AAV production

Small-scale purification of AAV produced with the AAV-MAX system

Introduction

The Gibco™ AAV-MAX Helper-Free AAV Production System is an adeno-associated virus (AAV) production system developed for scalable high-titer AAV production. The AAV-MAX system comprises a clonal HEK293F-derived suspension cell line adapted to a chemically defined production medium, a transfection reagent and booster, a production enhancer, and lysis buffer.

Here we present two strategies for downstream processing and purification of recombinant AAV vectors from 2 or 4 L of culture produced using the AAV-MAX system. Strategy 1 is a more basic process that does not require the use of expensive equipment and filters and involves a straightforward clarification by centrifugation and concentration by tangential flow filtration (TFF), which is ideal for early discovery or bench-scale scouting studies (Figure 1).

Strategy 2 involves depth filters for clarification, a TFF process with higher AAV concentration, and a buffer-exchange step to stabilize the AAV. Strategy 2 is scalable to much larger production volumes (Figure 1). Using strategy 1 and 2, we achieved 70% and 49% total process recovery, respectively, of a recombinant AAV serotype 6 (rAAV6) vector using Thermo Scientific™ POROS™ AAVX affinity resin, demonstrating excellent recovery of AAV vectors produced using the AAV-MAX system at typical lab scales.

Materials and methods

AAV production

rAAV6-GFP was produced using the complete AAV-MAX system kit (Table 1) in two 2.8 L shake flasks for strategy 1 and in four 2.8 L shake flasks for strategy 2, according to the AAV-MAX user guide (Pub. No. MAN0019619).

Lysis and nuclease treatment

At the time of harvest (3 days post-transfection), 10X Gibco™ AAV-MAX Lysis Buffer was added to 2 L of rAAV6 culture to a final 1X concentration. During lysis, $MgCl_2$ was added to a final concentration of 2 mM along with Thermo Scientific™ Pierce™ Universal Nuclease to a final concentration of 90 U/mL to digest DNA not encapsidated in rAAV particles. The culture was placed in a 37°C incubator on a shaker platform set at 125 rpm for 2 hours.

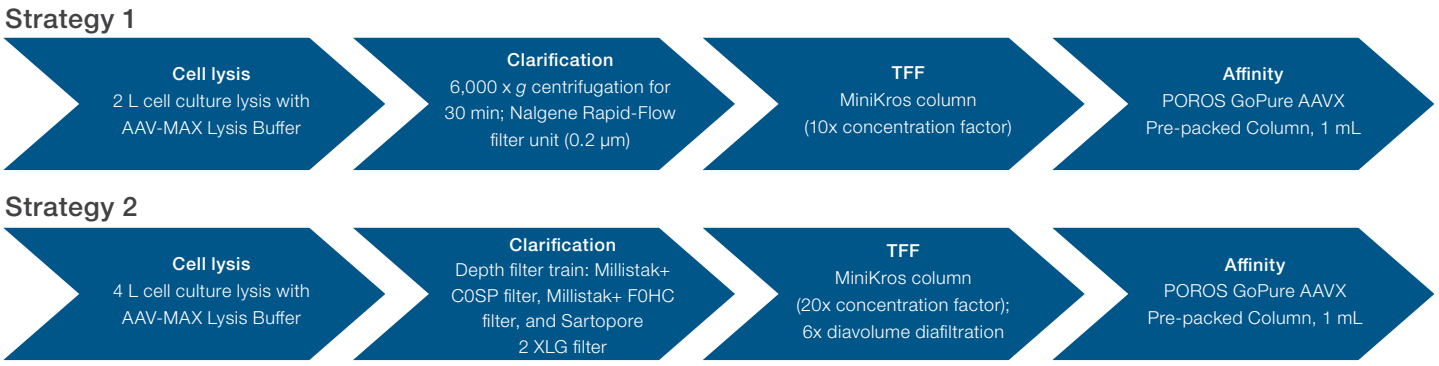

Figure 1. Overview of the two purification strategies.

Table 1. Materials used for AAV production and purification.

Process step	Material description	Cat. No.
AAV production	AAV-MAX Helper-Free AAV Production System Kit	A51217
Lysis and nuclease treatment	AAV-MAX Lysis Buffer (10X)	A50520
	1 M $MgCl_2$	J61014.AK
	Pierce Universal Nuclease	88702
	Thomson Optimum Growth 2.8 L Flask (fishersci.com)	50-112-006
Centrifugation clarification	Nalgene Rapid-Flow Sterile Disposable Filter Units with PES Membranes (0.2 µm)	567-0020
	Vacuum pump (Welch)	I31-0176
	Sorvall LYNX 6000 Superspeed Centrifuge	75006590
	Nalgene PPCO Centrifuge Bottles	3120-1000
Depth filter clarification	Masterflex L/S pump (fishersci.com)	07522-20
	Masterflex platinum-cured silicone tubing, size 25 (fishersci.com)	96440-25
	Tubing clamps or hemostat	16100118
	Millistak+ HC Pro Depth Filter, C0SP (MilliporeSigma)	MC0SP027H1
	Millistak+ Pod Depth Filter, F0HC (MilliporeSigma)	MF0HC027H1
	Sartopore 2 XLG filter (Sartorius)	5441307G4-SS-B
Tangential flow filtration	Masterflex L/S pump (fishersci.com)	07522-20
	Masterflex platinum-cured silicone tubing, size 25 (fishersci.com)	96440-25
	Tubing clamps or hemostat	PL19
	Tube hosecock	05847Q
	Reagent bottles (Corning)	431432
	MiniKros hollow-fiber TFF module (Repligen)	S02-E100-05-N
	SciLog pressure sensors (Parker)	080-699PSX-3P-5
	SciLog pressure monitor (Parker)	SP0820J-1329
Affinity chromatography	ÄKTA Pure 25 FPLC system (Cytiva)	NA
	POROS GoPure AAVX Pre-packed Column	A36652
	Various buffers	NA

Strategy 1: centrifugation clarification and TFF

Nuclease-treated cell lysate was centrifuged at 6,000 x *g* for 30 minutes at 20°C. The supernatant was decanted into a Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Unit (0.2 µm), and vacuum was applied to begin filtration. After overnight storage at 2–8°C, the clarified lysate was re-clarified using the same centrifugation clarification and filtration protocol before being loaded onto a MiniKros™ hollow-fiber tangential flow filter (HFTFF) column (Table 2). The new hollow fiber filter was rinsed with 2 mL of deionized (DI) water per cm^2 of filter area to flush the storage glycerol from the filter, then equilibrated with 1 mL of affinity equilibration buffer per cm^2 of filter area. The clarified lysate was recirculated through the HFTFF at a shear rate of 2,000 s^{-1} (368 mL/min). Using a hosecock, back pressure was applied and adjusted to the recirculation line to achieve a transmembrane pressure (TMP) of 5 psi throughout the process. Once the material was concentrated 10x, the material was collected. A 1.4x hold-up volume amount of affinity equilibration buffer was used to chase the HFTFF, and it was combined with the HFTFF product. The combined chase and HFTFF product was centrifuged and filtered using a Nalgene Rapid-Flow Sterile Disposable Filter Unit (0.2 µm), following the aforementioned clarification protocol.

Strategy 2: depth filter clarification and TFF

Millistak+™ C0SP and F0HC depth filters were flushed with a minimum of 100 L/m^2 of filter area (2.7 L) of DI water or until all air was purged from filters, at a flux rate of 600 L/m^2/hr (270 mL/min). The outlet of the Millistak+ C0SP filter was connected to the inlet of the F0HC filter, and the outlet of the F0HC filter was connected to the inlet of the Sartopore™ 2 XLG filter. The entire filter train was equilibrated with 1.5x depth filter hold-up volume (975 mL) of clarification buffer at a flow rate of 150 L/m^2/hr (67.5 mL/min). The nuclease-treated cell lysate was pumped through the entire filter train at a flow rate of 150 L/m^2/hr (67.5 mL/min), then chased with 1.5x total hold-up volume (1,008 mL) of clarification buffer at the same flow rate. After overnight storage at 2–8°C, the clarified lysate was re-clarified using a Sartopore 2 XLG filter before being loaded onto a MiniKros HFTFF column (Table 2). The new hollow fiber filter was rinsed with 2 mL of DI water per cm^2 of filter area to flush the storage glycerol from the filter, then equilibrated with 1 mL of affinity equilibration buffer per cm^2 of filter area. The clarified lysate was recirculated through the HFTFF at a shear rate of 2,000 s^{-1} (368 mL/min). Using a hosecock, back pressure was applied and adjusted to the recirculation line to achieve a TMP of 5 psi throughout the process. Once the material was concentrated 20x, the material was buffer exchanged. The buffer exchange was achieved by slowly adding 6 diavolumes of affinity equilibration buffer 2 to the sample reservoir while maintaining the 20x concentrated volume. After buffer exchange, the material was collected. A 1.4x hold-up volume amount of affinity equilibration buffer was used to chase the HFTFF, and it was combined with the HFTFF product. The combined chase and HFTFF product was filtered using a Sartopore 2 XLG filter.

Table 2. TFF parameters.

Parameter	Setting
Sample recirculation shear rate	2,000 s^{-1}
Transmembrane pressure (TMP)	<5 psi
Concentration factor	10x or 20x
Buffer exchange volume	6 diavolumes
Tubing size	25

Affinity purification

The ÄKTA™ FPLC system was sanitized with 0.5 N NaOH, contact time of 30 min, prior to purification. A Thermo Scientific™ POROS™ GoPure™ AAVX Pre-packed Column (0.5 x 5 cm, 1 mL) was preconditioned with 5 column volumes (CV) of DI water, followed by 5 CV of 0.2 M phosphoric acid with a contact time of 15 minutes, then 5 CV of 6 M guanidine HCl, 5 CV of 2 M NaCl, 5 CV of wash 1 buffer, and finally 10 CV of affinity equilibration buffer 1 for strategy 1 and affinity equilibration buffer 2 for strategy 2, all run at a flow rate of 300 cm/hr (Table 3). Next, the filtered HFTFF product was loaded onto the column at a flow rate of 300 cm/hr (corresponding to 1 minute of residence time). After the filtered HFTFF product was loaded, the column was washed with 10 CV of equilibration buffer followed by three different intermediate washes, each 5 CV, and then a final 10 CV of equilibration buffer. The rAAV6 was eluted using a glycine buffer at pH 2.5. The POROS GoPure AAVX column was stripped and sanitized with 7 CV of 0.2 M phosphoric acid with a contact time of 15 minutes, followed by 5 CV of 6 M guanidine HCl, 5 CV of affinity equilibration buffer, 5 CV of 2 M NaCl, 10 CV of DI water, and then 5 CV of 20% ethanol for storage.

Quantification of viral genomes by ddPCR

AAV titers in viral genomes per mL (vg/mL) were determined by droplet digital PCR (ddPCR) using primers and probe targeting the GFP gene of interest. Prior to ddPCR analysis, samples were subjected to nuclease treatment to remove non-encapsidated DNA. Then, viral genome titers were determined by running serial dilutions of each sample on a QX200™ Droplet Digital PCR System (Bio-Rad).

Determination of percentage of full capsids

Capsid titers were determined using AAV6 ELISA kits (Progen), according to the manufacturer's instructions. Percentage of full AAV6 particles in elution fractions was determined by dividing the genome titer by capsid titer.

Quantification of transducing units by flow cytometry

Twenty-four hours after seeding HT1080 cells into wells of a 96-well plate, medium was removed, and the cells were coinfected with serial dilutions of rAAV6-GFP process samples and adenovirus 5 helper virus. Transducing units per mL (TU/mL) for each sample was calculated based on the number of GFP-positive cells per well as determined by flow cytometry.

Turbidity measurements

All turbidity measurements were made using 10 mL of sample on a Thermo Scientific™ Orion™ AQUAfast AQ3010 Turbidity Meter.

Table 3. Affinity purification method.

Method	Step	Buffer or solution	Inlet	Outlet	CV	Flow rate (cm/hr)	Flow rate (mL/min)	Incubation time (min)
Pre-use clean-in-place (CIP)	Rinse	Process water	B6	Waste	5	300	0.98	–
	Strip 1	0.2 M phosphoric acid	B3	Waste	5	300	0.98	15
	Regeneration	6 M guanidine HCl	B2	Waste	5	300	0.98	–
	Strip 2	2 M NaCl	B1	Waste	5	300	0.98	–
	Wash 1	50 mM Tris, 1.5 M NaCl, 0.01% Pluronic™ F-68 surfactant, pH 7.2	A2	Waste	5	300	0.98	–
	Affinity equilibration	Buffer 1: 100 mM Tris, 150 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2 Buffer 2: 100 mM Tris, 500 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2	A1	Waste	5	300	0.98	–
Affinity processing	Affinity equilibration	Buffer 1: 100 mM Tris, 150 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2 Buffer 2: 100 mM Tris, 500 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2	A1	Waste	5	300	0.98	–
	Load	Affinity load	S1	Out 1	Variable	300.14	0.98	1 min residence time
	Affinity equilibration	Buffer 1: 100 mM Tris, 150 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2 Buffer 2: 100 mM Tris, 500 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2	A1	Waste	10	300	0.98	–
	Wash 1	50 mM Tris, 1.5 M NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2	A2	Out 2	5	300	0.98	–
	Wash 2	50 mM Tris, 1.5 M urea, 0.01% Pluronic F-68 surfactant, pH 9.0	A3	Out 3	5	300	0.98	–
	Wash 3	50 mM Tris, 1% Tween™ 20 surfactant, 0.01% Pluronic F-68 surfactant, pH 9.0	A4	Out 4	5	300	0.98	–
	Affinity equilibration	Buffer 1: 100 mM Tris, 150 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2 Buffer 2: 100 mM Tris, 500 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2	A1	Waste	10	300	0.98	–
	Elution	75 mM glycine, 50 mM NaCl, 500 mM arginine, 0.01% Pluronic F-68 surfactant, pH 2.5	A5	Out 5	5	300.14	0.98	1 min residence time
	Strip 1	0.2 M phosphoric acid	B3	Out 6	5	300	0.98	–
Post-use CIP	Strip 1	0.2 M phosphoric acid	B3	Waste	2	300	0.98	15
	Regeneration	6 M guanidine HCl	B2	Waste	5	300	0.98	–
	Affinity equilibration	Buffer 1: 100 mM Tris, 150 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2 Buffer 2: 100 mM Tris, 500 mM NaCl, 0.01% Pluronic F-68 surfactant, pH 7.2	A1	Waste	5	300	0.98	–
	Strip 2	2 M NaCl	B1	Waste	5	300	0.98	–
	Rinse	Process water	B6	Waste	5	300	0.98	–
	Rinse	Process water	A6	Waste	5	300	0.98	–
	Storage	20% ethanol	A7	Waste	5	300	0.98	–

Results

Two different strategies were tested for processing AAV6 cultures prior to affinity chromatography. For strategy 1, centrifugation and filtration were used to create clarified lysate, followed by a 10x concentration over a HFTFF. For strategy 2, a series of depth filters were used to create clarified lysate, followed by a 20x concentration and 6-diavolume buffer exchange over a HFTFF. The results for each strategy are described below.

Strategy 1: centrifugation clarification

The lysed and nuclease-treated AAV6 culture had a turbidity of 302 nephelometric turbidity units (NTU) and a titer of 7.85 x 10^{13} vg/L of cell culture (Figures 2 and 3). After centrifugation and filtration over the 0.2 µm Nalgene Rapid-Flow filter unit, the turbidity was reduced to 16.49 NTU, and the process had a step yield of 94% with a titer of 7.36 x 10^{13} vg/L of cell culture (Figures 2 and 3). The 10x concentration over the HFTFF took 53 minutes to process with an average permeate flux of 28 liters per meter per hour (LMH) (Figure 4). After concentration over the HFTFF, the turbidity increased to 131 NTU and there was no loss of titer observed during this step. After centrifugation and filtration of the HFTFF product over the 0.2 µm Nalgene Rapid-Flow filter unit, the turbidity was reduced to 103 NTU. During affinity loading, the UV A_{280} signal reached above 2,000 mAU, indicating a large amount of protein was passed over the column (Figure 5). However, the maximum precolumn and delta column pressures remained below 0.05 MPa during the entire loading process. The purified affinity product produced 5.49 x 10^{13} vg/L of cell culture with a step yield of 74% recovery and an overall process yield of 70% recovery (Figure 3). Infectivity was maintained throughout the purification process; both the extracted cell culture and affinity products had infectivity values of 3.32 x 10^{3} vg/TU (Figure 6). This purification did not include an enrichment step for full capsids, so the percentage of full capsids remained relatively constant over the various steps of the purification process in a range of ~20–30% (Figure 7). Using SDS-PAGE, the affinity product showed the expected size and quality of capsid proteins (Figure 8).

Strategy 2: depth filter clarification

The lysed and nuclease-treated AAV6 culture had a turbidity of 442 NTU and a titer of 1.18 x 10^{14} vg/L of cell culture (Figures 2 and 3). Clarification through the Millistak+ C0SP, Millistak+ F0HC, and Sartopore 2 XLG filter train took 74 minutes. The maximum pressure on the Millistak+ C0SP filter reached 2.42 psi. The maximum pressure on the Millistak+ F0HC filter reached 1.34 psi. And the maximum pressure on the Sartopore 2 XLG filter reached 0.8 psi (Figure 9). The clarification step reduced turbidity to 1.03 NTU and had a step yield of 70% with a titer of 8.29 x 10^{13} vg/L of cell culture (Figures 2 and 3). The 20x concentration over the HFTFF took 52.6 minutes to process with an average permeate flux of 36.8 LMH (Figure 4). The 6-diavolume buffer exchange over the HFTFF took a total of 22.9 minutes with an average permeate flux of 21.4 LMH.

After concentration and diafiltration over the HFTFF, the turbidity increased to 12.8 NTU, and there was minimal loss of titer with a step yield of 76% (Figures 2 and 3). After filtration of the HFTFF product over the Sartopore 2 XLG filter, the turbidity was reduced

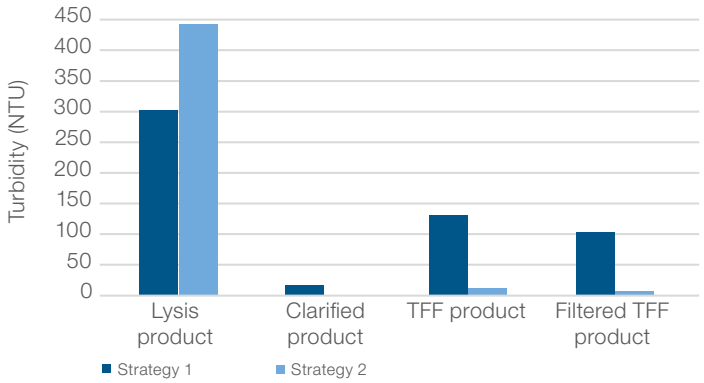

Figure 2. Turbidity at multiple process steps. Turbidity was measured using an Orion AQUAfast AQ3010 Turbidity Meter at each process step. Lower turbidity is observed post-clarification of the strategy 2 sample.

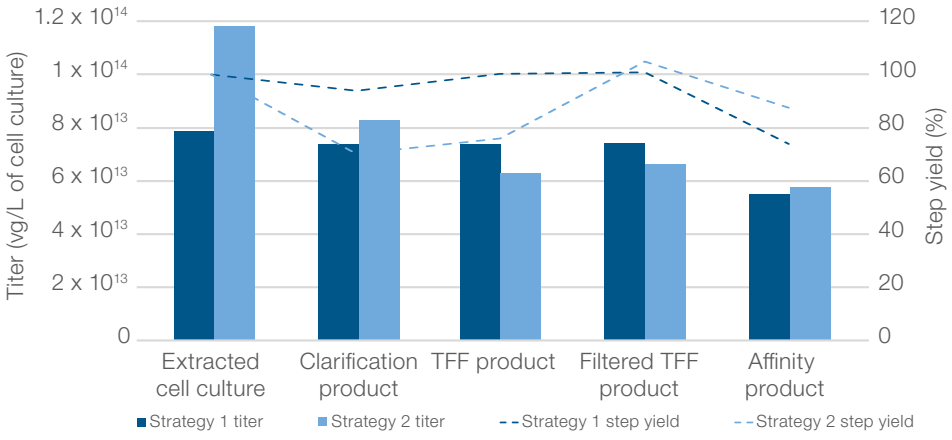

Figure 3. Titer and step yield of cell cultures at each process step. Titers (vg/mL) were determined by ddPCR, and vg/mL was multiplied by total volume in mL at each process step to determine total vg. Total vg was then divided by the total liters of cell culture that were processed at each step to determine the titer in vg/L of cell culture. This value enables comparison of the titers from both purification strategies at each process step regardless of the volume of material processed at each step. The change in titer is used to determine the step yield at each process step.

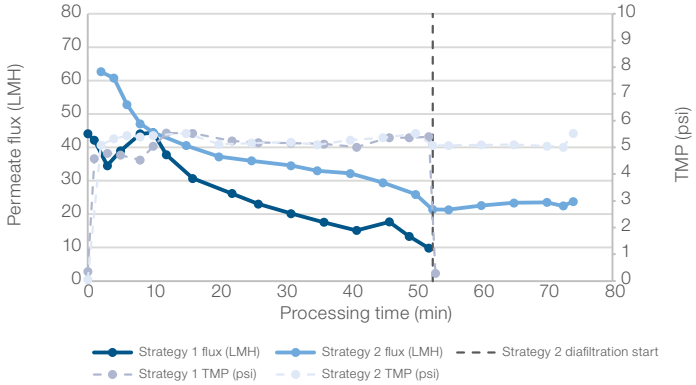

Figure 4. TFF permeate flux and filter transmembrane pressure over processing time. Pressure sensors were attached to the inlet, retentate, and permeate of the filter. Transmembrane pressure (TMP) was calculated by taking the average between the inlet and retentate pressure and subtracting the permeate pressure. A TMP of 5 psi was targeted using a hosecock to adjust the back pressure on the retentate. During TFF, the permeate was collected and weighed every 2–5 minutes. The permeate weight in kg (correlating to liters) was divided by the hours of processing time to determine the permeate flow rate in liters per hour. The permeate flow rate was then divided by the filter area in m^2 to determine the permeate flux in liters per meter per hour (LMH). For both strategies, the permeate flux decayed as the concentration factor increased. For strategy 2, the flux stabilized during buffer exchange as the concentration remained constant at this step. The permeate flux was notably higher throughout the process for strategy 2, indicating a cleaner feed material.

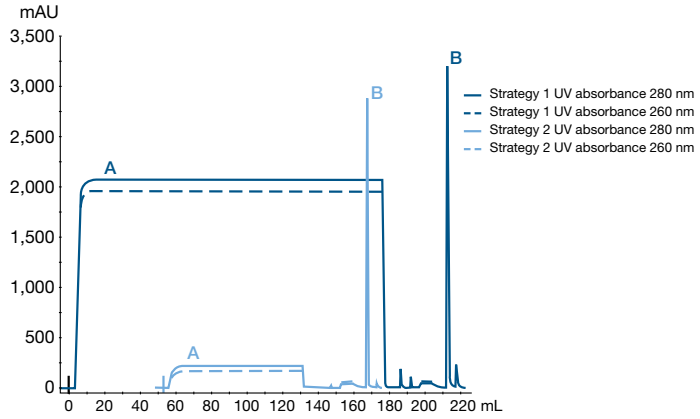

Figure 5. Affinity chromatography over 1 mL POROS GoPure AAVX Pre-packed Column. UV absorbance at 260 nm and 280 nm was measured using the ÄKTA FPLC system during affinity chromatography. The initial broad peak (A) correlates to contaminating proteins flowing through the affinity column without binding. The tall and sharp peak (B) correlates to the captured AAV being eluted from the column.

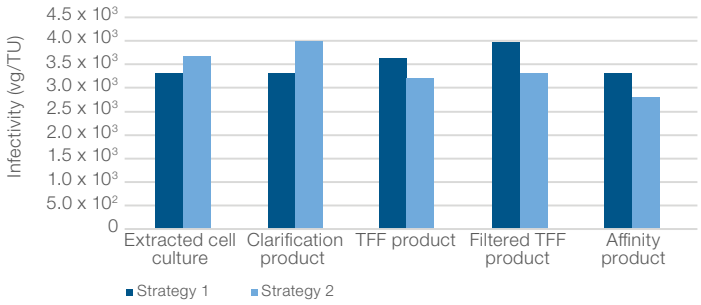

Figure 6. Infectivity at each process step. Infectious titer (TU/mL) was determined by flow cytometry, and vg/mL was determined by ddPCR. Values for vg/TU were calculated by dividing the vg/mL titer by the infectious titer.

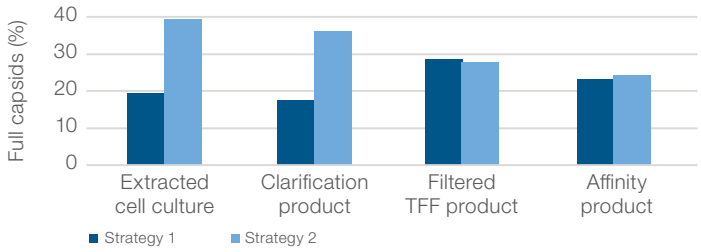

Figure 7. Percentage of full capsids at each process step. Values for vg/mL were determined by ddPCR, and values for capsids/mL were determined by ELISA. The percentage of full capsids was calculated by dividing the vg/mL by capsids/mL at each process step.

to 7.47 NTU. There was no product loss during this filtration. During affinity loading, the UV A_{280} signal reached only 215 mAU, indicating some protein was being passed over the column (Figure 5). The maximum precolumn and delta column pressures remained below 0.07 MPa during the entire loading process.

The purified affinity product produced 5.78 x 10^{13} vg/L of cell culture, providing an overall process yield of 49% (Figure 3). Infectivity was maintained throughout the purification process for the extracted cell culture (3.68 x 10^3 vg/TU) and the affinity product (2.79 x 10^3 vg/TU) (Figure 6). This purification did not include an enrichment step for full capsids, so the percentage of full capsids remained relatively constant over the various steps of the purification process in a range of ~25–40% (Figure 7). Using SDS-PAGE, the affinity product showed the expected size and quality of capsid proteins (Figure 8).

Conclusions

Two strategies are presented for bench-scale (2–4 L of cell culture) purification of rAAV6 produced using the AAV-MAX system. Both strategies produce high-quality and high-yield rAAV6 with overall yields of 70% for strategy 1 and 49% for strategy 2. The lower overall yield

Strategy 1	
Lane	Description
1	SeeBlue Plus2 standard
2	Affinity load
3	Affinity flow-through
4	Affinity wash 1
5	Affinity wash 2
6	Affinity wash 3
7	Affinity product
8	Affinity strip
9	SeeBlue Plus2 standard

Strategy 2	
Lane	Description
1	SeeBlue Plus2 standard
2	SeeBlue Plus2 standard
3	Affinity load
4	Affinity flow-through
5	Affinity wash 1
6	Affinity wash 2
7	Affinity wash 3
8	Affinity product
9	Affinity strip
10	SeeBlue Plus2 standard

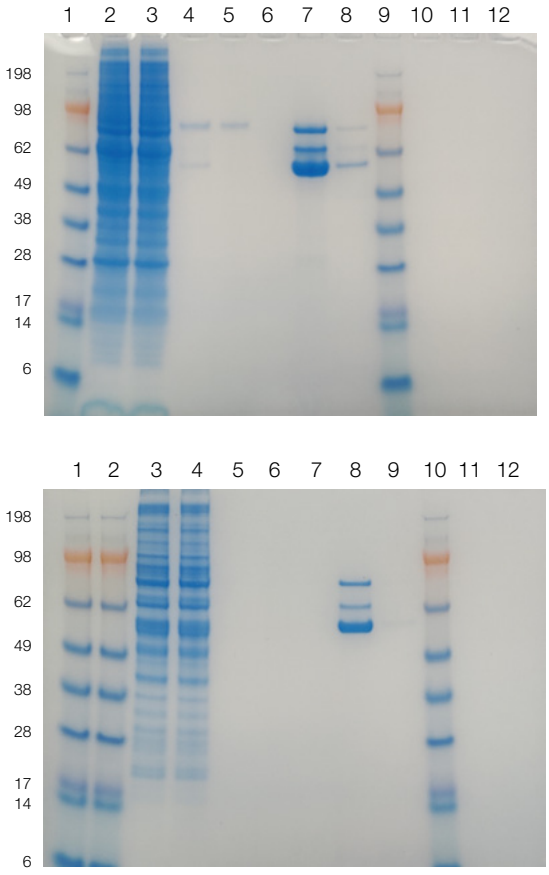

Figure 8. SDS-PAGE of samples from the affinity process step. The affinity step samples and Invitrogen™ SeeBlue™ Plus2 Pre-stained Protein Standard were loaded onto an Invitrogen™ NuPAGE™ 4–12% Bis-Tris gel under reducing conditions and run for 35 minutes at 200 V. The gel was then stained using Invitrogen™ SimplyBlue™ SafeStain.

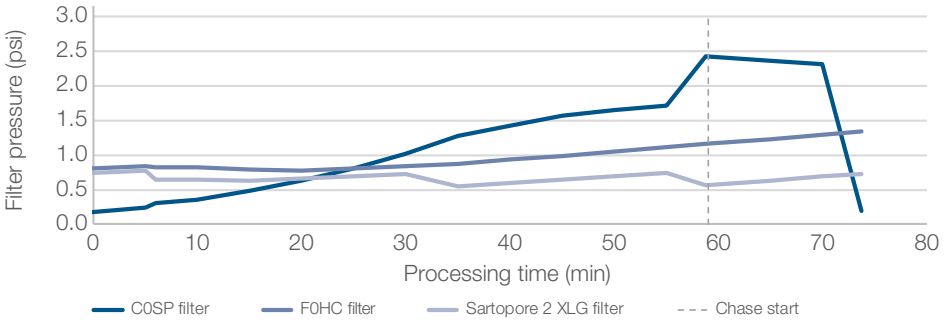

Figure 9. Filter pressure during processing for strategy 2. Three pressure sensors were added to the filter train during processing. Pressure sensor 1 was added before the Millistak+ C0SP filter. Pressure sensor 2 was added between the Millistak+ C0SP and Millistak+ F0HC filters. And pressure sensor 3 was added between the Millistak+ F0HC filter and Sartopore 2 XLG filter. Pressure readings were recorded every 5 minutes during processing. The delta pressure between sensor 1 and sensor 2 was used to determine the Millistak+ C0SP filter pressure. The delta pressure between sensor 2 and sensor 3 was used to determine the Millistak+ F0HC filter pressure. The pressure reading from sensor 3 was used to determine the Sartopore 2 XLG filter pressure. Pressure above 10 psi would indicate clogging of the filter. None of the filter pressures exceeded 2.5 psi at any time during the run.

observed in strategy 2 is due to higher product loss during depth filtration when compared to centrifugation clarification. A notable difference in the two strategies is that strategy 2 produced a significantly cleaner intermediate product as evident by lower turbidity values, faster TFF flux during concentration, and a lower A_{280} value in the affinity flow-through. The turbidity of the clarified product and TFF product of strategy 2 were 94% and 90% lower than the turbidity of the clarified product and TFF product of strategy 1, respectively. The TFF flux during concentration was also 33% higher for strategy 2 than strategy 1, and the A_{280} value in the affinity flow-through was 89% lower for strategy 2 than strategy 1. Despite this difference, the final affinity products for both strategies were comparable in infectivity, percentage of full capsids, and yield. We recommend using strategy 1 for initial discovery or small-scale scouting studies. Strategy 2 is suitable for process development work since the depth filtration step is scalable using any stackable or interlocking filter system and filter holder.

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Appendix: 2 L downstream protocol

Lysis

1. At the time of harvest, add 10X lysis buffer to a final 1X concentration in culture (222 mL).
2. Add 1 M $MgCl_2$ to a final concentration of 2 mM in culture (4.45 mL).
3. Add nuclease to a final concentration of 90 U/mL in culture (Benzonase™ Nuclease or Pierce Universal Nuclease) (0.8 mL of Pierce Universal Nuclease).
4. Stir the lysate to ensure components are mixed.
5. Pour the lysate into two 2.8 L flasks.
6. Shake the lysate at 37°C for 2 hr at 125 rpm.

Clarification with centrifugation

1. Centrifuge the lysate at 6,000 x *g* for 30 min.
2. Decant the AAV-containing supernatant into a 0.2 µm Nalgene Rapid-Flow Sterile Disposable Filter Unit.
3. Apply vacuum and collect the clarified lysate.
4. Proceed directly to tangential flow filtration, or store the clarified sample at 2–8°C overnight.

Clarification with depth filters

Assembling and equilibrating the filter train:

1. Attach tubing to the inlet, vent, and outlet of the Millistak+ C0SP filter with a pressure sensor placed before the inlet.
2. Rinse the Millistak+ C0SP filter.
   A. With the outlet line clamped and the vent line open, begin flushing the C0SP filter with deionized (DI) water at a flow rate of 600 L/m²/hr (270 mL/min). To ensure all air is removed from the filter, clamp both the outlet and vent lines and allow filter pressure to rise to <10 psi before opening the vent line again. Continue until no air bubbles can be seen leaving the filter vent line. Tapping on the filter by hand or with a rubber mallet can also help remove air pockets from the filter.
   B. While flushing the C0SP filter with DI water at a flow rate of 600 L/m²/hr (270 mL/min), clamp the vent line, and open the outlet line. To ensure all air is removed from the filter, clamp both the outlet and vent lines and allow filter pressure to rise to <10 psi before opening the outlet line again. Continue until either 100 L/m² of filter area (2.7 L) of DI water has been passed through the filter or until no air bubbles can be seen leaving the filter vent line, whichever comes later.
3. Rinse the Millistak+ F0HC filter.
   A. Attach the outlet line from the C0SP filter to the inlet of the F0HC filter with a pressure sensor placed just before the inlet. Attach tubing to the vent and outlet of the F0HC filter.
   B. With the outlet line clamped and the vent line open, begin flushing the C0SP and F0HC filters with DI water at a flow rate of 600 L/m²/hr (270 mL/min). To ensure all air is removed from the F0HC filter, clamp both the F0HC outlet and vent lines and allow filter pressure to rise to <10 psi before opening the F0HC vent line again. Continue until no air bubbles can be seen leaving the F0HC vent line. Tapping on the filter by hand or with a rubber mallet can also help remove air pockets from the filter.

C. While continuing to flush the C0SP and F0HC filters with DI water at a flow rate of 600 L/m^2/hr (270 mL/min), clamp the F0HC vent line and open the F0HC outlet line. To ensure all air is removed from the F0HC filter, clamp both the F0HC outlet and vent lines and allow filter pressure to rise to <10 psi before opening the F0HC outlet line again. Continue until either 100 L/m^2 of filter area (2.7 L) of DI water has been passed through the filters or until no air bubbles can be seen leaving the filter vent line, whichever comes later.

4. Equilibrate the filter train.

   A. Attach the outlet line from the F0HC filter to the inlet of the Sartopore 2 XLG filter with a pressure sensor placed just before the inlet. Attach tubing to the outlet of the Sartopore 2 XLG filter.

   B. With the Sartopore 2 XLG vent open, begin flowing equilibration buffer through all three filters at a flow rate of 150 L/m^2/hr (67.5 mL/min). Close the Sartopore 2 XLG vent once the filter is visually filled with fluid, and continue to flow a total of 1.5x depth filter hold-up volume (975 mL) of buffer through the filters.

Clarification processing:

1. Pump the entire nuclease-treated cell lysate through the filter train at a flow rate of 150 L/m^2/hr (67.5 mL/min). Pressure should not exceed 10 psi.
2. Chase the product with 1.5x total filter train hold-up volume (1,008 mL).

Tangential flow filtration (TFF)

If previously clarified lysate was stored at 2–8°C overnight, it may be turbid and require re-clarification using a centrifugation protocol or a Sartopore 2 XLG filter.

Assembling and equilibrating a new hollow fiber:

1. Attach tubing to the hollow fiber with pressure sensors on retentate, permeate, and sample line (to monitor transmembrane pressure).

   - Ps refers to the tube running directly from the sample to the bottom of the hollow fiber.
   - Pp refers to the permeate tube coming out of the side of the hollow fiber at the top, which will go to waste.
   - Pr refers to the retentate tube coming out of the top of the hollow fiber, which will recirculate the sample.

2. Rinse the hollow fiber filter.

   A. With the Pp line clamped, rinse the hollow fiber with a minimum of 0.25 mL/cm^2 of DI water, directing the Pr line to waste.

   B. Open the Pp line and adjust back pressure on the Pr line until flow is approximately equal through both Pr and Pp lines.

   Note: Do not allow the column to exceed its maximum pressure, and keep the transmembrane pressure (TMP) at or below 5 psi.

   C. Rinse the hollow fiber with a minimum of 2 mL/cm^2 of DI water, directing both the Pr and Pp lines to waste.

3. Test the integrity of the hollow fiber filter.

   A. Empty all DI water from the hollow fiber (noting the hold-up volume at this time).

   B. With the Pr line clamped and the Pp line open, slowly pump air into the hollow fiber until a TMP of ~5–7 psi is achieved.

   C. Note the TMP drop over 1 minute. A value of 2 psi/min is passing.

4. Equilibrate the hollow fiber filter.

   A. Place both Ps and Pr lines into the desired equilibration buffer.

   B. Open the Pp line and adjust back pressure on the Pr line until flow is approximately equal through both Pr and Pp lines.

   C. Rinse the hollow fiber with a minimum of 1 mL/cm^2 of equilibration buffer, directing the Pp line to waste.

TFF processing:

1. Concentrate the sample.

   A. Place the Pr and Ps lines into the sample reservoir.

   B. Attach the Pp line to a waste container on a scale.

   C. Run the peristaltic pump (sample flow) at 2,000 shear (368 mL/min for MiniKros hollow-fiber module, Cat. No. S02-E100-05-N).

   D. With the Pp line clamped, allow the sample to recirculate for a minimum of 3 minutes.

   E. Open the Pp line and adjust back pressure on the Pr line until a TMP of 5 psi is achieved.

   F. Using an auxiliary pump, add the sample to the sample reservoir, maintaining a volume of 80% capacity.

   G. Continue to adjust back pressure on the Pr line, maintaining a TMP of 5 psi, and add the sample to the sample reservoir until the sample is concentrated 10–20x.

2. Perform buffer exchange of the sample (optional).

   A. Using the auxiliary pump, begin adding equilibration buffer to the sample reservoir, maintaining concentrated volume.

   B. Continue to add equilibration buffer until 6 diavolumes of buffer have been added.

3. Recover the sample and chase product.

    A. Remove all back pressure from the Pr line and clamp the Pp line.

    B. Allow the concentrated and buffer-exchanged sample to recirculate for a minimum of 3 minutes.

    C. Remove the Ps line from the sample reservoir, blowing air through the hollow fiber to collect all the sample in the sample reservoir.

    D. Place both Pr and Ps lines in a bottle with 1.4x hold-up volume of equilibration buffer and allow the equilibration buffer to recirculate for a minimum of 3 minutes.

    E. Remove the Ps line from the bottle, blowing air through the hollow fiber to collect all the equilibration buffer in the bottle.

    F. Combine the chase with the sample.

Affinity purification

1. Place the chosen inlet lines into their correct purification buffers and equilibrate the lines.
2. Attach the POROS GoPure AAVX column to the column valve.
3. Run equilibration buffer through the column until UV, conductivity, and pH traces stabilize.
4. Run the precolumn conditioning method.
5. Place the sample line into the filtered TFF product/affinity load.
6. Run the affinity processing method.
7. Neutralize the affinity product with 1/20 volume of neutralization buffer.
8. Run the post-column CIP method.

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