Affinity chromatography

AVB Sepharose™ High Performance

AVB Sepharose High Performance is an affinity chromatography resin designed for the purification of adeno associated virus (AAV). Adeno associated viruses (AAV) are of increasing interest as potential vectors for gene therapy. To enable the use of AAV in clinical applications, an efficient and high-quality production process is needed, including downstream purification. The purification process needs to be robust, with high yields, high purity, and low leakage of ligand. In current purification protocols density gradient centrifugation is typically used, followed by several chromatography steps, giving a process with low yield and poor scalability.

Benefits of AVB Sepharose HP include:

- Efficient, industrial-scale purification of adeno-associated viruses (AAV) of several subclasses by affinity chromatography
- Reduced regulatory concerns (due to non-mammalian derived product) in the production of AAV for clinical applications
- High selectivity and excellent scalability

Resin characteristics

AVB Sepharose High Performance is based on a highly cross-linked 6% agarose matrix, which enables rapid processing of large sample volumes. The ligand is attached to the base matrix via a long, hydrophilic spacer arm to make it easily available for binding of the virus (Fig 1).

The AAV affinity ligand was developed with technology from BAC BV (now part of Thermo Fisher Scientific Inc.). Ligand manufacturing, including fermentation and subsequent purification/formulation, is performed in the absence of mammalian components. The ligand itself was developed using Camelidae-derived, single-domain antibody fragments from the immune response of llamas towards the target AAV. The gene of the selected protein was cloned into a yeast cell expression system.

Table 1 summarizes the main characteristics of AVB Sepharose High Performance.

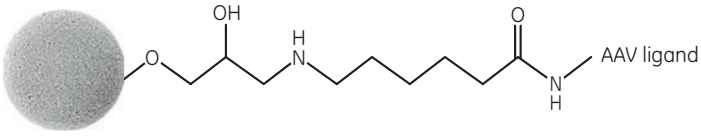


Fig 1. Partial structure of AVB Sepharose High Performance.

Table 1. Main characteristics of AVB Sepharose High Performance

Matrix	Cross-linked agarose, 6%, spherical
Particle size, d_{50v}*	34 µm
Ligand	Recombinant protein (M_r 14 000) produced in *Saccharomyces cerevisiae*. Binds AAV of subclasses 1, 2, 3, and 5
Total binding capacity†	Typically > 10^{12} genome copies/mL of chromatography resin
Flow velocity	Up to 150 cm/h at 30 cm bed height at 20°C using buffers with the same viscosity as water at < 0.3 MPa (3 bar)
pH stability, operational‡	3 to 10
pH stability, CIP§	2 to 12
Working temperature¶	4°C to 30°C

* Median particle size of the cumulative volume distribution.

† Protein in excess is loaded in 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0 on Tricorn™ 5/50 column. The binding capacity is obtained by measuring the amount of bound and eluted protein in 0.1 M sodium acetate, 0.5 M NaCl, pH 2.5.

‡ pH interval where the resin can be operated without significant change in function.

§ pH interval where the resin can be subjected to cleaning in place (CIP) or sanitization in place without significant change in function.

¶ Recommended long-term storage conditions: 2°C to 8°C, 20% ethanol.

Principles

Affinity chromatography is one of the chromatographic methods for purification of a specific molecule or a group of molecules from complex mixtures. The technique offers high selectivity and usually high capacity for the target molecule. As affinity chromatography is a binding technique, the sample volume does not affect the separation. Diluted samples can be applied, although capacity is commonly somewhat lower with more diluted sample.

The immobilized ligand adsorbs the target molecule under suitable binding conditions. Under suitable elution conditions, the target molecule is desorbed. These conditions depend on the target molecule, feed composition, and the chromatography resin, and they must be evaluated together with other chromatographic parameters (e.g., sample load, flow velocity, bed height, regeneration, cleaning-in-place, etc.) to establish the conditions that will bind the largest amount of target molecule in the shortest time and with the highest product recovery.

Regeneration should restore the original function of the resin. Depending on the nature of the sample, regeneration is normally performed after each cycle, followed by re-equilibration in start buffer. In order to prevent build-up of contaminants over time, more rigorous protocols may have to be applied (see cleaning in place [CIP] and sanitization in place [SIP]).

Application

When using AVB Sepharose High Performance the AAV can be applied directly from clarified AAV vector cell lysate. Conventional buffers (e.g., PBS, Tris, citrate) may be used for loading, washing, and elution. Virus binds to the column at around neutral pH and is typically eluted by lowering the pH, for example in the range of pH 2 to 5 (see *Screening of different elution conditions*). Since AAV is sensitive to highly acidic conditions (1), it is important to minimize the exposure to low pH during elution. Therefore, collected elution fractions should be neutralized immediately.

Screening of different elution conditions

Although AAV is efficiently eluted at low pH, the virus is sensitive to highly acidic conditions (1). Therefore, we designed a study to determine whether alternative buffers could be used for elution. Seven elution buffers were prepared (Table 2). Microplates were used to facilitate the screening of various elution conditions. Three elution conditions were further evaluated using AVB Sepharose High Performance column chromatography. Low pH elution provided the highest virus yields using column chromatography; subsequent high pH elution with arginine provided a minimal increase in yield. Although the virus yield after column chromatography was lower with high pH buffer and arginine compared with low pH buffer, the purity of the virus was comparable.

Elution buffer formulation

Published reports (2–4) and internal data were used for guidance in elution buffer formulation (Table 2). Arginine was included because it has been shown to enhance elution of antibodies from Protein A and from an antigen-conjugated agarose column (2) and to increase the recovery of enzymes in dye-affinity chromatography (3). Arginine seems to improve recovery and separation of proteins by reducing interaction of the protein with the column. In addition, it reduces protein aggregation (2). As far as we know, arginine has not previously been used to elute virus. $MgCl_2$ (2.5 M) was selected for inclusion because it has been used to elute AAV virus from an immuno-affinity column (4).

Overview of microplate experiments

Microplates were manually filled with 20 µL of AVB Sepharose High Performance or base matrix (Sepharose High Performance) per well. Liquid was removed between steps with centrifugation for 2 min at 290 × g. AAV sample (rAVV1, 7×10^{10} viral genomes/mL) was loaded at 200 µL per well. Equilibration, sample loading, and washing were identical for all wells. Two elution strategies were evaluated. In one strategy, one of the seven buffers listed in Table 2 was used for all three elutions. In another strategy, EB1 was always used for the first elution, followed by two elutions with one of the other buffers from Table 2. All eluted samples were analyzed by measuring the absorbance at 280 nm; samples of particular interest were further analyzed by AAV1 ELISA according to the manufacturer's instructions (PRAAVI; Progen Biotechnik GmbH, Heidelberg, Germany).

Procedure for microplate experiments

The following steps were performed:

1. Equilibration (3 times) with 200 µL of equilibration buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) per well.
2. Loading of 200 µL of rAAV1 sample per well. Microplates were incubated for 15 min on a shaker at 1100 rpm.
3. Washing (3 times) with 200 µL of wash buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) per well.
4. Elution (3 times) with 200 µL of elution buffer per well. Two different elution strategies were used (see above).

Table 2. Elution buffers evaluated in microplate experiments

EB1	0.1 M sodium acetate, 0.5 M NaCl, pH 2.5
EB2	0.1 M sodium acetate, 0.5 M NaCl, 0.5 M arginine, pH 10.0
EB5	20 mM Tris-HCl, 2.5 M $MgCl_2$, pH 8.0
EB6	0.1 mM sodium acetate, 2.5 M $MgCl_2$, pH 2.5
EB7	0.1 M glycine, 0.5 M NaCl, pH 3.0
EB8	20 mM Tris-HCl, 0.5 M NaCl, 0.5 M arginine, pH 10.8
EB9	1.5 M NaCl, 0.02% (w/v) Tween™ 80, 50% (v/v) ethylene glycol, 20 mM L-histidine, 20 mM $CaCl_2$, pH 6.5

Results from microplate experiments

In all cases, substantially more AAV was eluted from AVB Sepharose High Performance than from the base matrix, Sepharose High Performance. The base matrix did not show bind and elute properties. Instead, it retarded the AAV. The screening on microplates indicated that the yield of AAV1, based on A_{280} readings, was highest with three elutions of EB8 (20 mM Tris-HCl, 0.5 M arginine, pH 10.8). In microplates, it was not possible to elute all the virus with low pH buffer (EB1). Using high pH elution with arginine (EB2) as a second step helped to elute material left on the gel after low pH elution.

Overview of column experiments

Columns were packed with 1 mL of AVB Sepharose High Performance at a flow rate of 214 cm/h. rAAV1 was eluted using three different conditions: low pH followed by high pH with 0.5 M arginine; high pH with 0.5 M arginine; or high pH with 1.0 M arginine. Eluted virus was detected using AAV1 ELISA (Progen). Fractions of interest were further analyzed using SDS-PAGE.

Chromatographic results and yield determination

Figure 2 shows the chromatographic results for low pH elution followed by high pH elution with 0.5 M arginine. Note that EB8 buffer was used instead of EB2 because of the higher buffering capacity of Tris at high pH. Fractions 2, 3, and 4 were collected after low pH elution, and fraction 7 was collected after high pH elution. The flowthrough and fractions were analyzed separately by AAV1 ELISA. Almost all of the bound virus (120% of 130% total recovered virus) was eluted after the initial low pH elution. In contrast to the results in the microplate experiments, only a small amount (6%) of the bound virus was eluted with the second, high pH elution containing arginine.

Chromatographic results for high pH elution containing 0.5 M arginine are shown in Figure 3. Eluted virus was 72% of 73% total recovered virus.

A third elution condition used high pH buffer containing 1.0 M arginine (Fig 4) to determine if a higher arginine concentration would improve yields. Eluted virus was 62% of 64% total recovered virus. Based on AAV1 ELISA results. Therefore, the increased arginine concentration did not improve recovery of the virus.

Column:	Tricorn 5/50 (1 mL column volume)
Resin:	AVB Sepharose High Performance, 1 mL
Sample:	rAAV1 (7.0 × 10^{10} viral genomes/mL) filtered through 0.2 µm filter
Sample load:	20 mL
Equilibration, loading, and wash buffer:	20 mM Tris-HCl, 0.5 M NaCl, pH 8.0
First elution buffer:	EB1 (0.1 M sodium acetate, 0.5 M NaCl, pH 2.5)
Second elution buffer:	EB8 (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M arginine, pH 10.5)
Flow rate:	153 cm/h
System:	ÄKTA™ system

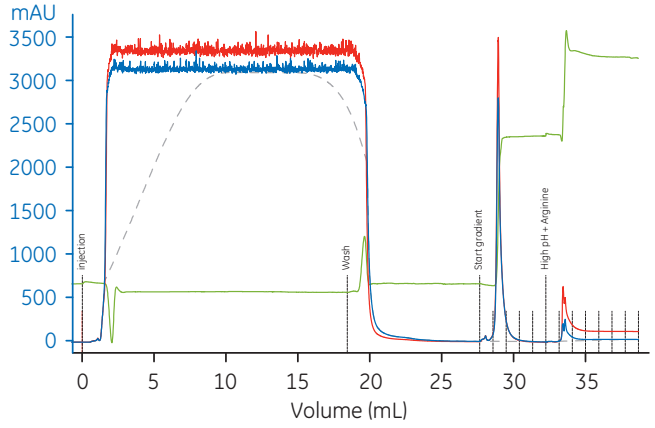


Fig 2. Purification of AAV on AVB Sepharose High Performance using low pH elution followed by high pH elution with 0.5 M arginine. The absorbance at 260 and 280 nm is shown in red and blue, respectively. Conductivity is shown in green. Fractions (1 mL) were collected after the gradient was started.

Column:	Tricorn 5/50 (1 mL column volume)
Resin:	AVB Sepharose High Performance, 1 mL
Sample:	rAAV1 (7.0 × 10^{10} viral genomes/mL) filtered through 0.2 µm filter
Sample load:	20 mL
Equilibration, loading, and wash buffer:	20 mM Tris-HCl, 0.5 M NaCl, pH 8.0
Elution buffer:	EB8 (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M arginine, pH 10.5)
Flow rate:	153 cm/h
System:	ÄKTA system

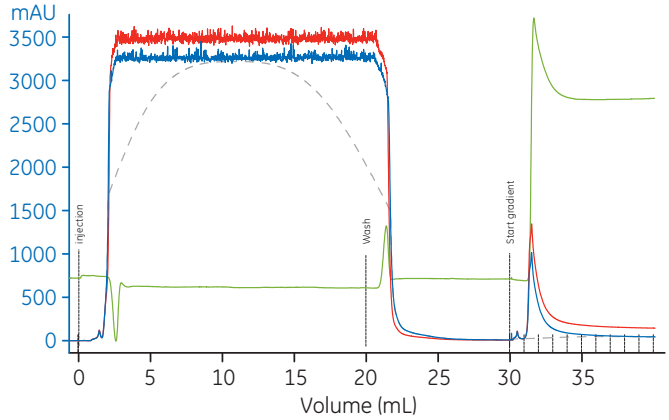


Fig 3. Purification of AAV on AVB Sepharose High Performance using high pH elution with 0.5 M arginine. The absorbance at 260 and 280 nm is shown in red and blue, respectively. Conductivity is shown in green. Fractions (1 mL) were collected after the gradient was started.

Column:	Tricorn 5/50 (1 mL column volume)
Resin:	AVB Sepharose High Performance, 1 mL
Sample:	rAAV1 (7.0×10^{10} viral genomes/mL) filtered through 0.2 µm filter
Sample load:	20 mL
Equilibration, loading, and washing buffer:	20 mM Tris-HCl, 0.5 M NaCl, pH 8.0
Elution buffer:	20 mM Tris-HCl, 0.5 M NaCl, 1.0 M arginine, pH 10.8
Flow rate:	153 cm/h
System:	ÄKTA system

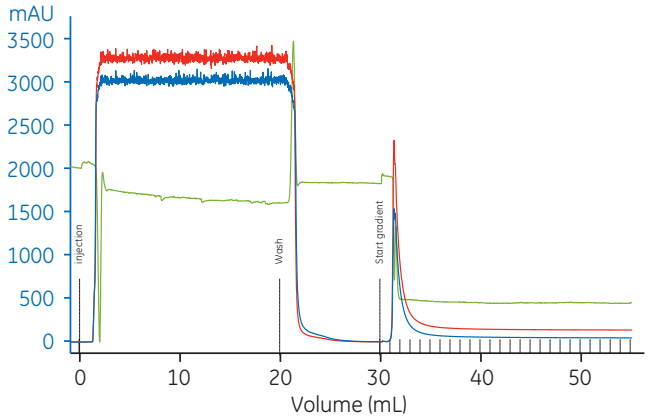


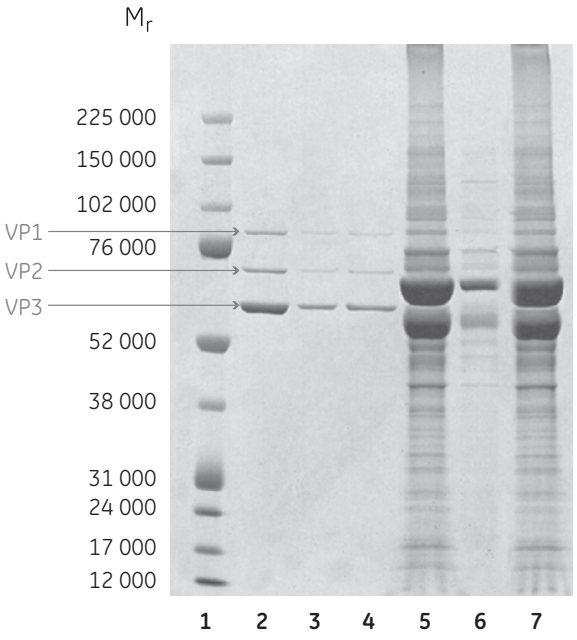
Fig 4. Purification of AAV on AVB Sepharose High Performance using high pH elution with 1.0 M arginine. The absorbance at 260 and 280 nm is shown in red and blue, respectively. Conductivity is shown in green. Fractions (1 mL) were collected after the gradient was started.

SDS-PAGE results

SDS-PAGE (Fig 5) shows only the three AAV viral capsid proteins, VP1, VP2, and VP3 (at M_r 87 000, 73 000, and 62 000 respectively) in fractions eluted from AVB Sepharose High Performance columns using low pH buffer or high pH buffer containing 0.5 M arginine. These results indicate that high purity AAV is eluted in a single step regardless of the elution buffer used in these studies.

Conclusions of this study

These data indicate that low pH elution provides the highest yield of virus from AVB Sepharose High Performance affinity columns. Although yields were lower with high pH elution and 0.5 or 1.0 M arginine, these results show that high pH elution buffer containing arginine yields highly pure AAV. Therefore, high pH elution is a viable alternative for purifying virus that is sensitive to low pH.



Lanes

1. Molecular weight marker
2. Fraction 2 from low pH elution (Fig 2)
3. Fraction 7 from high pH elution (0.5 M arginine) that followed low pH elution (Fig 2)
4. Fraction 2 from high pH elution (0.5 M arginine; Fig 3)
5. Flowthrough (Fig 2)
6. Wash (Fig 2)
7. Loaded sample

Fig 5. SDS-PAGE results of AVB Sepharose High Performance column chromatography using two different elution conditions.

Stability

The ligand is linked to the Sepharose High Performance base matrix via a stable amide bond. In a study where AVB Sepharose High Performance was stored at 40°C at different pH values for one week it was shown that the leakage is low between pH 2 and 12 (Fig 6). At higher pH there is a leakage of both carbon and nitrogen, indicating hydrolysis of the ligand.

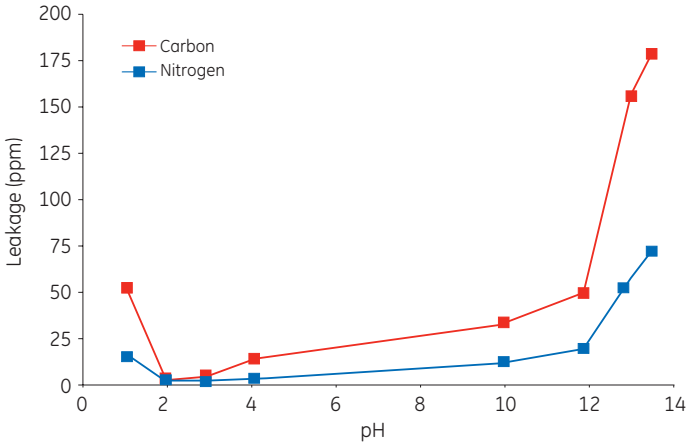


Fig 6. Stability of AVB Sepharose High Performance at different pH.

For stability in commonly used cleaning and sanitization solutions, studies have been performed on KappaSelect and LambdaFabSelect resins, two other products employing Thermo Scientific™ CaptureSelect™ affinity ligands (Thermo Fisher Scientific). The results from these studies are expected to be valid for AVB Sepharose HP as well, except for stability at high pH, which is somewhat enhanced for LambdaFabSelect compared with other CaptureSelect ligand-containing products. For results, see data file for the respective product.

Leakage assay

For determination of ligand leakage from AVB Sepharose HP resin, the Thermo Scientific CaptureSelect AVB Sepharose HP Leakage ELISA Kit (Thermo Fisher Scientific) can be used.

Cleaning in place (CIP) and sanitization in place (SIP)

A cleaning or sanitization protocol should be designed for each application, as the efficiency of the protocol is strongly related to the feedstock and other related operating conditions. The recommended protocol comprises initial strip of the resin at low pH, and then subjecting the resin to NaOH of low concentration for cleaning. Lastly, PAB (120 mM phosphoric acid, 167 mM acetic acid, 2.2 % v/v benzyl alcohol) is used for final sanitization of the resin. PAB solution is sensitive to light and should be freshly made not to damage the resin.

PAB solution should be stored in a dark bottle and kept no longer than for a week. PAB solution has a pH of < 2, and resin stability can be limited in prolonged exposure at such a low pH.

1. 0.1 M citric acid, pH 2.1; 10 min; 13 CV
   10 CV PBS, pH 7.4
2. 10 mM NaOH, pH 12; 15 min; 19 CV
   10 CV PBS, pH 7.4
3. PAB; 15 min; 19 CV

Equilibrate the resin using equilibration buffer prior to next purification cycle.

Storage

The recommended storage conditions are 20% ethanol at 4°C to 8°C. AVB Sepharose High Performance is supplied pre-swollen in a 20% ethanol solution.

References

1. Wu, N. *et al.* Production of viral vectors for gene therapy applications. *Curr. Opin. Biotechnol.* **11**, 205–208 (2000).
2. Ejima, D. *et al.* Improved column chromatography performance using arginine. *American Biotechnology Laboratory* 16–18 (Feb 2007).
3. Arakawa, T. *et al.* Improved performance of column chromatography by arginine: dye-affinity chromatography. *Protein Expr. Purif.* **52**, 410–414 (2007).
4. Summerford, C. *et al.* Viral receptors and vector purification: new approaches for generating clinical-grade reagents. *Nat. Med.* **5**, 587–588 (1999).

Interesting reading

Smith R. H., *et al.* A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells. *Mol. Ther.* **17**, 1888–1896 (2009).

Ordering information

Product	Quantity	Product code
AVB Sepharose High Performance	75 mL	28411201
	1 L	28411202
	5 L	28411203
	10 L	28411204
Prepacked HiTrap™ column	5 × 1 mL	28411211
	1 × 5 mL	28411212

Related literature

AVB Sepharose High Performance Regulatory Support	File on request
Affinity Chromatography Handbook	18102229
Affinity Columns and Media, Selection Guide	18112186

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