REPLIGEN

AVIPure® AAV8 Affinity Resin Process Development Guide

Introduction

Adeno-associated virus (AAV) is increasingly recognized as an ideal choice for delivery of gene therapy modalities. To realize the therapeutic benefits of AAV, an efficient manufacturing process that delivers sufficient purity for use in a clinical setting is necessary. Current AAV production methods are characterized by low viral titers and high HCP levels; consequently, downstream purification processes must deliver high yields at high purity, and short processing times.

AVIPure® AAV8 Affinity Resin is characterized by high binding capacities, high yields and the ability to process lysate at high volumetric flow rates, enabling capture from dilute feed streams. In addition, the resin is alkali stable enabling the repeated use of 0.5 M NaOH for cleaning-in-place (CIP) and sanitization applications. These attributes assure that use of this resin will result in improved process economy at any scale of operation.

AVIPure® AAV8 Affinity Resin consists of a high affinity recombinant protein ligand coupled to a highly cross-linked agarose base matrix developed for bioprocess applications. Attachment of the ligand to the base matrix through a long flexible spacer ensures ligand accessibility and subsequently leads to high binding capacities. The engineered affinity of the ligand ensures highly specific binding of AAV8 at neutral pH, while enabling elution at low pH (*e.g.,* pH 2.0). Elution at higher pH can be possible, but additives will need to be added to assure reasonable yields. The inert agarose base matrix shows minimal nonspecific binding, leading to a high purity of recovered AAV8. Furthermore, the agarose base bead of the AVIPure® AAV8 Affinity Resin base matrix enables rapid processing of large volumes of lysate without excess pressure drop over the packed column.

This application note describes the recommended operating conditions and process optimization methodology for the resin. The methodology described can be applied to determine optimal capture conditions for dilute and concentrated feed, and for determination of preferred CIP conditions for the capture step using AVIPure® AAV8 Affinity Resin.

Recommended chromatographic conditions

Optimal conditions for purification of AAV8 using AVIPure® AAV8 Affinity Resin must be determined empirically for each AAV8 construct. However, as a starting point, the chromatography method summarized in Table 1 is recommended. To assure comparability of each cycle, it is also recommended to perform a sanitization step prior to the first use of the resin. The step should be equivalent to the CIP step used before resin storage.

Table 1. Recommended purification protocol for AVIPure® AAV8 Affinity Resin to purify viral vectors from concentrated lysate

Step	Column volumes	Residence time (min)	Suggested buffer
Equilibration	5	4	50 mM Tris, 400 mM NaCl, pH 7.5
Load	Titer dependent	1 – 8	-
Wash 1	5	4	Equilibration buffer
Elution	5	6 or 4[a]	50 mM Glycine, 150 mM NaCl, pH 2
Strip	2	4	Process specific (e.g., pH <2)
CIP	5	6	0.5 M NaOH
Re-equilibration	8	4	Equilibration buffer

[a] From the process efficiency perspective, an increase in residence time (reduction of flowrates) during elution step results in lower buffer consumption and more concentrated pools.

In the case of AAV8 purification from unconcentrated feeds, the same protocol as described in Table 1 can be used but the residence time for load can be reduced to 1 minute.

Effect of residence time on dynamic binding capacity

Figure 1 shows partial breakthrough curves obtained when AVIPure® AAV8 Affinity Resin was loaded with purified AAV8 capsids. The breakthrough curves were obtained at 1-, 2-, 3-, and 4-minute residence times. Dynamic binding capacities at 5% breakthrough extracted from Figure 1 are listed in Table 2. These capacities are some of the highest reported for AAV affinity resins, varying from 4.8×10^{17} vp/L to over 1.7×10^{18} vp/L at 1- and 4-minute residence times, respectively. The high capacities at short residence times yield very high resin productivities (See Table 2), which makes the AVIPure® AAV8 Affinity Resin an attractive bioprocess resin for purification of AAV8 capsids at various process configurations and scales, including processing of unconcentrated feed material. Note that the results presented in Figure 1 represent data obtained with a specific variant of AAV8, and while the trends shown can be considered characteristic for all types of AAV8 capsids, the absolute values for binding capacities should always be determined for specific AAV8 variants.

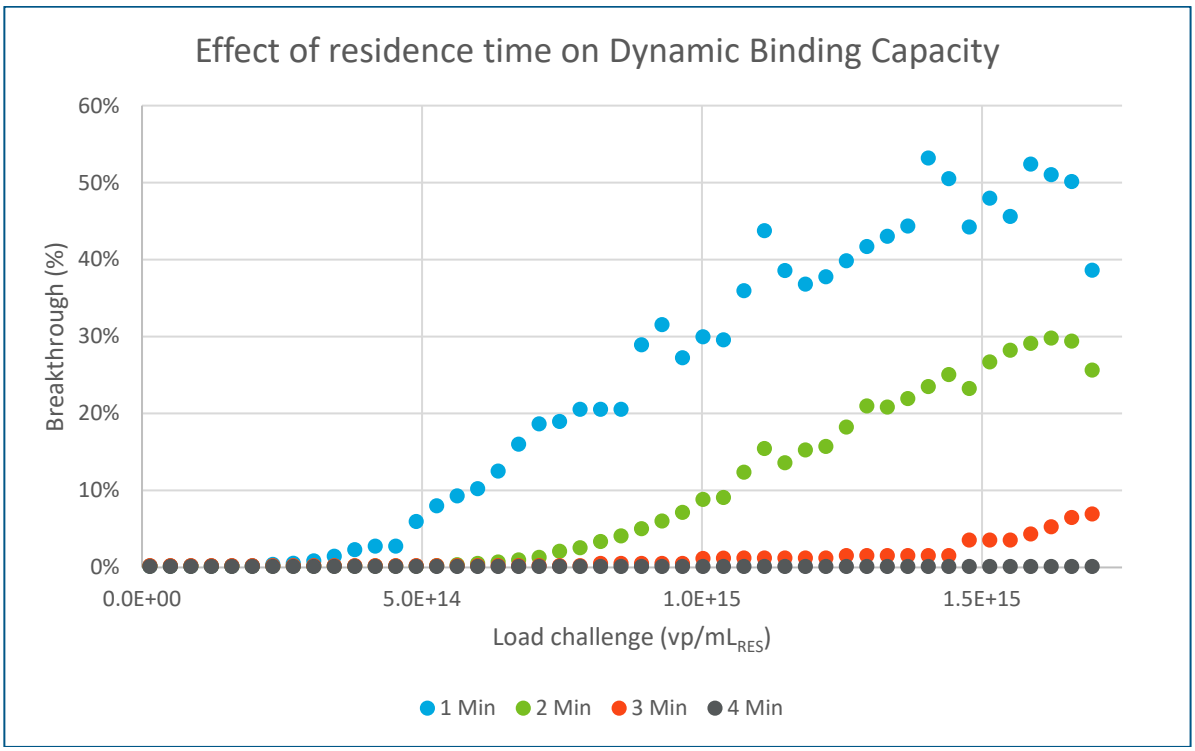

Figure 1. Effect of residence time on binding capacity of AVIPure® AAV8 Affinity Resin with AAV8 capsids. The study was carried out at 0.35 mL scale using purified AAV8 capsids (donated by an industrial collaborator) that were diluted to the desired concentration using 20 mM Tris, 400 mM NaCl, 0.01% poloxamer 188 at pH 7.5. The use of 0.01% poloxamer 188 was dictated by necessity to remove any non-specific adsorption of capsids to elements of chromatography system

Table 2. Dynamic binding capacities at 5% breakthrough for AVIPure® AAV8 resin obtained at various residence times

Residence time (min)	Feed concentration (vp/L)	$DBC_{5\%}$ (vp/L_{bed})	Productivity (vp/L_{bed})/h
1	2.6×10^{16}	4.8×10^{17}	1.9×10^{17}
2		8.9×10^{17}	2.4×10^{17}
3		1.6×10^{18}	2.7×10^{17}
4		$> 1.7 \times 10^{18}$	$> 2.8 \times 10^{17}$

Effect of capsid concentration on dynamic binding capacity

Upstream production of AAV viral vectors often results in low capsid concentration in the lysate. While the capsids can be concentrated and buffer exchanged prior to the capture step, direct loading of unconcentrated lysate may be preferred to eliminate a process step and the associated potential capsid loss. However, to process the large lysate volumes at low capsid titers, an affinity resin with high binding capacity at short residence times is required for efficient processing in a reasonable time.

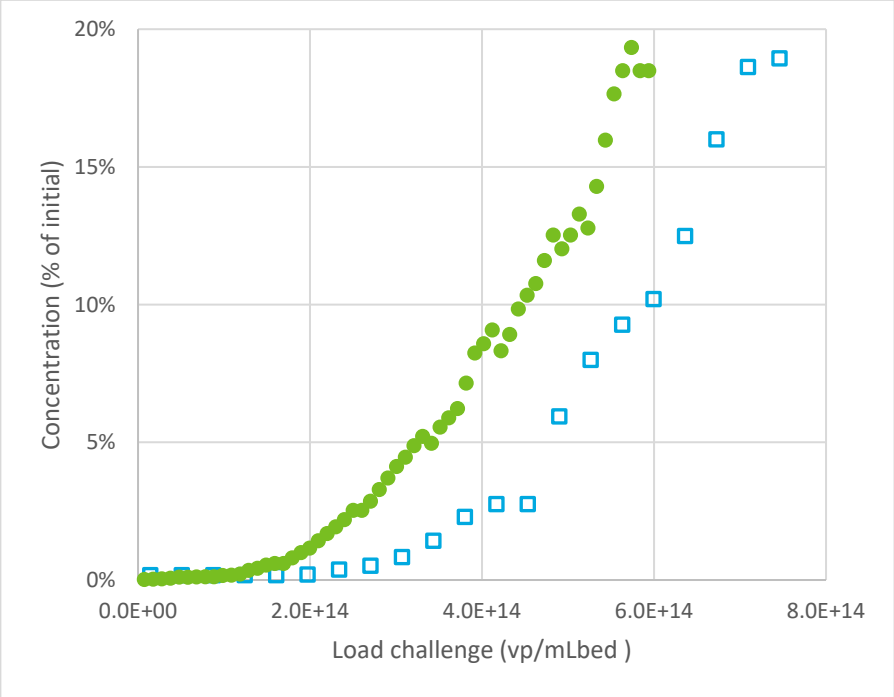

Figure 2. Partial breakthrough curves obtained at 1 minute residence time for two feed concentrations of 6.0×10^{11} vp/mL (1 ×, filled circles) and 2.6×10^{13} vp/mL (43 ×, open squares). The study was carried out at 0.35 mL scale using purified AAV8 capsids donated by our industrial collaborators that were diluted to the desired concentration using 20 mM Tris, 400 mM NaCl, 0.01% poloxamer 188 at pH 7.5.

The binding capacity of AVIPure® AAV8 Affinity Resin was evaluated at 6.0×10^{11} vp/mL and 2.6×10^{13} vp/mL, which represent titers typical of unconcentrated and concentrated lysate, respectively. To obtain the indicated concentrations, purified capsids were dia-filtered and diluted with 20 mM Tris, 400 mM NaCl, 0.01% poloxamer 188, pH 7.5. The residence time for both runs was 1 minute. For each run, the breakthrough curve was determined by collecting 1.5 column volume (CV) fractions of the column effluent and analyzing the fractions for total capsid using ELISA. The results obtained are shown in Figure 2. Binding capacities at 5% breakthrough for the unconcentrated and concentrated capsid samples were 3.9×10^{14} and 4.8×10^{14} vp/mL, respectively.

These data clearly demonstrate that the high DBC at low residence times of the AVIPure® AAV8 Affinity Resin can enable processing of lysate without a concentration step prior to affinity capture. The benefits of direct loading can include mitigation of potential yield losses during ultrafiltration and even shorter overall process times. With the unconcentrated feed, the load on the column can be increased by reducing the load safety factor.

Elution conditions

Identification of most suitable wash and elution conditions will ensure high purity and high yields from the affinity step, respectively. If use of the recommended elution conditions for AVIPure® AAV8 Affinity Resin (50 mM glycine, 150 mM NaCl, pH 2) does not result in full elution of adsorbed capsids, a screening study can be performed either in a column or microtiter plate format. From the amount of sample requirement perspective, the initial screening in the microtiter plate format is preferable. It allows for a quick assessment of the most promising conditions that can later be optimized using columns.

Below, we present an example of a wash and elution buffer screening study in microtiter plate format that can be used as a template for identifying the optimum wash and elution conditions when working with AAV8 capsids. However, considering that purity after an affinity step is already very high, identifying elution conditions should be the primary focus, and optimization of wash

conditions should be performed only if the standard conditions don't suffice from the eluted product perspective.

The following protocol described here and shown in Figure 3 works well for screening of elution conditions for use with AVIPure® AAV8 Affinity Resin. Dispense five microliters of the resin into a well of a microtiter plate containing 100 µL of equilibration buffer. Filter the plate and add 250 µL of feed containing 1×10^{13} vp/mL. After 60 minutes incubation, filter the plate and add 100 µL of wash (equilibration) buffer. After 5 minutes of incubation, filter the plate and perform another wash cycle. Following filtration of the second wash cycle, add 100 µL of elution solution. Incubate for 5 minutes and filter the plate, collecting the filtrate. Perform another elution cycle. Pool the two elution fractions and neutralize by adding 40 µL of 1 M Tris Base, 0.5 M NaCl, pH 9. Analyze the neutralized elution pool for AAV capsids using AAV8 capsid ELISA. UV absorbance can also be used as it can provide guidance as to which samples to analyze by ELISA. For most efficient elution buffers, most of the capsids should be found in the first elution cycle. Note that the effectiveness of elution buffers varies between different types of capsids. Users are encouraged to determine the optimal elution conditions for each of their capsids.

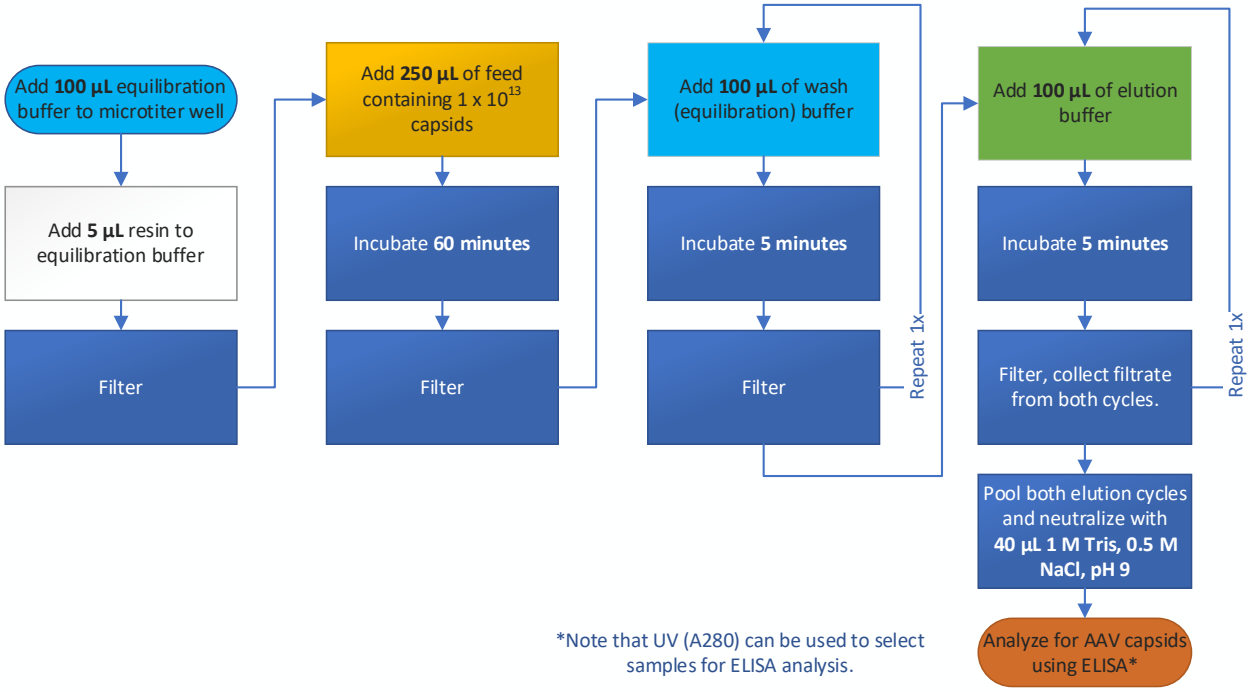

Figure 3: Visual representation of an elution condition screening protocol.

Pressure flow properties of the base beads

AVIPure® AAV8 Affinity Resin is based on a 50 µm highly cross-linked agarose matrix. While the rigidity of the base matrix enables process-relevant flowrates below equipment pressure limits, the compressible nature of agarose beads needs to be accounted for when designing the capture step with consideration for the column dimensions. The resin can be packed into large diameter columns (e.g., 30 cm in diameter), but the maximum allowable flow rate will need to be considered if the column is packed to higher bed heights. For instance, at 20 cm bed height the minimum residence time in larger diameter columns will be 5 minutes. Therefore, a wider, shorter bed is recommended if operating at faster flow rates.

Examples of pressure flow curves for bed heights between 5 and 20 cm are shown in Figure 4. The minimum recommended residence times for 5 and 20 cm bed heights are 1 and 5 minutes, respectively. Short residence times are recommended if viral particles are loaded without a prior concentration step.

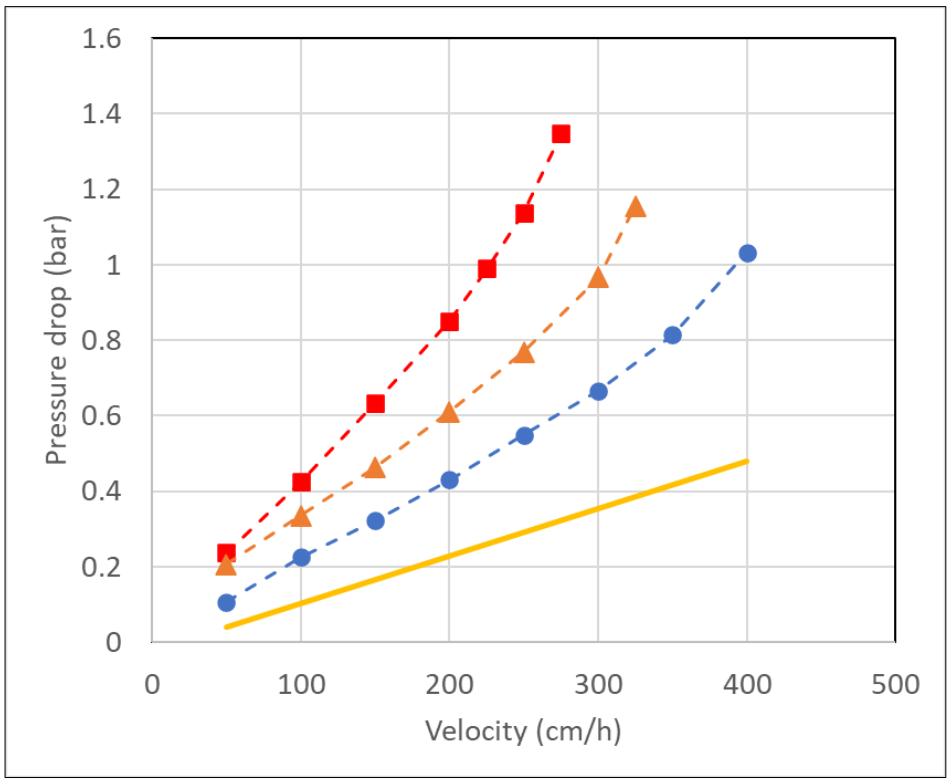

Figure 4. Pressure flow data for AVIPure® AAV8 affinity resin base bead in 30 cm diameter column packed to 20 cm (red squares), 15 cm (orange triangles), 10 cm (blue circles), and 5 cm (solid line, simulated data) bed heights at 1.15 compression factor. Data courtesy of Purolite® Ltd.

Development of an efficient cleaning in place (CIP) protocol

During CIP procedures cleaning solutions are applied to the column to remove precipitated or denatured material tightly bound to elements of the purification system such as resin, hardware, etc. From the resin reusability perspective, lack of stringent CIP at each cycle will lead to accumulation of undesired material, and increased probability of resin fouling that leads to limited ligand accessibility and increased backpressure during subsequent runs, eventually making the column unusable. Among various CIP agents, NaOH is preferred in bioprocess settings due to its low cost, ease of disposal, and ability to dissolve precipitated proteins, remove nucleic acids, saponify fats, and inactivate endotoxins. AVIPure® AAV8 Affinity Resin is an alkali-tolerant resin enabling the use of NaOH concentrations up to 0.5 M for CIP between cycles. Below, a resin cycling study

using real feed and 0.5 M NaOH as the CIP solution is described. The outline of the AVIPure® AAV8 Affinity Resin lifetime study is presented in Figure 5. The study was carried out at 0.35 mL scale using crude feeds containing AAV8 donated by an industrial collaborator. The feed had been concentrated 20-fold by tangential flow filtration (TFF). The following resin performance attributes were measured after each cycle: step yield defined as total capsids eluted compared with total capsids loaded, level of residual host cell protein (HCP), and level of residual host cell DNA (HCDNA). HCP and HCDNA were measured using ELISA and PicoGreen™, respectively. Total capsids were measured using HP-SEC.

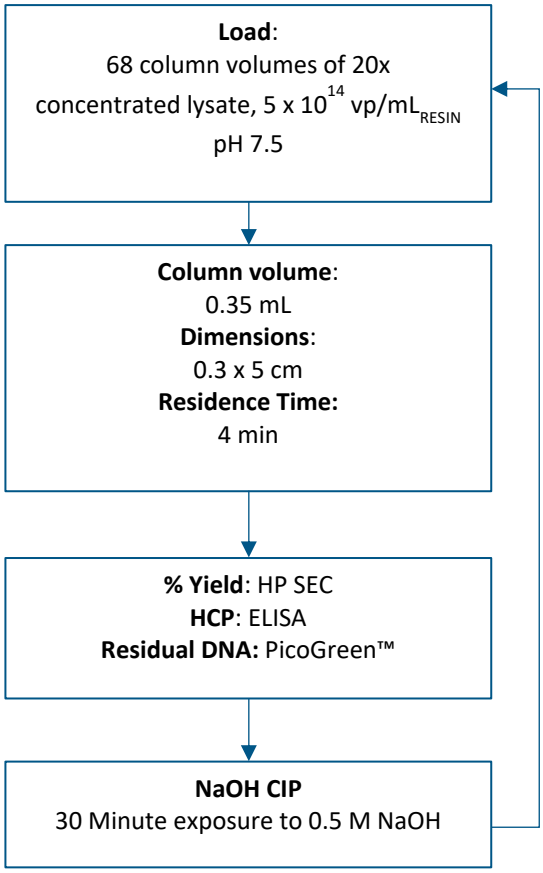

Figure 5. Outline of cycling studies.

Overlay of selected chromatograms from the study is shown in Figure 6 and the resin performance attributes monitored in the study (step yield, HCP and HCDNA reduction) are shown in Figure 7. The results clearly demonstrate the alkaline stability of AVIPure® AAV8 Affinity Resin. The resin retains ≥90% of its binding capacity after 20 CIP cycles with 30 minutes of exposure to 0.5 M NaOH per cycle. Removal of both the HCP and HCDNA is also unaffected by the extended NaOH exposure and remains at the average levels of 5- and 3-logs reduction for HCP and HCDNA, respectively. During the study, the column pressure drop was also monitored for each chromatography cycle, but no changes in the pressure drop were observed (results not shown).

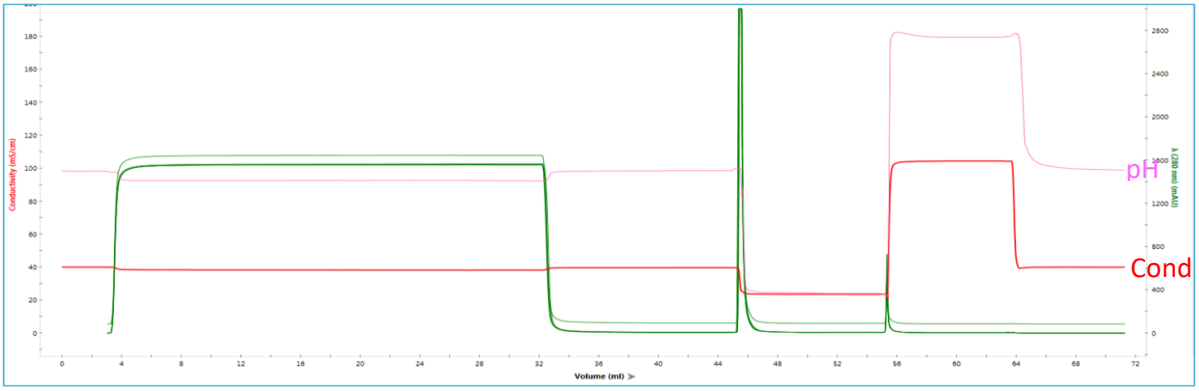

Figure 6: Overlay of chromatograms from cycles 1, 5, 10, 15 and 20. CIP regime used 30-minute contact time with 0.5 M NaOH after each cycle. Feed: HEK293 clarified lysate

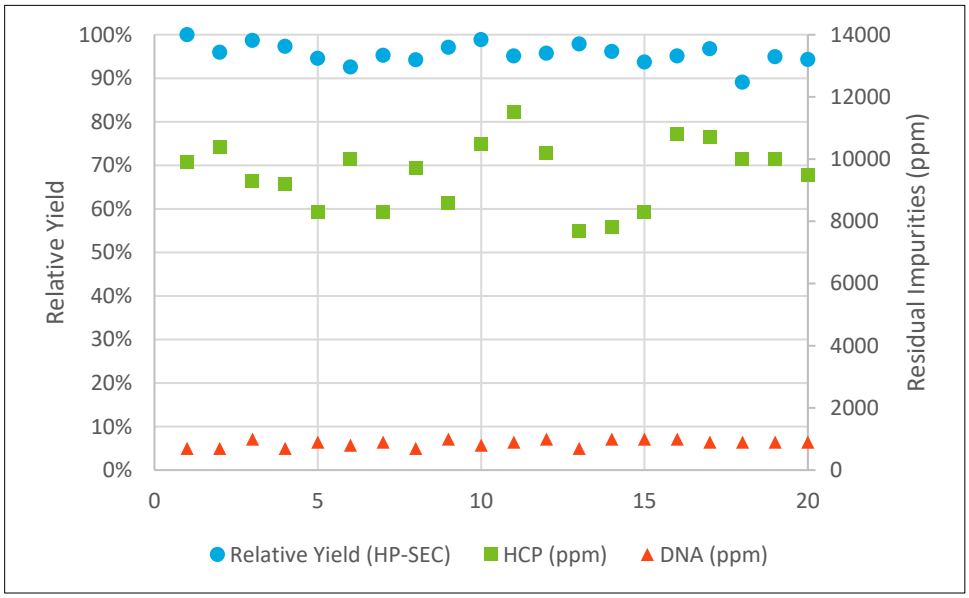

Figure 7. Effect of number of column reuses on relative yield, residual HCP, and residual HCDNA. CIP regime used 30-minute contact time with 0.5 M NaOH after each cycle. HCP and HCDNA levels in the feeds were 2.0×10^7 ppm and 3.7×10^3 ppm, respectively.

The level of stability shown in Figure 7 is unparalleled for affinity resins for AAV8 purification. For instance, based on the results obtained in this study, a 1.5 L column packed with AVIPure® AAV8 Affinity Resin can be used to purify not just one but twenty 2,000 L bioreactors, thus reducing the effective cost of the resin 20-fold.

A recommended CIP protocol for AVIPure® AAV8 Affinity Resin is provided below. It represents a good starting point for determination of the most efficient CIP protocol for the specific AAV8 purification process. With an optimized CIP protocol, the AVIPure® AAV8 Affinity Resin lifetime can be further increased even beyond the 20 cycles shown in the results presented above.

The following CIP protocol has been shown to work effectively for AVIPure® AAV8 Affinity Resin:

1. Wash the column with 5 column volumes of equilibration buffer
2. Perform CIP step using 0.5 M NaOH
    a. Apply 3 CV of CIP solution at 3 minutes residence time
    b. Perform a static hold for a total contact time of 15 minutes
    c. Apply 2 CV of CIP solution at 3 minutes residence time
3. Re-equilibrate the column with at least 5 column volumes of equilibration buffer

To identify the most desirable CIP conditions for a specific process scenario, concentration and contact time of NaOH exposure should be empirically determined to suit individual process requirements. Depending on the nature of the feed stock, different CIP regimes may provide an optimal balance of chromatographic performance and resin lifetime. For example, 0.1 M NaOH exposure for 15 minutes every cycle, with a 0.5 M NaOH exposure for 30 minutes every 10th cycle or at the end of each batch will further extend resin lifetime. CIP with 0.5 M NaOH is recommended before long-term storage.

Before storing the column in storage solution (*e.g.,* 20% ethanol or 2% benzyl alcohol), the column should be neutralized, for example with equilibration buffer.

Conclusions

AVIPure® AAV8 Affinity Resin is an alkaline-stable affinity chromatography resin developed for simple, one-step purification of adeno-associated virus (AAV8) vectors directly from lysate. AVIPure® AAV8 ligand has been engineered for enhanced alkali stability, enabling the repeated use of 0.5 M NaOH for cleaning-in-place (CIP) and sanitization applications. Compared to existing AAV affinity chromatography resins outside of the AVIPure® family, AVIPure® AAV8 Affinity Resin delivers an order of magnitude increase in resin lifetime and promises to dramatically decrease resin costs.

Process development workflow followed a standard approach consisting of determination of dynamic binding capacity at 1- and 4-minute residence times and nonconcentrated and concentrated feeds, identification of optimum wash and elution conditions, and finally determination of efficient CIP protocol based on NaOH.

AVIPure® AAV8 Affinity Resin is available in bulk or in prepacked columns. A residual ligand kit ELISA assay is available from Cygnus Technologies (Part number F1005). Regulatory Support File for the AVIPure® AAV8 Affinity Resin is available upon request.

Ordering information

Items listed here are available through the Repligen e-store (store.repligen.com) for most regions. You can also contact your sales representative or customer service for sales, or the email addresses for the regions listed below:
US: customerserviceUS@repligen.com
EU: customerserviceEU@repligen.com
China: customerserviceCN@repligen.com

Bulk Resin Volume	AVIPure® AAV8
10 mL	100AAV8-10
25 mL	100AAV8-25
50 mL	100AAV8-50
100 mL	100AAV8-100
250 mL	100AAV8-250
1000 mL	100AAV8-1000

Column Type	ID [mm]	H [mm]	V [mL]	AVIPure® AAV8
OPUS® MiniChrom®	5	50	1.0	23051106
OPUS® MiniChrom®	11.3	50	5.0	23051107
OPUS® MiniChrom®	8	100	5.0	23051104-100

Column Type	Rows	V [µL]	AVIPure® AAV8
OPUS® RoboColumn®	8	200	23051108R
OPUS® RoboColumn®	8	600	23051108R-30

Description	AVIPure® AAV8
Residual Ligand ELISA Assay, available through Cygnus Technologies	F1005

Residual ligand assay kits for AVIPure® AAV affinity resins are available through Cygnus Technologies (https://www.cygnustechnologies.com/); 1-910-454-9442; orders@cygnustechnologies.com.