



# AVIPure® AAV9 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 AAVs 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® AAV9 Affinity Resin is characterized by high binding capacities, high yields and the ability to process lysate at high volumetric flow rates, hence 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® AAV9 Affinity Resin consists of a high affinity synthetic peptide 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 AAV9 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 AAV9. Furthermore, the agarose base bead of the AVIPure® AAV9 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® AAV9 Affinity Resin.

### Recommended chromatographic conditions

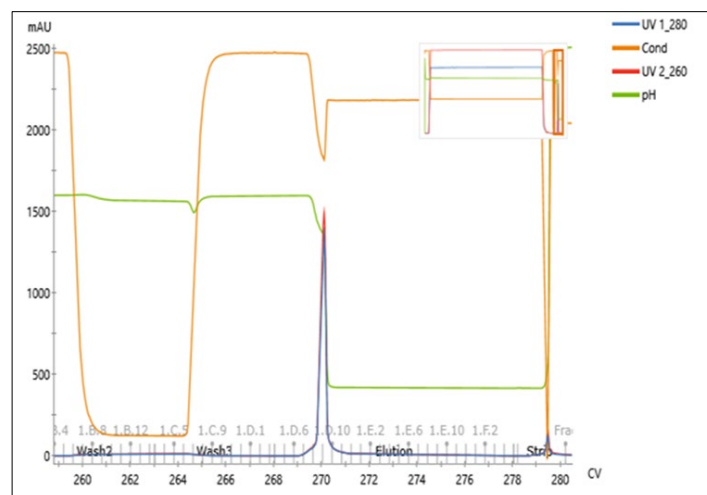
Optimal conditions for purification of AAV9 using AVIPure® AAV9 Affinity Resin must be determined empirically for each AAV9 construct. However, as a starting point, the chromatography method summarized in [Table 1](#) is recommended. To assure comparability of each cycle, it is also recommended to perform 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® AAV9 Affinity Resin to purify viral vectors from concentrated lysate

Step	Column volumes	Residence time (min)	Suggested buffer
Sanitization	3 - 5	4 - 6	0.5 M NaOH
Equilibration	8	4	50 mM Tris, 400 mM NaCl, pH 7.5 Generally, can be matched to lysis buffer
Load	Titer dependent	4	-
Wash 1	5	4	Equilibration buffer
Wash 2 (if needed)	5	4	50 mM Tris, 50 mM octanoic acid, 0.5 M urea, pH 8.0
Wash 3	2	4	Equilibration buffer
Elution	5	6 or 4 <sup>a</sup>	50 mM glycine, 150 mM NaCl, pH 2; upflow elution may improve recovery. (Neutralize with 1 M Tris, pH 9)
Strip	2	4	Process specific (e.g., pH < 2)
CIP	5 or 1	6 or 30	0.5 M NaOH; total contact time should be 30 min
Re-equilibration	8	4	Equilibration buffer
Long term storage	3	3	2% benzyl alcohol or 18 - 20% ethanol at 8° C

<sup>a</sup> 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 AAV9 purification from unconcentrated feeds, the same protocol as described in [Table 1](#) can be used but the residence time for equilibration, load, washes, and re-equilibration can be reduced to match the residence time of the load, which can be as short as 1 minute. A typical chromatogram obtained with AVIPure® AAV9 Resin is shown in Figure 1. A very sharp elution peak is one of characteristic features of this resin.

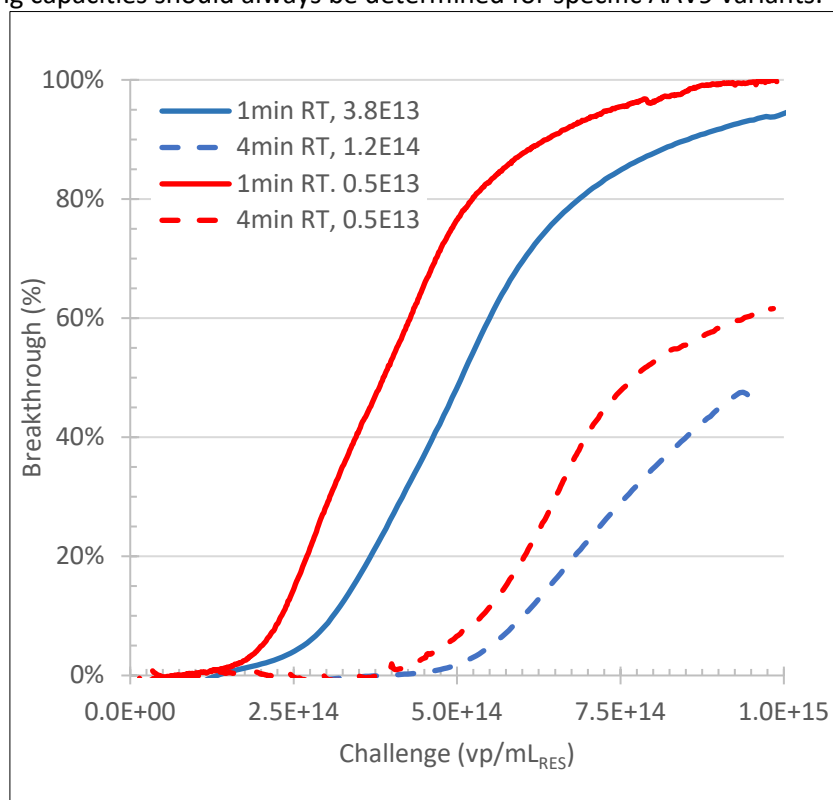


**Figure 1.** Example of chromatogram obtained with AVIPure® AAV 9 Resin loaded with nonconcentrated lysate containing AAV9 capsids. Load: 250 CV with  $2.5 \times 10^{11}$  vp/mL at 1 min residence time.

**Effect of residence time and capsid concentration on dynamic binding capacity**

[Figure 2](#) shows breakthrough curves obtained when AVIPure® AAV9 Affinity Resin was loaded with purified AAV9 capsids. The breakthrough curves were obtained at 1- and 4-minute residence times and at two feed concentrations representing unconcentrated and concentrated feeds. Dynamic binding capacities at 10% breakthrough extracted from [Figure 2](#) are listed in [Table 2](#). These capacities are some of the highest reported for AAV9 Affinity Resins, varying from  $2.0 \times 10^{17}$  to

over  $7 \times 10^{17}$  vp/L at 1- and 4-minute residence times for low and high concentration feeds, respectively. The high capacities at short residence times yield very high resin productivities, which makes the AVIPure® AAV9 Affinity Resin an attractive bioprocess resin for purification of AAV9 capsids at various process configurations and scales, including processing of unconcentrated feed material. Note that the results presented in [Figure 2](#) represent data obtained with a specific variant of AAV9, and while the trends shown can be considered characteristic for all types of AAV9 capsids, the absolute values for binding capacities should always be determined for specific AAV9 variants.



**Figure 2.** Effect of residence time and feed concentration on binding capacity of AVIPure® AAV9 Affinity Resin with. Solid and dashed lines represent data obtained at 1-minute and 4-minutes residence time, respectively. Red color represents data for a low feed concentration, and the blue lines represent data obtained with high concentrations load. The study was carried out at 0.2 mL scale using purified AAV9 capsids 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 is necessary at small scale to remove any non-specific adsorption of capsids to elements of chromatography system.

**Table 2.** Dynamic binding capacities at 10% breakthrough for AVIPure® AAV9 Resin obtained at various residence times

Residence time (min)	Feed concentration (vp/L)	DBC <sub>10%</sub> (vp/L <sub>bed</sub> )
1	$5.0 \times 10^{15}$	$2.0 \times 10^{17}$
4		$5.5 \times 10^{17}$
1	$3.8 \times 10^{16}$	$3.0 \times 10^{17}$
4		$7.0 \times 10^{17}$

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.

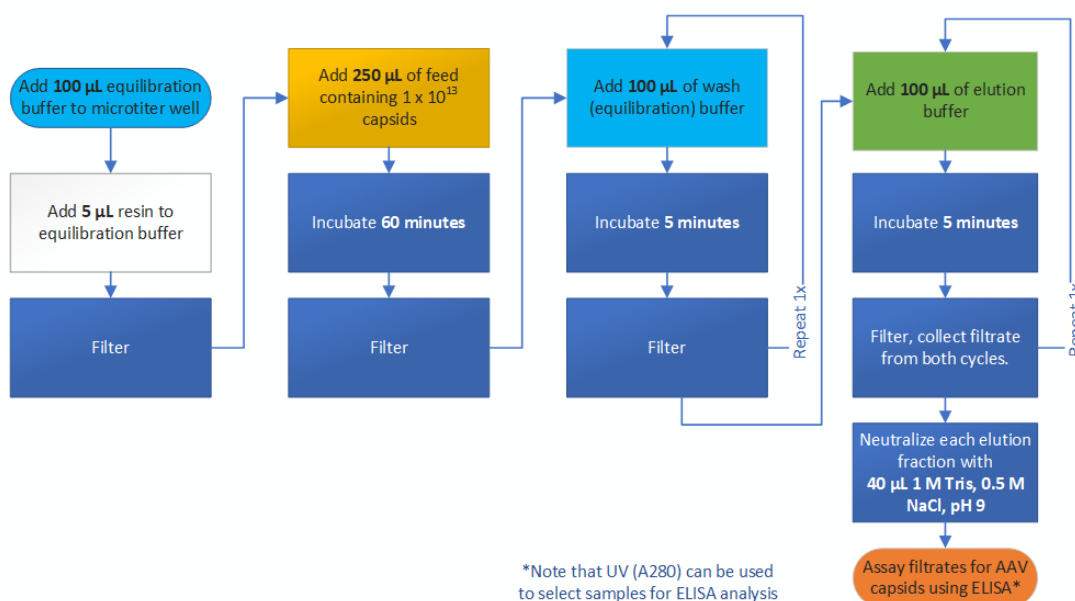
The data in [Figure 2](#) clearly demonstrate that the high DBC at low residence times and at low capsid concentrations of the AVIPure® AAV9 Affinity Resin can enable processing of lysate without a concentration step prior to affinity capture. The benefits of direct loading can include mitigation of potential yield losses during ultrafiltration and even shorter overall process times.

### 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® AAV9 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 perspective of the amount of sample required, 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 AAV9 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 resulting purity in the eluted product using the standard conditions don't suffice.

The following protocol described here and shown in [Figure 3](#) works well for screening of elution conditions for use with AVIPure® AAV9 Affinity Resin. Dispense five microliters of the resin into a well of a microtiter plate containing 100  $\mu\text{L}$  of equilibration buffer. Filter the plate and add 250  $\mu\text{L}$  of feed containing  $1 \times 10^{13}$  vp/mL. After 60 minutes incubation, filter the plate and add 100  $\mu\text{L}$  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  $\mu\text{L}$  of elution solution. Incubate for 5 minutes and filter the plate, collecting the filtrate. Perform another elution cycle. Neutralize elution fractions by adding 40  $\mu\text{L}$  of 1 M Tris Base, 0.5 M NaCl, pH 9. Analyze the neutralized elution pool for AAV capsids using AAV9 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.

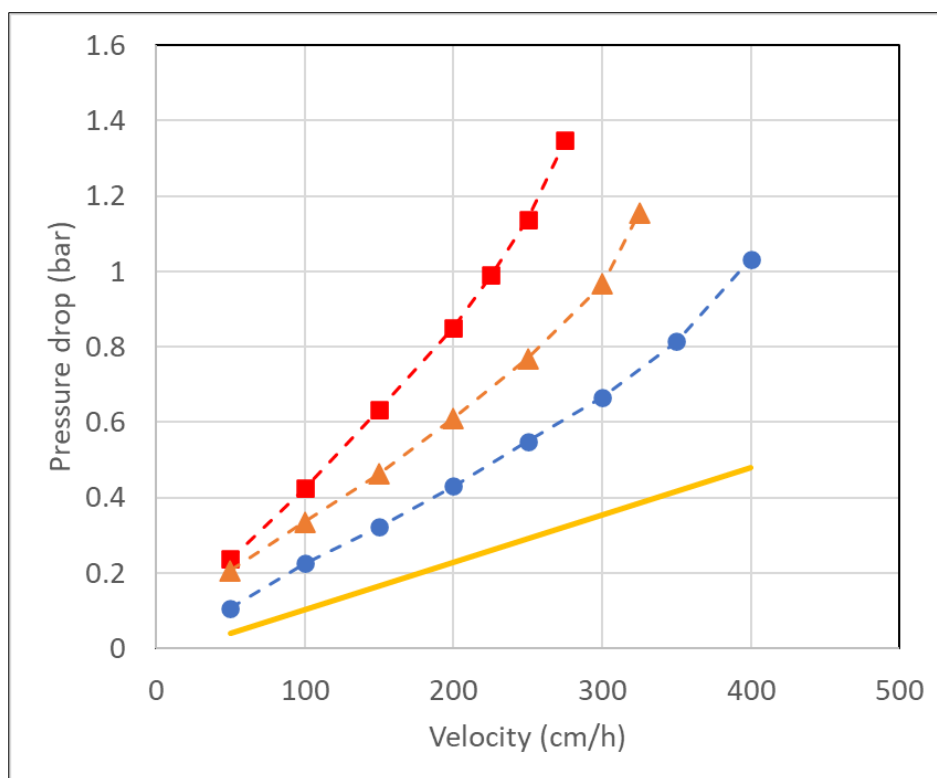


**Figure 3.** Visual representation of an elution condition screening protocol.

## Pressure flow properties of the base beads

AVIPure® AAV9 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. Such configurations can be accommodated with Repligen OPUS Pre-packed Columns.

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.

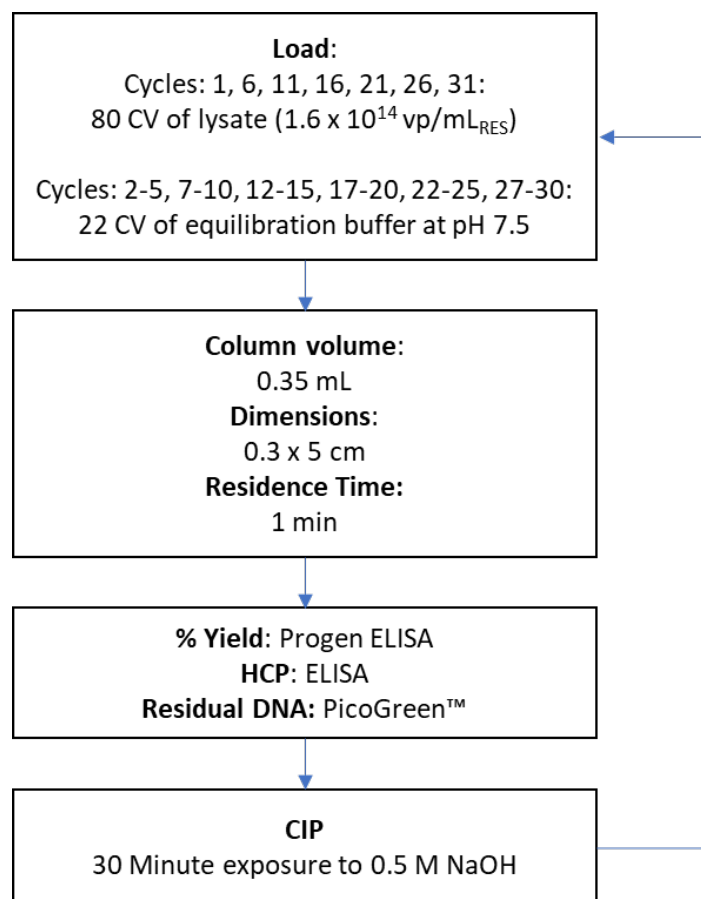


**Figure 4.** Pressure flow data for AVIPure® AAV9 Affinity Resin base bead in 30 cm diameter column packed to 20 cm (red squares), 15 cm (orange triangles), 10 cm (blue circles), and 5 cm (solid line, simulated data) bed heights at 1.15 compression factor. Data courtesy of PuroLite® Ltd.

## Development of an efficient cleaning in place (CIP) protocol

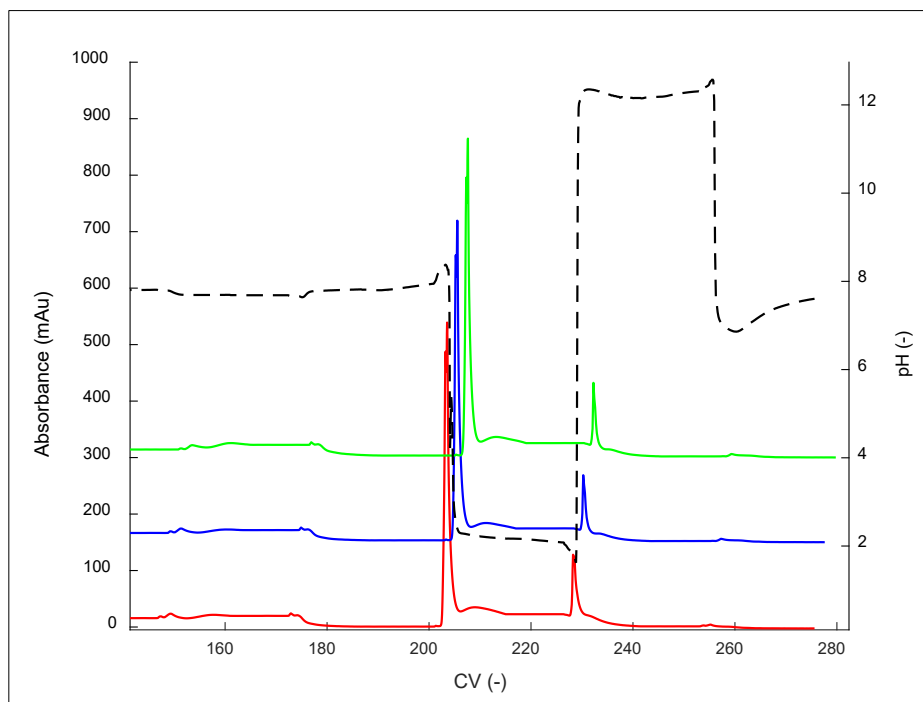
During CIP procedures cleaning solutions are applied to the column to remove precipitated or denatured material tightly bound to elements of the purification system such as resin, hardware, etc. From the resin reusability perspective, lack of stringent CIP at each cycle will lead to accumulation of undesired 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® AAV9 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® AAV9 Affinity Resin lifetime study is presented in [Figure 5](#). The study was carried out at 0.35 mL scale using crude feeds containing AAV9 capsids. 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 Progen ELISA.

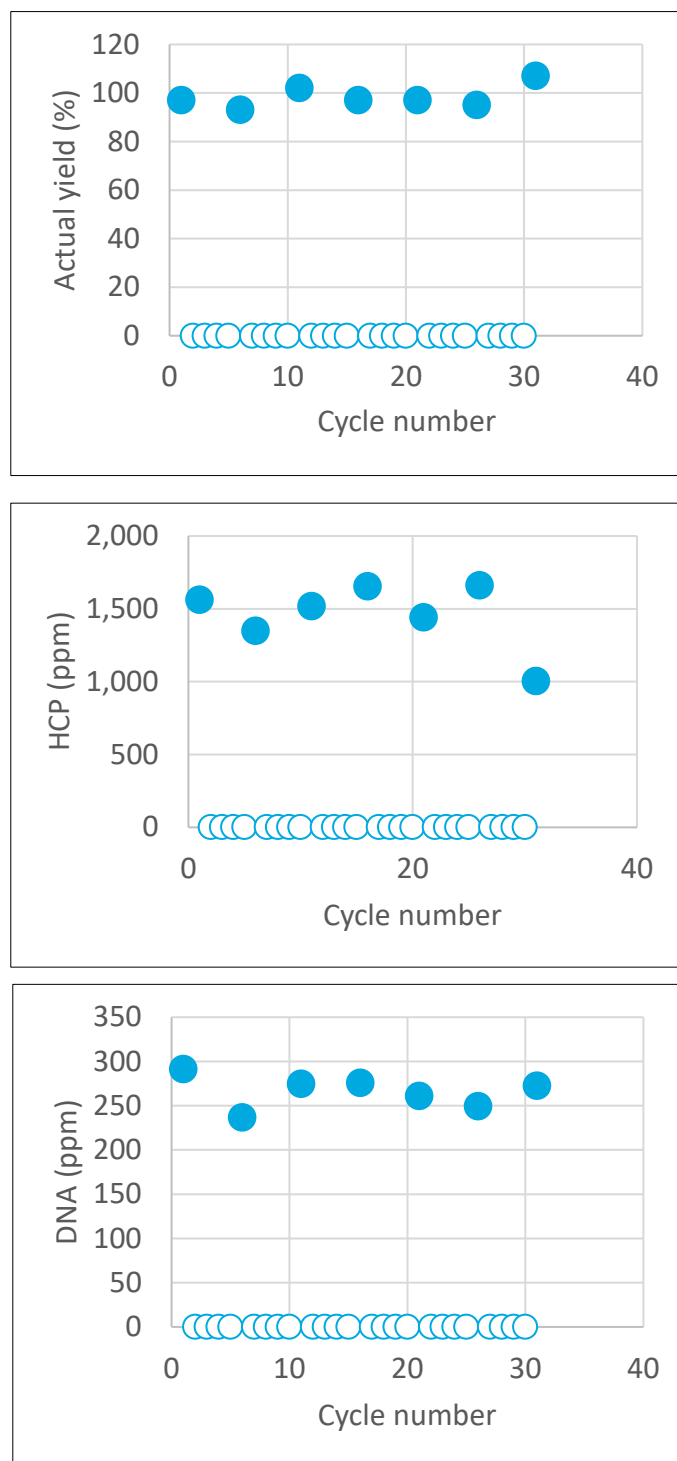


**Figure 5.** Outline of AVIPure® AAV9 lifetime studies.

Overlay of chromatograms from cycles run with the real feed is shown in [Figure 6](#). Summary of the resin lifetime study is shown in [Figure 7](#), where the resin performance attributes are plotted against the 0.5 M NaOH CIP cycle number. The study was performed by loading the column with the real feed every 5<sup>th</sup> cycle (*i.e.*, each load cycle was followed by four cycles consisting of CIP and equilibration steps). In total, by the end of the study the resin was exposed to 0.5 M NaOH for 15 hours. The results clearly demonstrate the alkaline stability of AVIPure® AAV9 Affinity Resin. The resin retains ≥ 90% of its binding capacity after 30 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 elution and CIP parts of chromatograms from cycles 1 (red curve), 16 (blue curve), and 31 (green curve) recorded during the AVIPure® AAV9 Affinity Resin lifetime study. The curves are shifted diagonally for clarity. CIP regime used: 30-minute contact time with 0.5 M NaOH after each cycle. Feed: HEK293 clarified lysate. The black dashed curve represents pH trace recorded during first cycle.



**Figure 7.** Effect of number of 30 minutes long 0.5M NaOH CIP cycles on relative yield, residual HCP, and residual HCDNA measured for real feed cycles (solid symbols). HCP and HCDNA levels in the feeds were  $2.9 \times 10^5$  ppm and  $7.1 \times 10^5$  ppm, respectively. Open symbols represent CIP/Equilibration cycles and are presented for clarity.

The level of stability shown in [Figure 7](#) is unparalleled for other commercial affinity resins for AAV9 purification. For instance, based on the results obtained in this study, a 1.5 L column packed with AVIPure® AAV9 Affinity Resin can be used to purify not just one but *thirty* 2,000 L bioreactors, thus reducing the effective cost of the resin 30-fold.

A recommended CIP protocol for AVIPure® AAV9 Affinity Resin is provided below. It represents a good starting point for determination of the most efficient CIP protocol for the specific AAV9 purification process. With an optimized CIP protocol, the AVIPure® AAV9 Affinity Resin lifetime can be further increased even beyond the 30 cycles shown in the results presented above.



The following CIP protocol has been shown to work effectively for AVIPure® AAV9 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 10<sup>th</sup> 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® AAV9 Affinity Resin is an alkaline-stable affinity chromatography resin developed for simple, one-step purification of adeno-associated virus (AAV9) vectors directly from lysate. AVIPure® AAV9 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® AAV9 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® AAV9 Affinity Resin is available in bulk or in prepacked columns. A residual ligand kit ELISA assay is available from Cygnus Technologies (Part number F970). Regulatory Support File for the AVIPure® AAV9 Affinity Resin is available upon request.

## Ordering information

Items listed here are available through the Repligen E-Store ([store.repligen.com](https://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](mailto:customerserviceUS@repligen.com)

EU: [customerserviceEU@repligen.com](mailto:customerserviceEU@repligen.com)

China: [customerserviceCN@repligen.com](mailto:customerserviceCN@repligen.com)

Bulk resin volume	AVIPure® AAV9
10 mL	100AAV9-10
25 mL	100AAV9-25
50 mL	100AAV9-50
100 mL	100AAV9-100
250 mL	100AAV9-250
1000 mL	100AAV9-1000

Column type	ID [mm]	H [mm]	V [mL]	AVIPure® AAV9
OPUS® MiniChrom®	5	50	1.0	23051206
OPUS® MiniChrom®	11.3	50	5.0	23051207
OPUS® MiniChrom®	8	100	5.0	23051204-100

Column type	Rows	V [μL]	AVIPure® AAV9
OPUS® RoboColumn®	8	200	23051208R
OPUS® RoboColumn®	8	600	23051208R-30

Description	AVIPure® AAV9
Residual Ligand ELISA Assay, available through Cygnus Technologies	F970

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](mailto:orders@cygnustechnologies.com).

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