

AVB Sepharose™ High Performance

AVB Sepharose High Performance is an affinity chromatography medium (resin) designed for the purification of adeno associated virus (AAV). Key characteristics of AVB Sepharose High Performance include:

- Fast, one-step purification of adeno associated viruses of several subclasses
- Prepacked HiTrap™ columns for simple operation with a syringe, pump, or chromatography system
- Excellent scalability

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.

Medium 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, an M_r 14 000 recombinant protein, is attached to the base matrix via a long, hydrophilic spacer arm to make it easily available for binding of the virus (Fig 1).

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

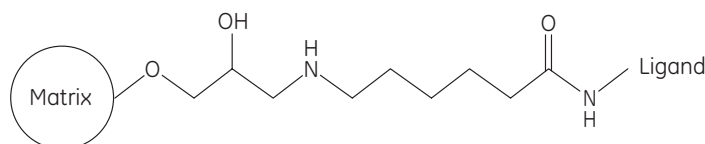


Fig 1. Partial structure of AVB Sepharose High Performance.

Table 1. Main characteristics of AVB Sepharose High Performance

Matrix	Cross-linked 6% agarose
Average particle size	34 μm
Ligand	M_r 14 000 recombinant protein produced in <i>S. cerevisiae</i> . Binds AAV of subclasses 1, 2, 3, and 5
Capacity	Typically $> 10^{12}$ genome copies/ml of chromatography medium
Recommended flow rate	Up to 150 cm/h at 30 cm bed height
Maximum back pressure	0.3 MPa, 3 bar
pH stability	
Long term	3–10
Short term	2–12

Functional principles

Affinity chromatography exploits an immobilized ligand that adsorbs a specific molecule or group of molecules under suitable binding conditions and desorbs them under suitable elution conditions. These conditions depend on the target molecule, feed composition, and chromatography medium, and must be studied together with other chromatographic parameters (e.g., sample load, flow velocity, bed height, regeneration, cleaning-in-place) to establish the conditions that will bind the largest amount of target molecule, in the shortest time and with the highest product recovery.



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.

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

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 room temperature at different pH values for one week it was shown that the leakage is low between pH 2 and 12 (Fig 2). At higher pH there is a leakage of both carbon and nitrogen, indicating hydrolysis of the ligand.

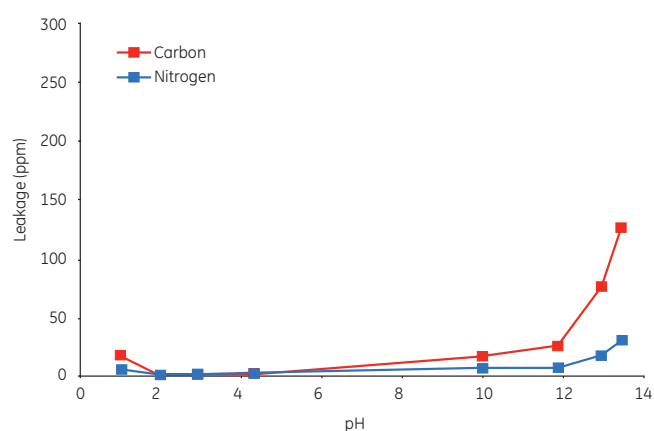


Fig 2. Stability of AVB Sepharose High Performance at different pH.

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. Nine elution buffers were prepared (Table 2); two of these (EB3 and EB4) were not evaluated because they formed precipitates and required heating prior to each use. 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 μ l 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 \times g. AAV sample (rAVV1, 7×10^{10} viral genomes/ml) was loaded at 200 μ l 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 μ l of equilibration buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) per well.
2. Loading of 200 μ l of rAAV1 sample per well. Microplates were incubated for 15 min on a shaker at 1100 rpm.
3. Washing (3 times) with 200 μ l of wash buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) per well.
4. Elution (3 times) with 200 μ l 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 A₂₈₀ 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 3 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.

Column: Tricorn™ 5/50 (1 ml column volume)
Medium: AVB Sepharose High Performance, 1 ml
Sample: rAAV1 (7.0 × 10¹⁰ viral genomes/ml)
Sample load: 20 ml
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: ÄKTAexplorer™ 10

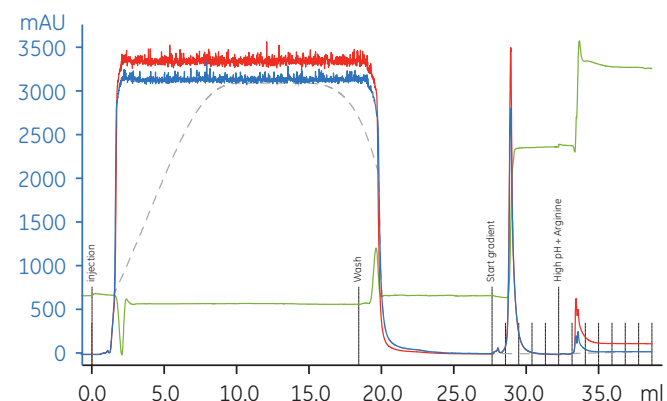


Fig 3. Purification of AAV on AVB Sepharose High Performance using low pH elution followed by high pH elution with 0.5 M arginine. The absorbance at 260 and 280 nm is shown in red and blue, respectively. Conductivity is shown in green. Fractions (1 ml) were collected after the gradient was started.

Chromatographic results for high pH elution containing 0.5 M arginine are shown in Figure 4. Eluted virus was 72% of 73% total recovered virus.

Column: Tricorn 5/50 (1 ml column volume)
Medium: AVB Sepharose High Performance, 1 ml
Sample: rAAV1 (7.0×10^{10} viral genomes/ml)
Sample load: 20 ml
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: ÄKTAexplorer 10

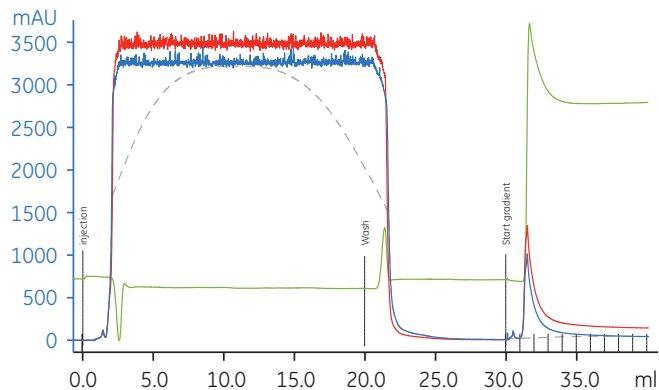


Fig 4. Purification of AAV on AVB Sepharose High Performance using high pH elution with 0.5 M arginine. The absorbance at 260 and 280 nm is shown in red and blue, respectively. Conductivity is shown in green. Fractions (1 ml) were collected after the gradient was started.

A third elution condition used high pH buffer containing 1.0 M arginine (Fig 5) 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)
Medium: A VB Sepharose High Performance, 1 ml
Sample: rAAV1 (7.0×10^{10} viral genomes/ml)
Sample load: 20 ml
Wash 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: ÄKTAexplorer 10

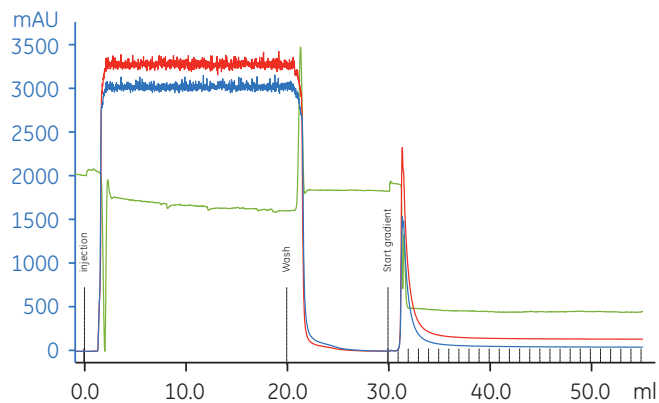
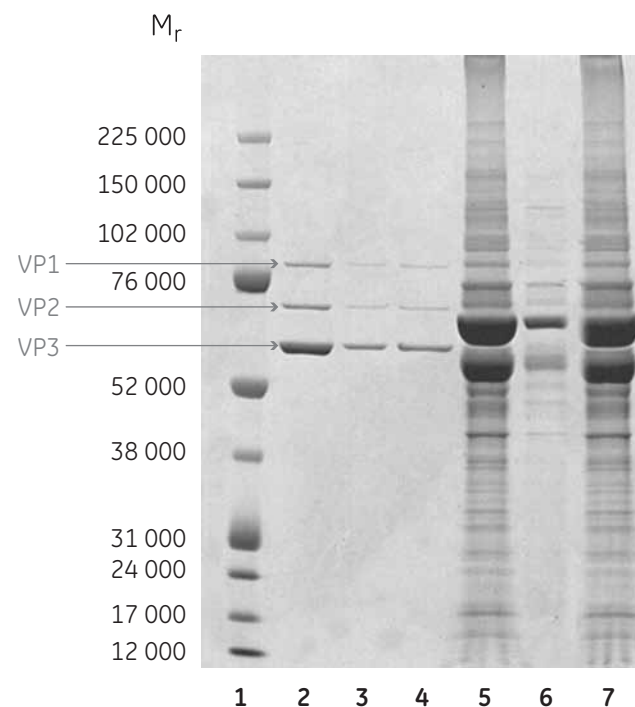


Fig 5. Purification of AAV on AVB Sepharose High Performance using high pH elution with 1.0 M arginine. The absorbance at 260 and 280 nm is shown in red and blue, respectively. Conductivity is shown in green. Fractions (1 ml) were collected after the gradient was started.

SDS-PAGE results

SDS-PAGE (Fig 6) 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.



Lanes

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

Fig 6. SDS-PAGE results of AVB Sepharose High Performance column chromatography using two different elution conditions.

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.

Cleaning-in-place and sanitization

A cleaning or sanitization protocol has to be designed for each application. A suggested cleaning protocol is to use a solution of low pH, for example 0.1 M phosphoric acid, alone or in combination with sodium chloride or ethanol. However, prolonged exposure (i.e., several days) to pH < 2 should be avoided due to a slow decomposition of the agarose matrix at low pH. Sodium hydroxide should be used with care due to the low stability of the medium under alkaline conditions.

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	Code no.
AVB Sepharose High Performance*	75 ml	28-4112-01
AVB Sepharose High Performance*	1 l	28-4112-02
Prepacked HiTrap column*	5 x 1 ml	28-4112-11
Prepacked HiTrap column*	1 x 5 ml	28-4112-12

* This product is part of our Custom Designed Media program. If you are interested in large-scale quantities, please contact your local GE Healthcare representative

Literature

Affinity Chromatography Handbook	18-1022-29
Affinity Columns and Media, Selection Guide	18-1121-86

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