BIOTECH

Application Note

USD3598

Pall's 100 kDa Omega™ Cassettes for Recombinant Adeno-Associated Virus (rAAV) Processing

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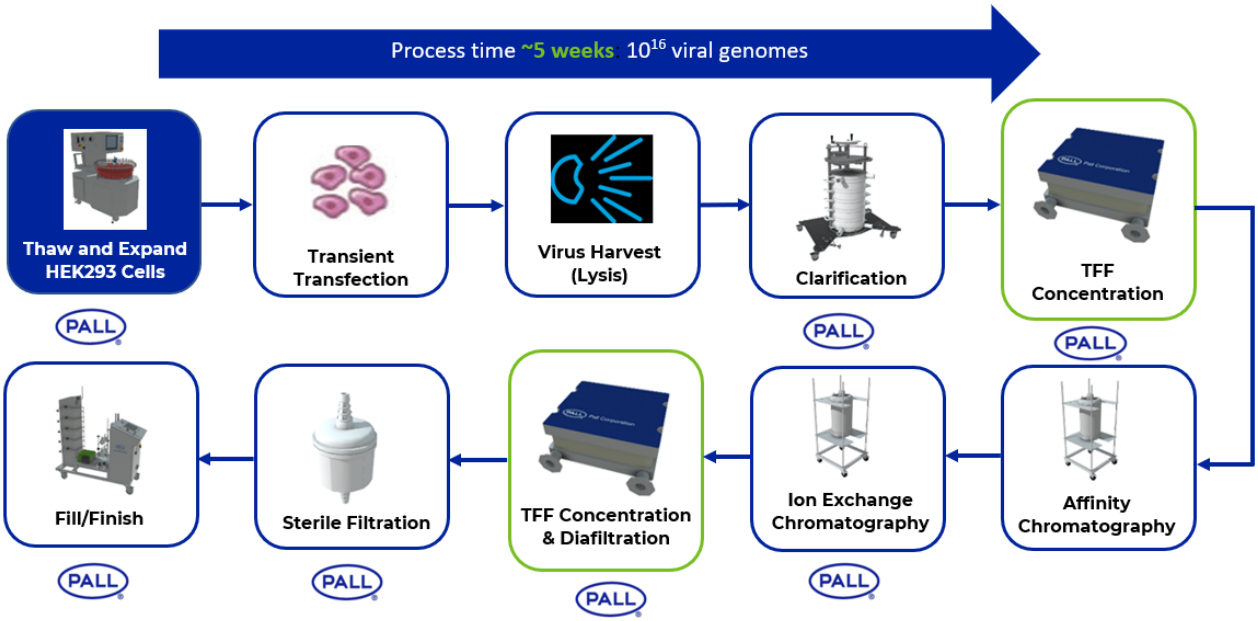
1 Introduction

In recent years there has been tremendous growth in the use of viral vectors in gene therapy, including the largest class of these vectors, recombinant Adeno-Associated Virus (rAAV). rAAV is a non-enveloped virus ~20 nm in diameter. It can package ~4.7 kilobases of DNA and shows relatively low immune response compared to retroviruses and adenoviruses. Furthermore, it is relatively stable under standard bioprocessing conditions.

To manufacture rAAV there are typically both pre- and post-chromatography steps that require Tangential Flow Filtration (TFF) to concentrate and diafilter the product (Figure 1). Both flat-sheet and hollow-fiber TFF formats can be used to achieve these goals. In either case there will be some process optimization required to maximize TFF performance. This could include parameters such as cross flow rate, transmembrane pressure (TMP), particle and volumetric loading, temperature, recovery procedure, and buffer conditions. Note that the approach for TFF process optimization is largely the same for all places a TFF step could be used in downstream processing. This application note will show some common process optimization studies and demonstrate the performance of Omega TFF flat sheet membranes for rAAV processing using an rAAV5 pre-chromatography concentration step.

Figure 1
rAAV manufacturing flow diagram



2 Material and Methods

2.1 Materials

TFF System:

- Cole-Parmer◆ Masterflex◆ L/S◆ series peristaltic pump
- OHAUS◆ scales
- PendoTech◆ PressureMAT◆ (PMAT) pressure sensor monitor
- Pall Cadence® single-use TFF modules with Omega polyethersulfone membrane: CSUM100T001 (100 kDa, 0.0093 m^2) and CSUM100T002 (100 kDa, 0.0186 m^2)
- Pall single-use cassette holder (PN: CSUH040)
- Silicone tubing: #16 (feed and retentate), #14 (permeate)
- Assorted Luer fittings
- 1x phosphate buffered saline (PBS) buffer and reverse osmosis deionized (RO/DI) water (0.2 µm pre-filtered)
- 2 mL Eppendorf◆ protein low binding sample tubes
- Pipettes

Feed Stock:

- rAAV5 from HEK293T cells grown in adherent iCELLis® Nano bioreactors (~9 x 10^9 gene copies (GC)/mL). Crude harvests were clarified through Pall PDK11 depth filters followed by Pall Supor® EKV sterilizing-grade filters prior to the TFF step.

2.2 TFF System Assembly and Preparation

For this application Pall's Cadence single-use (SU) TFF modules were mounted into a cassette holder and torqued to between 8 to 10 Nm (70 to 90 in-lbs.). (*When mounting a membrane cassette into a holder, care should be taken to avoid damaging the cassette by over torquing).* Prior to use, retentate and permeate hold-up volumes were determined. These values are used to determine the recirculation vessel volume target to reach the desired concentration factor, and the volume required for post-use recovery flushing. For a T01 module (0.0093 m^2) set-up we found a retentate hold-up volume of ~21 mL and a permeate hold-up volume of ~5 mL. The system was then flushed with >20 L/m^2 of RO/DI water and equilibrated with >20 L/m^2 of a 1x PBS buffer. The Cadence SU TFF modules are stored in water and gamma irradiated to be sterile and ready for use, requiring a minimal amount of flushing. For more information on pre-use conditioning and system assembly please refer to USD 2896[1] and USTR 2433b[2].

2.3 Normalized Buffer Permeability ($NBP_{20°C}$)

Generally, the normalized water permeability ($NWP_{20\,°C}$) of a TFF cassette is used as the basis for determining membrane recovery (i.e. how effectively the membrane was cleaned back to its original state). For this reason, an $NWP_{20\,°C}$ measurement is not required for SU modules which will not see multiple cycles, however it was employed here as a measure of fouling from the AAV feedstream. For this study, a 1x PBS buffer solution was used in lieu of water. This allowed for the TFF module and system to be preconditioned with the process buffer prior to the introduction of the rAAV feed. The system was run with the retentate closed and the feed pump was adjusted to target a TMP of 10 psi (0.69 bar). Permeate was collected in a separate vessel and flux was measured. All calculated buffer permeability rates were normalized to a temperature of 20 °C by applying a temperature correction factor (TCF 20°C). The temperature correction factors can be found in USTR 2433b[2].

Equation 1

$$NBP_{20°C} = \frac{Permeate\ Flux\ (LMH)}{Transmembrane\ Pressure\ (psi)} * TCF\ 20°C$$

2.4 Flux Excursion

An initial fouling check and the flux excursion work was done with the system in full recycle mode. A clarified rAAV5 pool was loaded into the TFF system feed tank with the retentate valve open and the permeate valve closed. For the initial fouling check the feed pump was started, and the speed adjusted until a TMP of 5 psi (0.34 bar) and a feed side ΔP of 10 psi (10 psi / 0.69 bar inlet, 0 psi / bar retentate) was achieved. Once the system flooded and the operating parameters were set, the permeate valve was opened and the pressures adjusted as needed. The permeate flux and operating conditions were recorded at 5-minute intervals for 15 minutes.

The flux excursion study was started by setting the crossflow rate at 7.5 L/m^2/min (LMM) with the retentate valve fully open. The permeate valve was then opened and the TMP adjusted to the first set of test values as indicated in Table 1 *(note: at high crossflow the starting TMP obtained with an unrestricted retentate may be higher than the initial target set point).* After the TMP was set, the system was run in total recycle for 60 seconds to allow for a steady state to be reached prior to recording the permeate flow. The TMP was then set to the next test value, and testing continued as described in the steps above. The flux excursion trial for each crossflow velocity was ended when the permeate rate did not increase for two consecutive TMP set points. At the end of the TMP excursion for each crossflow setting, samples were pulled from the recirculation pool and permeate line to measure for rAAV concentration using ddPCR. The system was then depolarized by closing the permeate valve, opening the retentate valve, and recirculating for >10 min.

2.5 rAAV Processing

Five batches of rAAV5 were produced from HEK293T cells grown in iCELLis Nano bioreactors using a serum-containing media. At harvest, the bioreactor supernatant was removed, the cells were lysed, and the bioreactor rinsed with a PBS solution. The supernatant, lysate, and wash pools were combined to create a crude harvest pool which was subsequently clarified through PDK11 depth filters and Supor EKV sterilizing-grade filters. One bioreactor batch was split and run through two separate TFF module, and the rest were processed through a single module. Cadence SU modules with 100 kDa Omega TFF membrane were flushed with RO/DI water and equilibrated with a 1x PBS buffer as described in Section 2.2. Membranes were then loaded to an average volumetric loading of 186.5 ± 8.5 L/m^2 with an average feed concentration of 7.3 x 10^9 ± 1.5 x 10^9 as shown in Table 3. The rAAV5 pools were concentrated to a target volumetric concentration factor (VCF) of 10x. The membranes were then depolarized by recirculating at 5 LMM for >10 minutes with the permeate valve closed and retentate valve fully open. The system was then drained of the rAAV5 pool and loaded with ~25 mL of 1x PBS. The buffer was recirculated for >10 min with the permeate closed and retentate open, then drained from the system and combined with the concentrated rAAV5 pool. Samples from the feed and concentrated pools were analyzed by Digital Droplet PCR (ddPCR) to get rAAV concentrations and calculate process yields.

If we assume no significant impact from loading on flux, and the flux at the end of the concentration to remain relatively constant over the diafiltration, then we can create rough estimates for how volumetric loading will impact process times using Equation 2. V_i is volumetric loading (L/m^2), VCF is the target concentration factor, DV is the target number of diavolumes, J bar is the time-averaged flux over the 10x concentration, and J_f is the flux at the end of the 10x concentration. Note that the time calculated here does not include time for pre-/post-use flushing or a post-use recovery chase. Alternatively, we can create a rough estimate for required filter area using Equation 3 where V_p is the starting pool volume.

Equation 2

$$t = \left[\frac{V_i * (VCF - 1)}{(VCF) * \bar{J}}\right] + \left[\frac{V_i * DV}{VCF * J_f}\right]$$

Equation 3

$$EFA = V_p * \frac{J_f * (VCF - 1) + \bar{J} * DV}{t * VCF * J_f * \bar{J}}$$

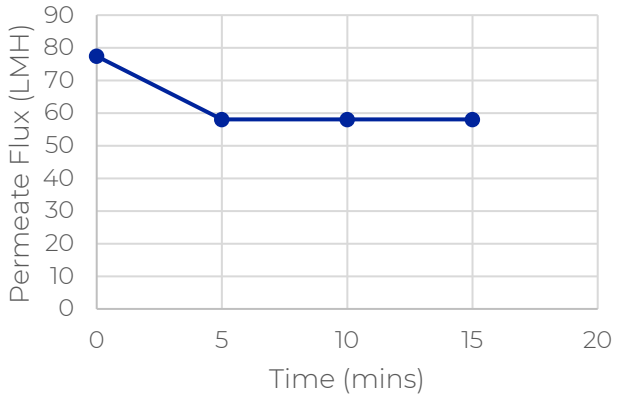
3 Results

3.1 Check for Feedstream Fouling

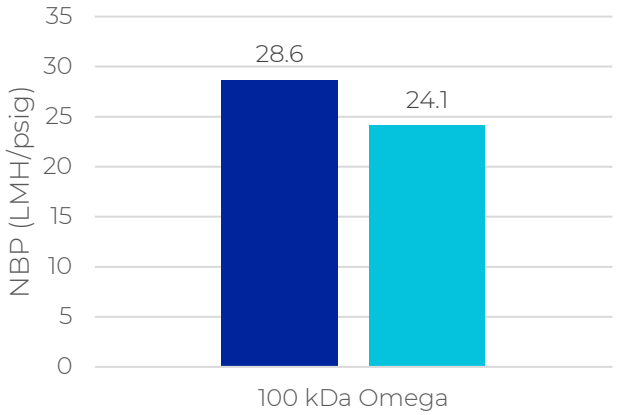
Prior to processing this rAAV5 clarified pool we decided to optimize the crossflow rate and TMP through a flux excursion study. This type of study can be complicated by any irreversible fouling of the membrane by the feedstock. A fouling check is therefore an important pre-requisite. As an initial check, the TFF system was operated in total recycle (retentate and permeate directed back to the process tank) for 15 minutes at low TMP. Results from the study showed an initial decline in permeate flux as the gel layer is formed, followed by a steady permeate flux for the remainder of the test as shown in Figure 2A. These results indicate that there was no significant short-term fouling observed from the rAAV5 clarified pool.

As a second check we loaded a clarified rAAV5 pool to 160 L/m^2 on a fresh module and concentrated it 10x. While these single-use TFF modules do not require a normalized water permeability (NWP) check pre-use, here we measured the normalized permeability of our equilibration buffer pre- and post-use as an indicator of fouling. It's important to highlight the post-use measurement was done without any cleaning or regeneration after the virus concentration. The results showed an 84% retention in permeability (Figure 2B), which is indicative of a low-fouling feedstream.

Figure 2
A: rAAV5 flux vs time in full recirculation.



B: Normalized buffer permeability (NBP; at 20 °C in 1x PBS) before and after a 10x concentration.



3.2 Permeate Flux vs TMP

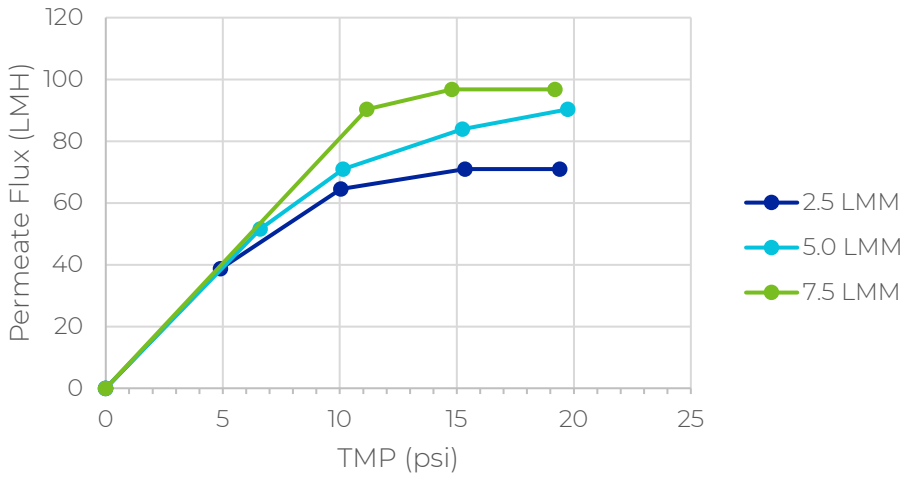
To optimize crossflow rate and TMP a flux excursion study was run with the clarified rAAV5 pool. The TFF system was set up to operate in total recycle (retentate and permeate lines directed back to the feed tank) and the crossflow rate set to the first condition in Table 1. The TMP was then ramped up until the permeate flux stabilized. The membrane was then depolarized, adjusted to the next crossflow rate, and the TMP excursion repeated.

Table 1
Flux vs. TMP excursion setpoints

Crossflow (L/m²/min)	Transmembrane Pressure Set Point (psi)					
7.5	5	10	15	20	25	30
5.0	5	10	15	20	25	30
2.5	5	10	15	20	25	30

The flux curves generated from the data collected are shown in Figure 3. We saw a moderate increase in flux with increasing crossflow rate. The critical TMP for this clarified rAAV5 feedstream (point at which permeate flux reaches a maximum) was ~10-15 psi (0.69 – 1.03 bar).

Figure 3
rAAV5 flux vs transmembrane pressure



At the end of each TMP excursion, retention of rAAV5 within the module was evaluated by ddPCR analysis of samples taken from the retentate pool and permeate line. Results from the analytics, provided in Table 2, show little virus passage, with retentions exceeding 99.7% at each crossflow rate evaluated. Virus concentrations in the retentate pools did vary between runs, highlighting the importance of proper depolarization and flushing in an optimized process. However, we did not observe a significant trend in retentate pool virus concentration vs crossflow rate, suggesting there was not significant virus loss due to shear at crossflow rates up to 7.5 L/m²/min.

Table 2
rAAV5 flux excursion retention / yield data

Excursion Start		Excursion End		
Initial Feed Concentration (GC/mL)	Crossflow Rate (L/m²/min)	Retentate Pool Concentration (GC/mL)	Permeate Line Concentration (GC/mL)	rAAV Retention %
8.70×10^9	7.5	7.25×10^9	6.25×10^6	99.9%
	5.0	8.83×10^9	2.33×10^7	99.7%
	2.5	6.77×10^9	1.83×10^7	99.7%

3.3 rAAV5 Processing

To evaluate the performance of the Omega 100 kDa modules in an rAAV application we ran six replicate TFF trials across five iCELLis Nano bioreactor batches producing an rAAV5 construct. Each batch was first clarified through depth and sterile filtration before loading onto the TFF modules. The TFF process included a RO/DI water flush and buffer flush followed by a 10x volumetric concentration of the clarified rAAV5 pool. After concentration the membrane was depolarized, drained, and flushed with a recovery buffer (as described). Table 3 shows the average volumetric loading and feed concentration across the six trials.

Step yield across the six trials showed strong rAAV recovery, averaging 91.4% (Table 3). The TMP, permeate flux, and VCF curves from a representative run are shown in Figure 4. As expected, flux declines initially as the polarized layer is formed on the membrane's surface, then declines more gradually as the product is concentrated. Time averaged flux across the 10x concentration was calculated and averaged 63.7 ± 5.6 L/m²/h (LMH) across the six trials.

We can use this flux to estimate process time for a similar 10x concentration over a range of loadings if we assume loading will not significantly impact process flux (Figure 5A). We can also build in an estimate for a hypothetical 10x concentration + 7 diavolume diafiltration process if we assume that the flux at the end of the concentration (~51.9 LMH) would remain relatively constant through the diafiltration (Figure 5A). In this process the addition of diafiltration roughly doubled the processing time with a 200 L/m² load translating to ~2.8 h for the concentration or ~5.5 h for the concentration and diafiltration. Note that these estimates do not include time required for any pre-/post-use flushing or recovery chase. Alternatively, we could select a processing time (e.g. 2 h or 4 h) and predict the required TFF filter area for a given load volume (Figure 5B). Here we see it would take ~14 m² to process a 2000 L batch ten-fold in a 2 h window (without diafiltration). These are very rough estimates but provide some guidance on where to start development given your expected load volumes and any manufacturing constraints around total membrane area or processing time.

Table 3
rAAV5 confirmation run data

	Average (n=6)	95% Confidence Interval
Loading (L/m²)	186.5	8.5
Feed concentration (GC/mL)	7.3×10^9	1.5×10^9
Flux (LMH)	63.7	5.6
Yield (based off genome copies)	91.4%	8.0%

Figure 4
10x concentration of rAAV5 clarified harvest

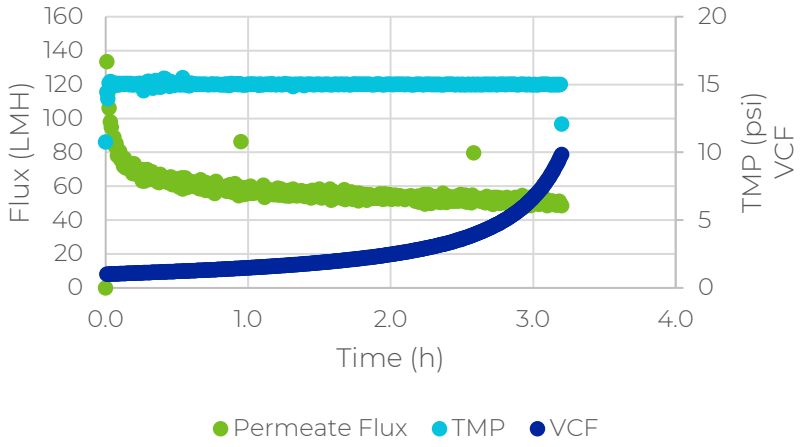
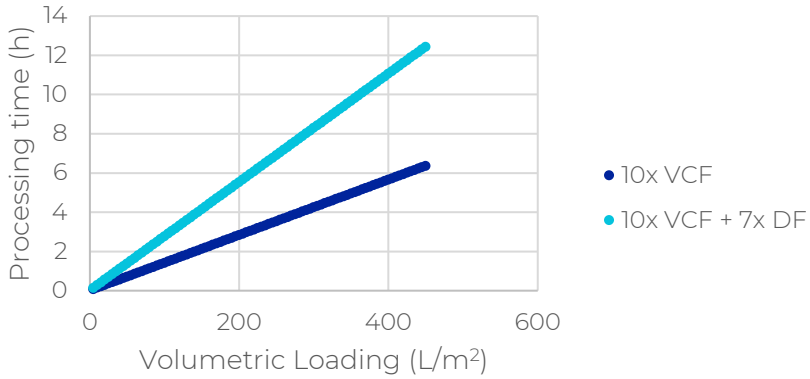
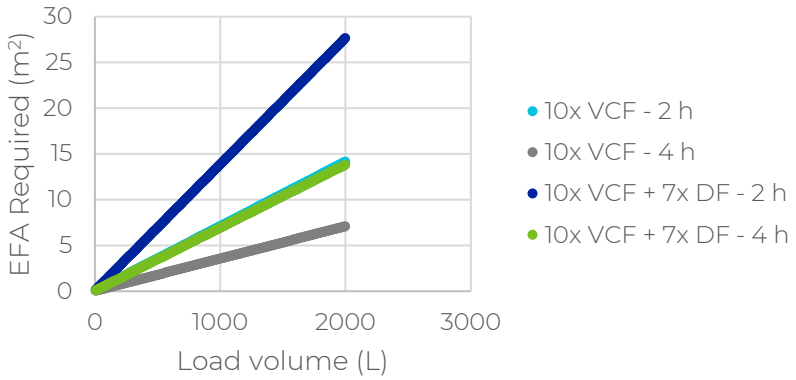


Figure 5
(A) Estimates of processing time vs. volumetric loading



(B) Required filter area vs load volume



4 Discussion

Here we have optimized a process and demonstrated the use of Pall's Cadence 100 kDa TFF modules in a standard rAAV downstream unit operation. First, we showed how crossflow rate and TMP can be optimized through a flux excursion study. We then showed that in a pre-chromatography application the Omega TFF membrane can retain >99.5% of an rAAV5 viral vector, and provide >90% yield in a ten-fold concentration step.

Note that while this work focused on an rAAV5 pre-chromatography step, we expect the virus retention and yields to translate to other rAAV serotypes and process steps. However, feedstock differences and process parameters will have small but significant impacts on filter performance. Operating parameters can be optimized through a series of short studies for each step employing TFF. Here we have shared data from a common method to optimize crossflow and TMP. The results showed the permeate flux was positively correlated with both TMP and crossflow rate and showed a critical TMP in the range of 10-15 psi (0.69 – 1.03 bar) within the crossflow range of 2.5 – 7.5 L/m^2/min. Note that to develop a robust manufacturing process there may be additional optimization work required, which could include development of the post-use recovery flush and/or buffer exchange conditions in a diafiltration application, but were outside the scope of this work.

The fluxes measured during the 10x concentration step were then used to make some process predictions and show how various process inputs and outputs can be targeted such as loading, filter area, and processing time. Pall's TFF portfolio includes the same Omega 100 kDa membrane built into both re-use and single-use cassette options that range from 93 cm^2 to 2.5 m^2. Re-use devices can be further stacked in parallel to process rAAV batches of greater than 2000 L. More information on the cassette options can be found in USD 2902a[3] and USD 2322c[4].

5 References

1. USD 2896 - Single-Use Tangential Flow Filtration (TFF) Modules with Omega Membrane.
2. USTR 2433b - T-Series TFF Cassettes with Omega Membrane Care and Use Procedures.
3. USD 2902a - Single-Use TFF Modules.
4. USD 2322c - T-Series TFF Cassettes with Omega Membrane.

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