A 3M urea cIEF gel solution was prepared by dissolving 1.8 g of urea (Sigma P/N U1250) in 7 mL of cIEF gel (P/N 477497). Once dissolved, the solution was

made up to a total of 10 mL with cIEF gel, mixed for 15 min, and then filtered using a 5 µm syringe filter. The 3 M urea-cIEF gel solution was degassed at 2,000 RCF with an Allegra X 15 R centrifuge (Beckman Coulter P/N 392933) and stored at 2-8° C.

### Sample Preparation

**Master Mix Solution.** Master mix solution was prepared by mixing the reagents with the following volumes: 200 µL of 3M urea-cIEF gel solution, 12 µL of ampholytes, 20 µL of cathodic stabilizer, 2 µL of anodic stabilizer, 2 µL of each pl marker. Buffer exchange may needed for AAV samples to reduce the current of focusing step and improve the life time of the capillary.

Different serotypes of AAV were analyzed to show the robustness of this methodology across various serotype. The serotypes used were as follows:

**Proprietary Serotype AAV Samples.** A set of two AAV samples of proprietary serotype were used. Sample #1 is the sample with enriched empty capsids while sample #2 is the sample with enriched full capsids. The concentration of these two samples was concentrated to approximately 2 mg/mL from 0.1 mg/mL using Amicon Ultra 0.5mL Centrifugal Filters (NMWL 10KDa) from EMD Millipore (PN UFC501096). 10 µL of each AAV sample was mixed with 240 µL of Master Mix solution and transferred to sample vials for analysis on a PA 800 Plus Pharmaceutical Analysis System. The ampholytes used in this master mix were Pharmalyte 3-10 Carrier Ampholytes from GE Healthcare Life Sciences (PN 17045601).

**AAV Serotype 5 Sample.** An AAV5-CMV-GFP(Cat# SL100819, Lot# AAV62019) sample from SignaGen Laboratories was also used with a titer ~1X 10<sup>13</sup> GC/mL. 3 µL of AAV sample was mixed with 24 µL of master mix and transferred to a nanoVial (SCIEX, P/N 5043467) for analysis on a PA 800 Plus Pharmaceutical Analysis System. The ampholytes used in this master mix were also Pharmalyte 3-10 Carrier Ampholytes from GE Healthcare Life Sciences.

**AAV Serotype 8 Sample.** Packaged AAV8 of pAV-CMV-GFP Empty Capsids sample with a titer of 5.10 X10<sup>12</sup>GC/mL was used as the AAV8 sample with enriched empty capsids, while Packaged AAV8 of pAV-CMV-GFP sample with titer of 1.10 X10<sup>13</sup>GC/mL was used as the AAV 8 sample with enriched full capsids. These two AAV 8 samples were purchased from Vigene Biosciences (Lot# 2019.09.12). 3 µL of each AAV8 sample was mixed with 24 µL of master mix and transferred to a nanoVial (SCIEX, P/N 5043467) for analysis. A mixture of Pharmalyte 3-10 wide pH range ampholyte from GE Healthcare Life Sciences ((PN 17-0456-01) and SERVALYT 6-8 narrow pH ampholyte which is from Serva Serving

Scientists (PN 42906.04) at a ratio of 4:2 were used as the ampholytes in the master mix solution.

**AAV Serotype 9 Sample.** An AAV9-CMV-GFP(Cat# SL100840) sample from SignaGen Laboratories was also used with a titer at  $3.12 \times 10^{13}$  GC/mL. 3  $\mu$ L of AAV 9 sample was mixed with 24  $\mu$ L of master mix and transferred to a nanoVial (SCIEX, P/N 5043467) for analysis on a PA 800 Plus Pharmaceutical Analysis System. The ampholytes used in this master mix were Pharmalyte 3-10 Carrier Ampholytes from GE Healthcare Life Sciences ((PN 17-0456-01) ) for wide pH range ampholytes analysis. A mixture of Pharmalyte 3-10 wide pH range ampholyte from GE Healthcare Life Sciences and SERVALYT 6-8 narrow pH ampholyte which is from Serva Serving Scientists (PN 42906.04) at a ratio of 4:2 were used as the ampholytes in the master mix solution for optimized analysis conditions.

### Instrument Setup

The instrumental setup parameters for the cIEF method on the PA800 Plus Pharmaceutical Analysis System are summarized in figures 3-8.

The “Initial Conditions” and “UV Detector Initial Conditions” were set up as indicated in Figure 3 and Figure 4, respectively. The same setup parameters were used for the conditioning, separation and shutdown methods.

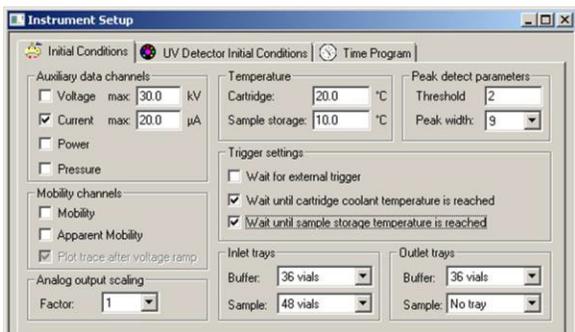


Figure 3. Initial Conditions

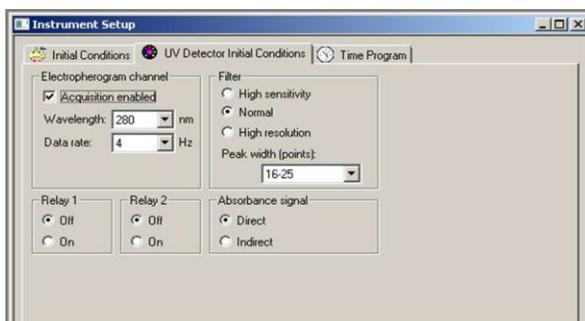


Figure 4. UV Detector Initial Conditions

Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1	Rinse - Pressur	50.0 psi	5.00 min	BI:F1	BO:B1	forward	CM rinse
2	Rinse - Pressur	50.0 psi	2.00 min	BI:B1	BO:B1	forward	H2O Rinse
3	Rinse - Pressur	50.0 psi	5.00 min	BI:E1	BO:B1	forward	Gel
4	Wait		0.00 min	BI:A1	BO:A1		Water dip
5							

Figure 5. Time Program for Conditioning Method

Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1	Rinse - Pressur	50.0 psi	3.00 min	BI:D1	BO:B1	forward, In / Out vial inc 7	CM rinse
2	Rinse - Pressur	50.0 psi	2.00 min	BI:B1	BO:B1	forward, In / Out vial inc 7	H2O Rinse
3	Inject - Pressur	25.0 psi	95.0 sec	SI:A1	BO:B1	Overside, forward	
4	Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 7	Water dip
5	Separate - Volta	25.0 kV	15.00 min	BI:C1	BO:C1	0.17 Min ramp, normal polarity, In / Out vial inc 7	
6	Separate - Volta	30.0 kV	30.00 min	BI:C1	BO:C1	0.17 Min ramp, normal polarity, In / Out vial inc 7	
7	Stop data						
8	Rinse - Pressur	50.0 psi	2.00 min	BI:B1	BO:B1	forward, In / Out vial inc 7	
9	Wait		0.00 min	BI:A1	BO:A1	In / Out vial inc 7	
10	End						
11							

Figure 6. Time Program for Separation Method

Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1	Rinse - Pressur	50.0 psi	2.00 min	BI:B1	BO:B1	forward	CM rinse
2	Rinse - Pressur	50.0 psi	5.00 min	BI:E1	BO:B1	forward	h2o Rinse
3	Lamp - Off						
4	Wait		0.00 min	BI:A1	BO:A1		
5							

Figure 7. Time Program for Shutdown Method

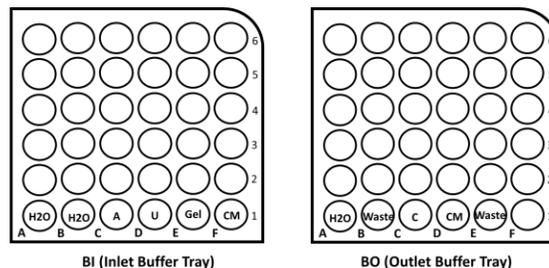


Figure 8. Buffer Tray Configuration

The time program for the conditioning method is illustrated in Figure 5, while the time programs for the separation and shutdown methods are described in Figure 6 and Figure 7, respectively.

The configuration for buffer tray setup is shown in Figure 8.

Each “H<sub>2</sub>O” vial was filled with 1.5 ml D.I. water. Waste vial was

filled with 1 ml D.I. water. "Gel" vial was filled with 1.2 mL cIEF gel

from the cIEF kit. Other vials were filled with 1.5 mL of solutions according to solution symbols.

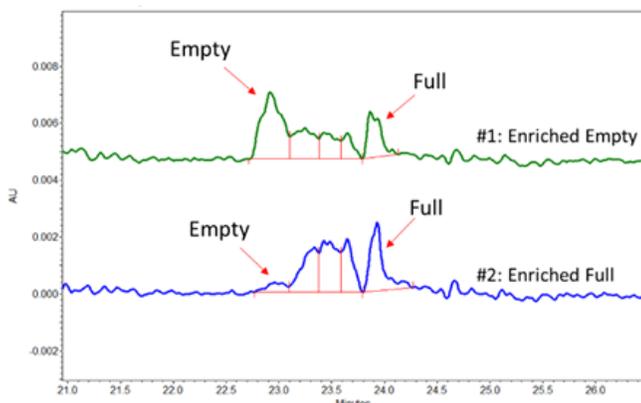
## AEX-HPLC Experimental

The AAV5-CMV-GFP (Cat# SL100819, Lot# AAV62019) sample from SignaGen Laboratories was also analyzed using a CIMac SO3-0.1 AAV Analytical Column from BIA separations (PN 110.6157-1.3) on an ACQUITY UPLC H-class PLUS System from Waters Corporation for the AEX-HPLC analysis following the instruction of AAV Analytical Column<sup>6</sup> for AAV full and empty capsid ratio comparison of orthogonal technologies

## Results and Discussion

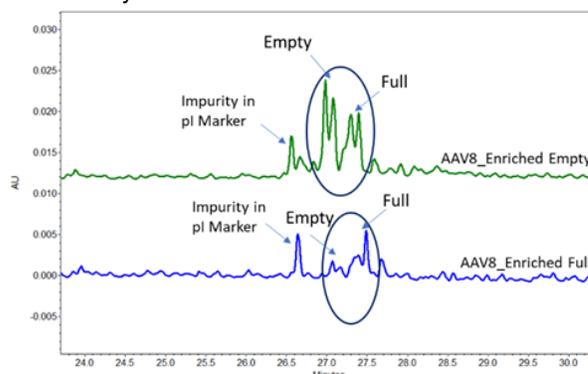
### Existing Methods to Separate AAV Full and Empty Capsids

There are multiple methods that have been used for the determination of the ratio of AAV full and empty viral capsids. One such approach is determining the percentage of the full capsids in the total capsids by dividing the number of genome vectors derived from the existing qPCR data by the total capsid number obtained from the ELISA data.<sup>7</sup> However, this method is limited by its insufficient data accuracy and precision. Another spectrophotometric based method uses the optical density of AAV samples at 260 nm and 280 nm in order to determine the protein and DNA content in the samples.<sup>8</sup> This approach is simple, rapid and easy to operate. However, it requires high purity of the AAV sample to minimize the interference of the impurities with UV absorbance at 260 nm and 280 nm. One of the traditional approaches for empty versus full capsid determination is AUC.<sup>3</sup> This technology is capable to separate full, partial, and empty capsids, but it has several drawbacks such as large sample quantity, high cost, the need of expert operators, the challenge for completing a QC release assay and a lengthy analysis time. TEM is another often used technology in the industry, and it could reliably count the full and empty particles as a population.<sup>4,5</sup>



**Figure 9. cIEF Results of AAV Samples of a Proprietary Serotype.**

However, it is very difficult to distinguish the partial capsids and it is too time consuming for data analysis to meet the need of timely quality control purposes. Ion exchange chromatography is also used for the product purification in the downstream process as well as the quantitative determination of AAV full/empty capsids.<sup>9</sup> It requires a large number of samples and the method is serotype-dependent. Furthermore, it could not distinguish partial capsids from full and empty capsids, and the full and empty capsids are not well resolved. This can result in inaccurate determination of the full and empty capsids ratio. Mass spectrometry based approach such as charge detection mass spectrometry (CDMS) has been recently shown to be able to separate full, partial and empty capsids.<sup>10</sup> However, this system is a non-commercial research system.



**Figure 10. cIEF Results of AAV Serotype 8 Samples. A is AAV8 Empty(E) and B is AAV8 Full (F).**

cIEF results of AAV samples of a proprietary serotype are shown as examples in Figure 9. Two samples of the same AAV product with different amounts of full and empty capsids were analyzed. Sample #1 was enriched with empty capsids, while sample #2 was enriched with full capsids. The cIEF profiles of the AAV samples were shown between pI marker 7.0 and 10.0. The empty capsid peak migrates at higher pI value while the full capsid peak migrates at lower pI value than the empty capsid. Meanwhile, some potential partial capsid peaks appeared to sit between those empty and full capsid peaks because of their moderate pI values. The cIEF profiles were consistent with those profiles obtained by analytical ultracentrifugation (data not shown).

For AAV samples with a pI difference close to or less than 0.1 pH unit between full and empty capsids, the wide-range pH ampholytes failed to provide sufficient baseline resolution for the accurate quantification of the full and empty capsids. A mixture of wide and narrow range pH ampholytes were therefore used to optimize the cIEF separation of AAV serotype 8 samples, whose pI difference was calculated to be about 0.1 pH unit between the full and empty capsid peaks. The dark blue circles in Figure 10, highlighted the empty and full capsid peaks of the AAV8 samples.

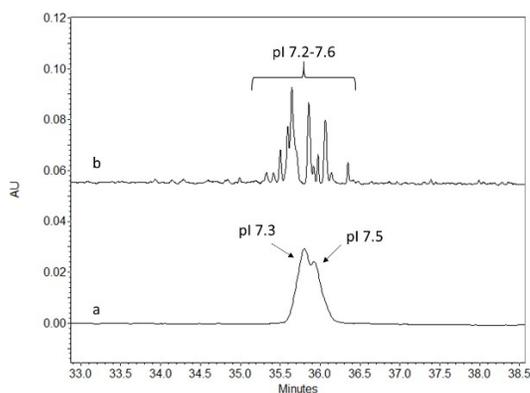
The single peak in front of the circle was identified as an impurity peak from one of the pI markers, since it was also observed in a blank injection with pI markers. Notably, higher intensity of empty capsid peaks was observed in the empty capsid-enriched sample, while higher intensity of full capsids peaks was observed in the full capsid-enriched sample as expected. It was demonstrated that the utilization of narrow pH range ampholyte 6-8 can provide excellent baseline resolution of the AAV full and empty peaks (Figure 10).

Multiple peaks were observed for the empty as well as full capsids, which could result from the charge heterogeneity of the capsids. Further experiments are needed to characterize these heterogeneous peaks.

**Table 1. Calculated pIs of Separated Peaks Using cIEF Method in Figure 9.**

ID	AAV sample #1		AAV sample #2	
	Peak #	pI	Peak #	pI
<b>Empty</b>	1	9.09	1	9.11
	2	8.95	2	8.99
	3	8.92	3	8.92
<b>Partial(possible)</b>	4	8.84	4	8.84
<b>Full</b>	5	8.73	5	8.73

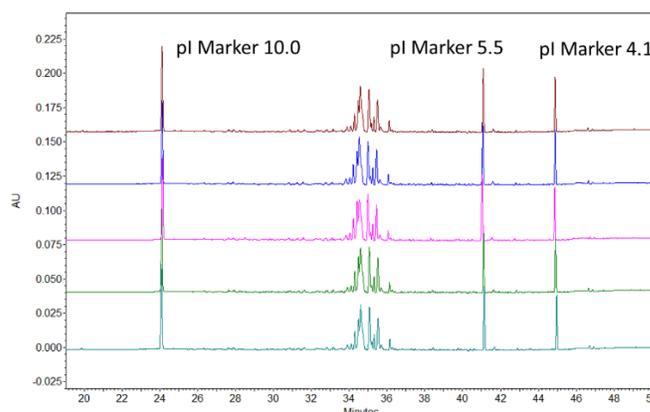
Similarly, a mixture of wide and narrow range pH ampholytes were used to optimize the cIEF separation of AAV serotype 9 sample. Instead of unresolved peaks observed at pI 7.3 and 7.5 from using the wide range pH ampholyte (Figure 11a), multiple resolved peaks between pI 7.2 to 7.6 were observed when using the mixture of pH ampholytes. Thus providing more valuable information on the abundance of partial/variants present with the full capsid.



**Figure 11.** Shown are the electropherogram a) cIEF analysis of the AAV9 sample with a) analysis using a wide pH ampholyte and b) a mix of wide and narrow ampholyte.

## Repeatability Analysis

To determine the analytical reproducibility of the method the AAV9 serotype sample was run 5 times to obtain the %RSD for the peak area and migration time. As shown in figure 12, we get excellent reproducibility with %RSD of  $\leq 5\%$  and  $\leq 2\%$  for the peak area and pI value, respectively.



**Figure 12.** 5 replicate analysis of Serotype AAV 9. The %RSD for peak area and pI are  $\leq 5\%$  and  $\leq 2\%$ , respectively.

## Distinct pI values for AAV identification

It is worth noting that the pI values of the AAV capsid peaks can be quantitatively determined based on the calibration curve of internal pI markers. The pI value of the AAV8 samples were approximately 7.1 (pI value data not shown in Figure 10) while the pI of the AAV samples with proprietary serotype were about 9.0 (Table 1). These results demonstrated that the pI values and profiles of the AAVs could be used for identification of different AAV vectors.

## Determination of AAV Full /Empty Capsids Ratio

The ratio of full/partial/empty capsids can be calculated based on the corrected peak areas of the separated capsid peaks in the cIEF electropherograms. The relative content of the full and empty capsids of AAV 8 samples separated in Figure 10, is summarized in Table 2.

**Table 2. F/E Capsid Determination of AAV8 Samples Separated in Figure 10.**

	Empty	Full
AAV8 E SMP	57%	43%
AAV8 F SMP	22%	78%

Full and empty capsids profile of AAV serotype 5 sample analyzed by cIEF is depicted by the red trace and compared to the AEX- HPLC profile denoted in the inset of Figure 2. As observed, the cIEF method could nicely resolve the full, the empty, and the partial capsid peaks; while the AEX-HPLC method showed poor resolution for the full and empty capsids. With the cIEF method, the partial capsids were able to be separated, whereas in the HPLC method it was not. Hence for the comparative study of the full and empty capsids ratio between these two orthogonal technologies, the sum of the area % of the full and partial capsid peaks in cIEF was counted as the full capsid peak in AEX-HPLC. And their absorbance at 280 nm for both technologies were evaluated for comparison. The results in Table 3, demonstrates that the ratio of the full and empty capsids determined by cIEF correlates well with that of the AEX-HPLC method.

As a note, the above comparison was based on the absorbance at 280 nm of AAV capsids, and it can over-estimate the percentage of full capsids due to the contribution of extra UV absorbance of the genetic materials in full capsids at 280nm . Hence a correction factor using the molar extinction coefficients of the full and empty at different wavelengths is needed to account for the over-estimation. For comparative analysis with other orthogonal techniques such as TEM and AUC, using this correction factor for cIEF analysis will improve the accuracy for the quantitative determination of full capsids for the various serotypes.

**Table 3. Comparison of AAV5 Full and Empty Capsids Determination Using cIEF and AEX-HPLC Methods.**

	Empty	Full *
<i>cIEF</i>	33%	67%
<i>AEX-HPLC</i>	31%	69%

**\* It is the sum of full and partial peaks for cIEF**

## Conclusions

Empty/ versus full is an important CQA that needs to be monitored throughout the development and production of the viral vector based therapy. While a number of techniques can be used for empty full analysis, there are still limitation to these workflows. This technical note demonstrates a robust cIEF-based method for the separation and analysis of AAV full and empty capsids of different serotypes. The pI profiles can be determined and used for AAV identification. The utilization of the optimal mixture of wide and narrow pH range ampholytes can efficiently improve the separation of AAV samples with small pI differences between full, partial and empty capsids. The sample analysis time for this method is rapid, less than 1 hour per sample. The analysis is performed on a well-validated and automated cIEF-based platform to obtain reliable and reproducible results across multiple serotypes. Making this method amenable for usage in release testing.

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