

Maximizing AAV Recovery on Affinity Capture: Yield Gains or Quality Compromise?

Increasing Affinity Step VG Recovery with a High Salt Wash Reduces Relative Protein Expression

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Introduction

Affinity resins for adeno-associated virus (AAV) purification are widely used as the primary capture step in many AAV manufacturing processes. Despite their broad implementation, cloning viral genome (VG) and capsid mass balances remains a persistent challenge. In our experience using POROS® CaptureSelect™ AAV9/AAVx with various serotypes (wt), VG mass balances of only 84% (AAV8), 80% (AAV9), 77% (AAV5), and 69% (AAV2) were obtained, even after the incorporation of an aggressive low-pH strip step.

We found that by modulating the conductivity of the wash step, recovery can be improved and nearly all VGs and capsids can be accounted for. However, the yield gain coming from these previously unrecoverable vectors raises a question regarding their suitability from a product quality perspective: because they required a different set of conditions than the majority of the other vectors to desorb, can we assume that they have the same product quality profile?

Methods

An AAV9 lysate derived from HEK293 cells grown in a 2L bioreactor and transfected with OXB's dual plasmid system encoding for Green Fluorescent Protein (GFP) was sterile filtered and divided into three equal aliquots. Each one was processed on POROS® CaptureSelect™ AAV9 Affinity resin using a series of wash steps with varying conductivities as depicted in **Table 1**. The Affinity products obtained were then loaded on an Anion Exchanger (POROS® HQ 50) for empty and full separation.

The Affinity and AEX Product and Strip fractions were characterized with respect to physicochemical attributes, including surface charge, hydrophobicity, empty-to-full capsid ratios, VP1 specific deamidation (N57), and in vitro potency to identify differences in the capsids coming from the various Affinity wash treatments.

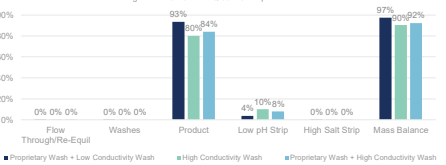
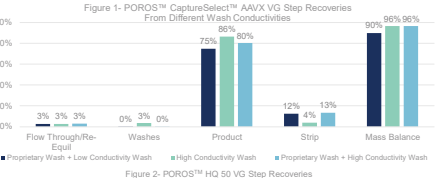
Affinity Step	Proprietary Wash + Low Conductivity Wash (Arm 1)	High Conductivity Wash (Arm 2)	Proprietary Wash + High Conductivity Wash (Arm 3)
Wash1	Proprietary Wash	None	Proprietary Wash
Wash2	50mM Tris, 100mM Sodium Chloride pH 8.0	30 mM Sodium Phosphate, 500 mM NaCl pH 7	30 mM Sodium Phosphate, 500 mM NaCl pH 7
	13 mS/cm	50 mS/cm	50 mS/cm
Elution	Glycine, Sodium chloride pH 2.7	Glycine, Sodium chloride pH 2.7	Glycine, Sodium chloride pH 2.7
Strip	Acetic Acid, Phosphoric Acid, pH 1.5	Acetic Acid, Phosphoric Acid, pH 1.5	Acetic Acid, Phosphoric Acid, pH 1.5

Table 1: Buffers used on POROS™ CaptureSelect™ AAV9 Affinity resin for the wash, elution, and strip steps. Wash buffers with different conductivities were used.

Results

Raising the Wash Step Conductivity Increased the Affinity Step VG Yield and Mass Balance

The POROS™ CaptureSelect™ AAVx runs where the wash conductivity was increased to 50 mS/cm (High Conductivity Wash, Arms 2 and 3) from 13 mS/cm (Low Conductivity Wash, Arm 1) resulted in a 5-11% improvement in VG recovery along with a near complete mass balance (96% Vs. 90%). Virtually no VGs were lost in all washes tested. Capsids recoveries (data not shown) largely mirrored that of the VGs. The arms with the proprietary wash had higher VG recoveries in the strip (12-13%) compared to 4% for the arm without it, suggesting that it delays the elution of some vectors.



Extra Vectors Recovered by Increasing the Affinity Wash Step Conductivity Didn't Translate to More Total VGs at the AEX step

The products from the three Affinity runs were further purified for full capsids enrichment with the anion exchanger POROS™ HQ 50. The extra vectors that were recovered in the Affinity runs with a high conductivity wash (Arms 2 and 3 in **Table 1**) didn't carry through to the AEX Products, with the total VGs obtained after this two column purification process being virtually identical at around 2.5E14 (**Table 2** AEX Product). So where did the extra VGs recovered in the Affinity Products of Arms 2 and 3 go?

Step	Proprietary Wash + Low Conductivity Wash (Arm 1)	High Conductivity Wash (Arm 2)	Proprietary Wash + High Conductivity Wash (Arm 3)
Affinity Product	2.9E14	3.4E14	3.3E14
AEX Product	2.5E14	2.4E14	2.4E14

Table 2: Total VGs Recovered in the Affinity and AEX Products

Extra Vectors Recovered by Increasing the Affinity Wash Step Conductivity Ended Up in the AEX Strip Fraction

The Affinity runs which incorporated a high conductivity wash step (Arms 2 and 3 in **Table 1**) and had resulted in an improved Affinity step recovery (**Figure 1**) ended up yielding higher AEX VG Strip recoveries (10% and 8%) than the low conductivity Affinity wash arm (Arm 1 in **Table 1**) (4%). This increase in VG recoveries in the AEX Strips was further corroborated by the increased A280 area (**Figure 3**).

How are the Capsids in the AEX Strip Different From Those in the AEX Product?

• Capsids in AEX Strip are More Negatively Charged Than Those in AEX Product

For the AEX step, the Affinity products were diluted to a set pH and conductivity to favor the binding of full capsids to the resin while discouraging that of empties. After a re-equilibration and a wash step (Flow Through/Re-Equi and Washes in **Figure 2**), the full capsids were recovered with an isocratic step of increased conductivity (Product in **Figure 2** and 'AEX elution peak' in **Figure 3**) after which the column was stripped first with a low pH buffer (Low pH Strip in **Figure 2** and 'AEX Low pH Strip' in **Figure 3**) followed by a high salt buffer (High Salt Strip in **Figure 2**). Because Poros™ HQ 50 is an anion exchanger (positively charged medium), we assume here that capsids (whether full or empty) that remained bound after the elution step and desorbed only in the low pH strip are likely more negatively charged.

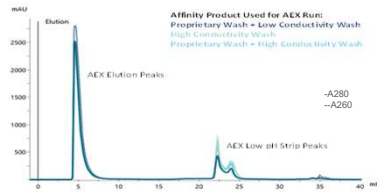


Figure 3: Overlay of the AEX Chromatograms. The Affinity Products loaded on AEX coming from a high conductivity wash arm had a higher VG Affinity step recovery and Mass Balance (Figure 1**) but also larger AEX Strip Areas.**

• Material in AEX Strip is Largely Composed of Empty Capsids

Figure 4 shows the packaging of the vectors found in the AEX Products and Strips derived from Analytical Ultracentrifugation. While the wash treatments used at the Affinity step had little impact on the % Fulls in the AEX Products (88-91% range), the proportion of full capsids in the AEX Strips increased for the Affinity arms with a high conductivity wash (Arms 2 and 3): 17% and 23% Vs. 14% for the low conductivity arm. This slight increase in full vectors parallels the increased VG recovery previously noted in the AEX Strip for Arms 2 and 3. Note however that the AEX strips are overwhelmingly composed of empty capsids (>71%).

• Capsids in AEX Strip Have a Protein Purity Level Similar to Those in AEX Product

Analysis of the AEX Products and Strips by capillary electrophoresis (CE) and host cell protein ELISA revealed that they were composed almost entirely of capsids protein (>90% purity) with no detectable host cell proteins (<10ng/ml HCP) for all products and strips (data not shown).

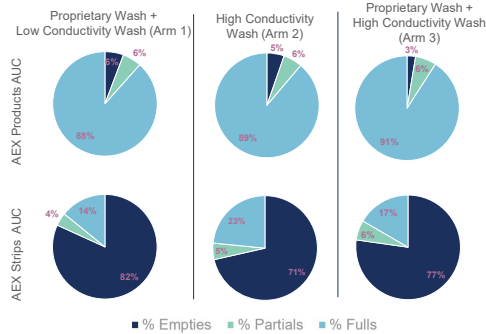


Figure 4: Analysis by Analytical Ultracentrifugation (AUC) of the vector packaging in the AEX Products and Strips

• Capsids in AEX Strip are More Deamidated than Those in AEX Product

Affinity Products, AEX Products and AEX Strips were analyzed by LC-MS to quantify their deamidation level at N57 (**Figure 5**). This VP1 specific post translational modification can confer an increased negative charge and has been linked to decreased transduction efficiency. The capsids in the AEX Strips were found to be much more deamidated (10-15%) than those in the AEX Products (2-3%). For all the arms, the deamidation level in the AEX products decreased by about half from that of the Affinity Products, suggesting that the conductivity at which the full capsids were eluted also selected those with a lower deamidation level.

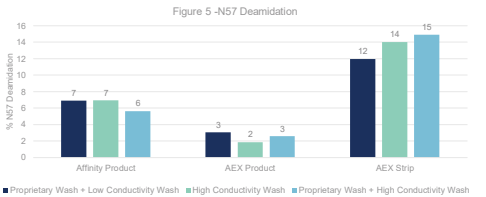


Figure 5: Comparison of Deamidation Levels of the Affinity Products, AEX Products, and AEX Strips at site N57 (VP1) (LC-MS)

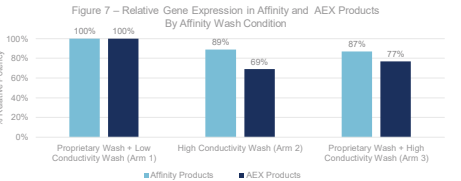
• Capsids in AEX Strip are More Hydrophobic than Those in AEX Product

The Affinity Products, AEX Products and Strips were analyzed by HIC-HPLC (hydrophobic interaction chromatography) to assess their hydrophobic profiles. In this method, samples are loaded in a high-salt buffer and separated using a linear gradient of decreasing salt concentration. Increased retention time corresponds to greater hydrophobicity (**Figure 6**). AEX products from the three arms eluted as a single peak at approximately 6 minutes. In contrast, the AEX strips displayed an additional peak at around 9 minutes, indicating the presence of more hydrophobic species. These secondary peaks were also observed in the Affinity Products and appear to have carried through to the AEX strips. Furthermore, the Affinity arms subjected to a high-conductivity wash showed AEX strips (and Affinity Products) with a larger proportion of this secondary, more hydrophobic peak relative to the 6-minute peak. This suggests that the high-conductivity wash on Affinity promotes increased desorption of hydrophobic material, potentially corresponding to the additional vectors that contributed to the increased recovery (**Figure 1**) at this step.

Figure 6: Overlay of the HIC-HPLC Chromatograms for the Affinity Products, AEX Products, and AEX Strips. Peaks at each chromatography step are normalized to the highest peak.

The High Conductivity Washes Depressed Gene Expression Levels of Affinity and AEX Products

GFP expression levels for the Affinity and AEX Products were compared to evaluate the impact of the different Affinity wash conditions on potency. HeLa RC32 cells (ATCC) were transduced with samples and GFP expression was quantified by fluorescence after 48 hours of incubation. The high-conductivity wash treatments (Arms 2 and 3) resulted in reduced gene expression relative to the low-conductivity wash condition (Arm 1). This reduction was observed in both the Affinity and AEX Products (**Figure 7**). For normalization, the Affinity Product from Arm 1 (proprietary wash + low-conductivity wash) was used as the reference for all Affinity Products, while the corresponding AEX Product from Arm 1 was the reference for all AEX Product samples. For example, the low conductivity wash arm of the AEX Product used as a reference was 100%, while its high salt counterparts were 69% and 77%. Unexpectedly, within each arm, the AEX Strips' GFP expression levels were above that of the AEX Products but importantly those from Arms 2 and 3 were still lower than Arm 1 (data not shown). The presumed reason behind the drop in potency between the Affinity Products and AEX Products for the high salt wash arms is the removal of these capsids (still present in the Affinity Product) which end up in the AEX strip. Also note that even the AEX strip from Arm 1 with the highest gene expression level only made up 4% of the total AEX VG mass balance (**Figure 2**).



Conclusions:

- Using a 50 mS/cm Vs. 13 mS/cm conductivity wash on POROS™ CaptureSelect™ AAVx before the elution step enabled up to an 11% improvement in VG step recovery and a nearly full VG mass balance (96% with an AAV9 feedstream). This boost in the Affinity step VG recovery however did not convert to more total VGs at the end of the purification process (post AEX chromatography).
- The extra VGs recovered at the Affinity step in the high conductivity wash arms ended up eluting in the AEX strip, suggesting that they have a different charge profile than those recovered in the AEX Product fraction.
- Compared to the AEX Product, our analysis found that these AEX strip capsids were:
 - More negatively charged
 - Composed of a higher portion of empty capsids
 - More deamidated (N57)
 - More hydrophobic
- The relative gene expression of the AEX Products and Strips of the high conductivity arms (Arms 2 and 3) was lower than those of the low conductivity arm (Arm 1), indicating that a high salt wash step on Affinity was detrimental to potency.

Discussion:

- Why did a high conductivity wash engender a boost in Affinity step recovery? Our analysis of the Affinity Products by HIC-HPLC (**Figure 6**) showed that the arms with a high conductivity wash also contained a higher area ratio of secondary (more hydrophobic 9 min) peak to the main (6 min) peak than the arms with a low conductivity wash - meaning that they have a higher content of hydrophobic material relative to the total material eluted. Since POROS™ CaptureSelect™ AAVx has a hydrophobic cross-linked poly(styrene-divinylbenzene) matrix, it is possible that the higher salt concentration of the high conductivity wash arms triggered the release of additional material hydrophobically bound to this matrix. The conductivity of the elution step being lower than that of the wash step may create a HIC-like separation mechanism where lowering the conductivity during the elution step engenders desorption of material not recoverable by only lowering pH.

- The presence of genome-containing particles in the AEX Products but also in the AEX Strips (**Figure 4**) suggests the presence of charge heterogeneous populations of full AAVs. Although the vectors found in the AEX Strips showed a higher negative charge, N57 deamidation, hydrophobicity, and proportion of empty capsids than those in the AEX Products, this experiment could not differentiate if all strip vectors were washed with these attributes - or only the empty capsids (which make up most of the strip contents). However, present these AEX strip vectors may be compared to their product fraction counterparts, their recovery would necessitate increasing the elution conductivity of the AEX step, which would introduce additional empty capsids into the AEX Product peak - a potential welcome risk for programs seeking the highest possible % of full AAVs. Furthermore, the VG gains would be minimal: the AEX Strip of the low conductivity wash arm (Arm 1) which had the highest relative potency level in this study (**Figure 7**) only accounted for 4% of the total VG Mass balance of the AEX step.

References

1. Gilek A, Simons J, Turner K, Goidanowicz L, Alvir M, Luck M, Wilson J. Deamidation of Amino Acids on the Surface of Adeno-Associated Virus Capsids Leads to Charge Heterogeneity and Altered Vector Function. *Molecular Therapy*. 2018; 28: 2848-2862

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