Simplified Lentivirus and AAV Clarification with TFDF®-based Intensified Virus Production

Application Note

Introduction

Previous studies showed that the use of Tangential Flow Depth Filtration (TFDF®) cell retention technique for perfusion cell culture provides increased viral vector production. This study, focusing on characterizing the clarification process post-TFDF, demonstrates that the implementation of TFDF-based process intensification not only delivers high virus production, but also provides simplified processes with reduced filtration requirements at commercial manufacturing scale.

Tangential flow depth filtration (TFDF) is a cell retention technology for intensified production of envelope viruses and viral vectors such as lentivirus (LV) and adeno-associated virus (AAV). The pore size of the TFDF filter enables continuous harvest of secreted LV and AAV capsids through the permeate during the virus production phase, while retaining all cells and most fine cell debris. The clarification process then requires only a secondary depth or membrane filtration. For intracellular viral vectors requiring cell lysis, the TFDF filter used for cell retention during the perfusion process can also be used to partially clarify the lysate, potentially reducing the surface area required during the clarification process.

This study was designed to identify and size post-TFDF filters that ensure complete clarification with high virus yield, throughput, and turbidity reduction.

The performance of the clarification filters from several suppliers was measured using three parameters:

- Virus yield (%): Amount of virus recovered following filtration
- Throughput (L/m^2): Volume of sample filtered up to 20 psi per square meter of filter
- Turbidity reduction (%): Reduction in the amount of turbidity following filtration

The filtration area required at commercial manufacturing scale was then estimated from the data generated at lab scale.

Materials and Methods

Filters

Post-TFDF filters were purchased from several suppliers.

Table 1. Filters

Supplier	Filter[1]	Material	Nominal Pore Size (µm)	Surface Area (cm^2)
A	1	Cellulose, resin	1.5 – 5	25
	2		0.2 – 0.8	25
B	1	Synthetic polyacrylic fibers, silica	0.55 – 9	23
	2		0.2 – 2	23
	3		≤0.1	23
C	1	Cellulose, Diatomaceous earth, resin	2 – 20	22
	2	Cellulose	0.2 – 0.4	22
	3	Polyethersulfone	0.2	20
D	1	Cellulose with inorganic filter aids	0.8 – 8	25
	2		0.4 – 0.8	25
	3		0.1 – 8	25

[1]Filters will be identified as follows: Supplier **A**, Filter **1:** filter **A-1**.

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Recombinant Viral Vectors

All recombinant AAV and LV viral vectors were produced through a triple or quadruple plasmid DNA (pDNA) transient transfection using HEK293F cells and chemical transfection reagents, such as Trans-IT VirusGEN® (Mirus Bio™) for AAV or FectoVIR®-LV (Polyplus Transfection™) for LV. Daily samples to monitor the viable cell density (VCD) and metabolites were taken until the day of harvest, typically day 3 or day 4 post transfection.

Feedstocks

Feedstocks from four process types were used:

- Crude HEK293 cells cultivated in batch mode (~3 x 106 cells/mL; >80% viablity) and producing excreted AAV8. Used as a reference process with no cell lysis.
- HEK293 cell lysate from batch mode, producing AAV9 with virus located both extra- (~50%) and intracellularly (~50%).
- TFDF permeate from cell culture without cell lysis (~10 x 106 cells/mL; >70% viability), for harvesting of excreted LV, AAV8, or AAV9 capsids.
- TFDF permeate after cell lysis for harvesting AAV9 capsids recovered from both extra- and intracellularly.

For AAV9 production, cells were lysed by spiking the bioreactor with a 10x lysis buffer to a final working concentration of 50 mM Tris-HCl (pH 8.0), 2 mM MgCl2, 1% Tween®-20. The lysate was treated with 20 – 25 U/mL of Benzonase® and quenched with 250 mM NaCl. Only the supernatant was collected for LV and AAV8 production, and no lysis was required.

Table 2. Feedstocks

Virus	Feedstock	Cell Density (cells/mL)	Viability (%)	Turbidity (NTU)	Cell Lysis	Virus Titer
AAV8	Whole cells	3×10^6	>80	200	No	1×10^{11} vp/mL
AAV8	TFDF permeate	10×10^6	>70	60	No	2.8×10^{11} vp/mL
AAV9	Lysate	10×10^6	>70	2020	Yes	2.9×10^{11} vp/mL
AAV9	TFDF permeate	10×10^6	>70	20	No	5.7×10^{11} vp/mL
LV	TFDF permeate	10×10^6	>70	25 – 56	No	2.2×10^7 TU/mL

Clarification

A KrosFlo® KR2i TFF System was used for the filtration tests as follows:

1. Fill filter with buffer and close vent.
    a. AAV: 50mM Tris, 250 mM NaCl, pH 8.3
    b. LV: 10 mM Histidine, 0.15 mM NaCl, pH 7.5
2. Flush with buffer at 150 LMH for a throughput of 50 L/m^2
    a. B filters: 300 LMH
3. Drain filter. Apply viral load at 115 LMH.
    a. Cell lysate: with stirring
    b. LV load and filtrate: on ice
4. Record turbidity, feed pressure, and permeate volume.
5. Continue loading until reaching 20 psi or 1 L of filtrate.
6. Recirculate 10 – 50 mL buffer through filter for ≥5 minutes.
7. Retain 1 mL samples of load, filtrate, and buffer chase.
    a. Freeze at -20°C until analysis.

Results

AAV TFDF Permeate Clarification

In this study, more than 90% of AAV8 capsids produced and secreted or excreted by HEK293 cells are present in the cell culture spent media. The possibility to continuously harvest the virus during the production phase in the permeate of the TFDF filter, without the need to lyse the cells, simplifies the cell clarification step and reduces the quantity of costly endonuclease to be used. The production of AAV9 leads to about half of the virus being excreted into the media and collected in the permeate of the TFDF filter. Like for AAV8, when using an intensified TFDF-based perfusion process, the harvest of the excreted virus is conducted throughout the entire virus production phase. The remaining intracellular part of virus produced is recovered from the bioreactor, after cell lysis, at the end of the production phase.

TFDF permeate feedstocks from AAV8 and AAV9 production cell cultures without cell lysis, representing manufacturing processes where AAV capsids are present in the media, were used to identify the best secondary filter to complete the clarification before proceeding to the purification steps.

The turbidity of the AAV8 and AAV9 TFDF permeate starting materials was measured at 60 and 20 NTU, respectively. This confirms that most of cell debris and particulates are retained by the 2 – 5 µm pore size TFDF filter. Since no cell lysis is used for these processes, and the TFDF-based perfusion maintains a cell viability at a higher level than batch cell culture, it is indeed expected that the TFDF permeate would be clean. But the post-TFDF turbidity value is still not low enough to directly move to purification steps. A secondary filtration, using sub-micron pore size filters, will further reduce the turbidity and prevent the fouling of the downstream purification media.

Four filters were used for the secondary clarification of the AAV8 and AAV9 TFDF permeates (Table 3). Throughput, turbidity reduction and yield were measured (Figure 1).

Table 3. Filters Used For the Clarification of AAV8 and AAV9 TFDF Permeate Feedstocks

Virus	Feedstock	Primary Clarification		Secondary Clarification	
		Filters	Pore Size Range (µm)	Filters	Nominal Pore Size Range (µm)
AAV8/AAV9	TFDF permeate	TFDF	2 – 5	A-2	0.2 – 0.8
				B-3	≤0.1
				C-2	0.2 – 0.4
				D-3	0.1 – 0.8

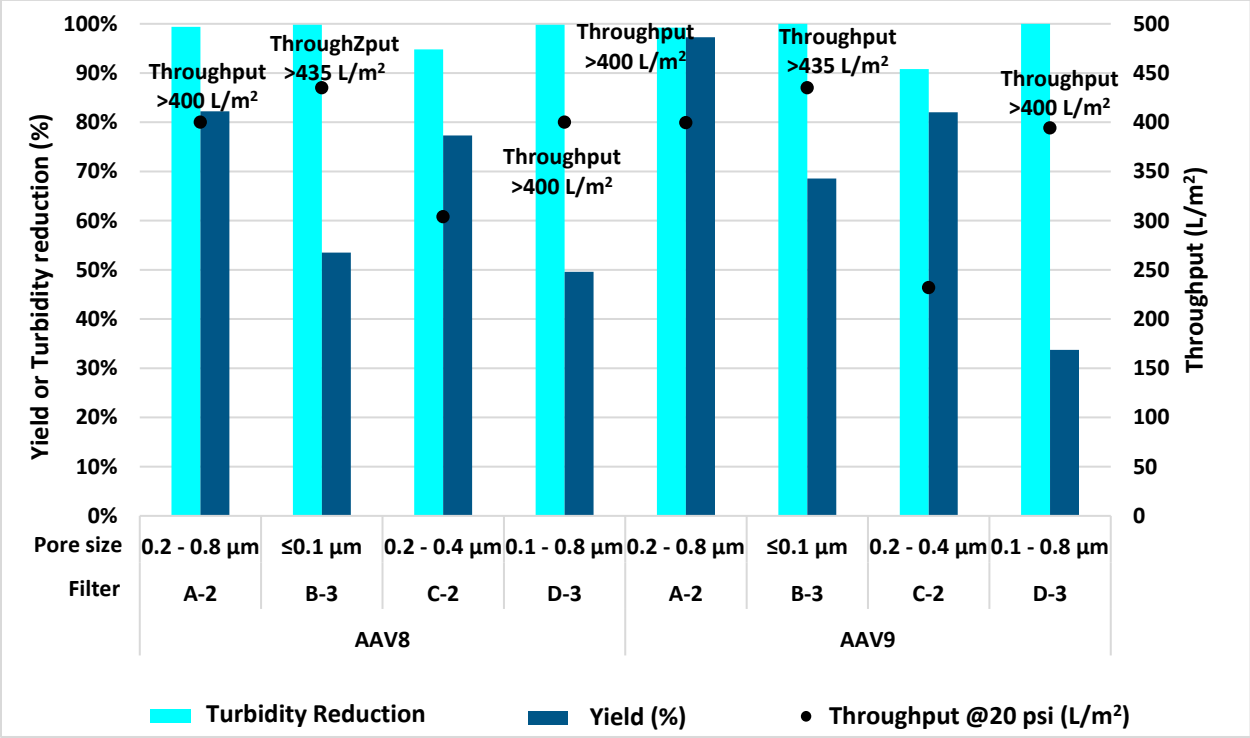

Figure 1. AAV8 and AAV9 TFDF Permeate Secondary Clarification: Filtration Throughput, Turbidity Reduction, and Yield

All filters tested reduced the turbidity to <5 NTU, making the filtrate ready to be processed downstream. For filters D-3, B-3, and A-2, all the sample volume available for the clarification test (~1 L) was filtered without reaching the 20 psi pressure limit. Throughput (400 – 435 L/m^2) was calculated based on the volume filtered, but the actual throughput should be considered higher. Filter C-2 throughput was significantly lower (~230 – 300 L/m^2) and is not, therefore, considered a good option for post-TFDF clarification of these feedstocks.

The virus yield of material recovered from filters D-3 (~30 – 50%) and B-3 (~55 – 70%), having the overall tighter pore sizes, were lower than other filters and are not, therefore, considered good post-TFDF secondary filtration options for this type of feedstock. The preferred secondary filter to complete the clarification of AAV8 or AAV9 TFDF permeates is filter A-2 with high yield (82% for AAV8 TFDF permeate feed, 97% for AAV9 TFDF permeate feed), high throughput (>400 L/m^2), and high turbidity reduction (100%, <5 NTU).

AAV8 Cell Culture Feedstock Clarification

To compare the performance of the TFDF-based process to that of a standard batch process, an AAV8 whole batch cell culture feedstock was clarified. The clarification filters used are presented in Table 4. Throughput, turbidity reduction, and yield were measured following each filtration (Figure 2).

Table 4. Clarification Filters for AAV8 Whole Cell Feedstock

Virus	Feedstock	Primary clarification	
		Filters	Nominal Pore Size Range (µm)
AAV8	HEK293 whole cells	B-1	0.55 – 9
		C-1	2 – 20
		D-1	0.8 – 8

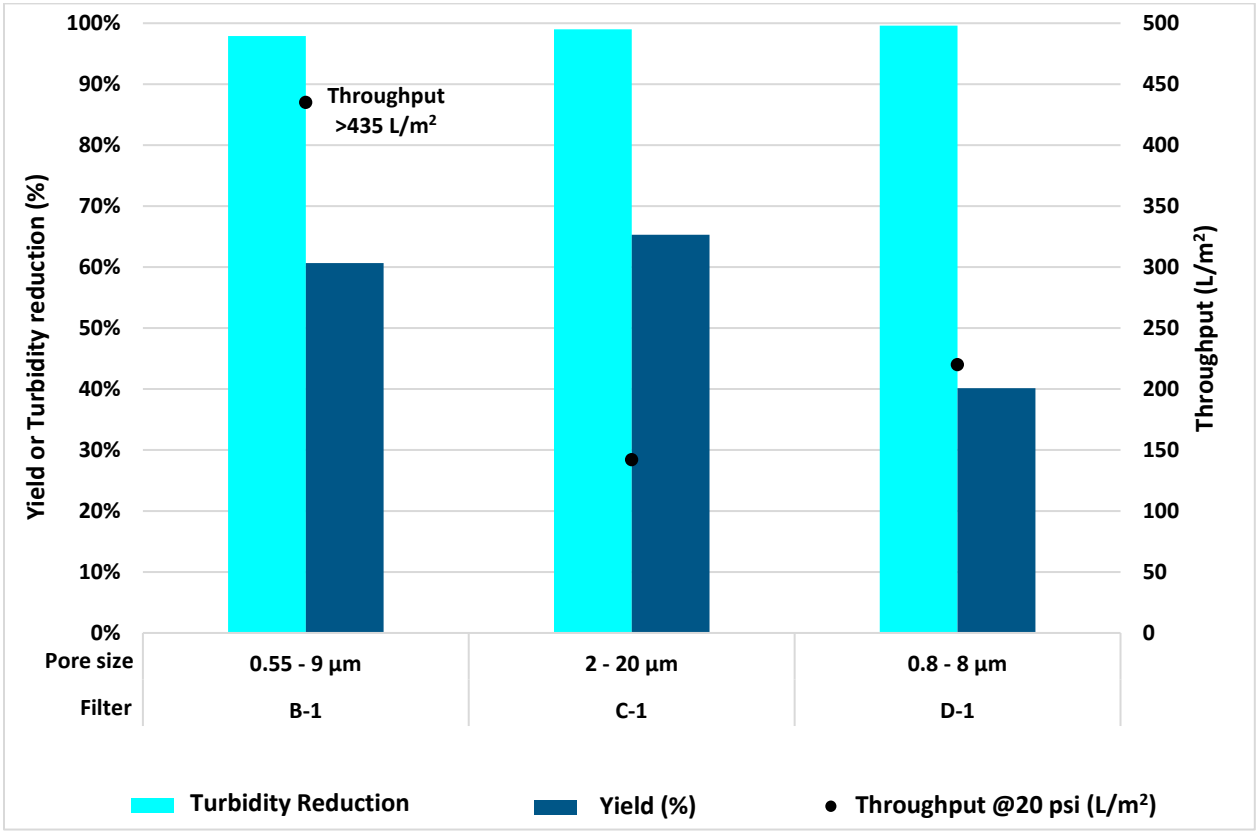

Figure 2. AAV8 Whole Cells Clarification: Turbidity Reduction, Yield, and Filtration Throughput

The turbidity of this AAV8 feedstock was only 200 NTU, which can be explained from the low cell density (~3 x 10^6 cells/mL) and high viability (~80%) of this batch cell culture. All three filters tested provided almost complete turbidity reduction with no need for a secondary clarification stage.

Filter B-1 provided a high throughput (>435 L/m^2), but filter C-1 throughput (142 L/m^2) was significantly lower. Filter C-1 recovery (65%) was slightly higher than that of filter B-1 (60%), but due to the high throughput, filter B-1 is considered the better option for this feedstock.

A comparison of the performance of filter A-2 for the AAV8 TFDF permeate and filter B-1 for the AAV8 whole cell feedstock shows similarities in turbidity reduction and throughput; however, the recovery was much lower for the batch process (60%) than the TFDF-based process (>80%).

In addition, a comparison of the pressure curves of the two filters shows significant differences. Although neither filter reached 20 psi after 1 L of feedstock filtered, the pressure during the whole cells clarification increased significantly (Figure 3). After filtering 1 L of feedstock, the pressure was recorded at 15 psi for filter B-1 compared to only 5 psi for filter A-2. This indicates that filter B-1 would have reached the 20 psi pressure limit much sooner than filter A-2. An exponential regression from the data recorded on each filter gives a good estimate of the total volume filtered on each filter at the pressure limit of 20 psi. The throughput calculated from the exponential regression for the filtration of AAV8 whole cell feedstock on filter B-1 is 490 L/m^2 compared to 685 L/m^2 for the filtration of AAV8 TFDF permeate with filter A-2.

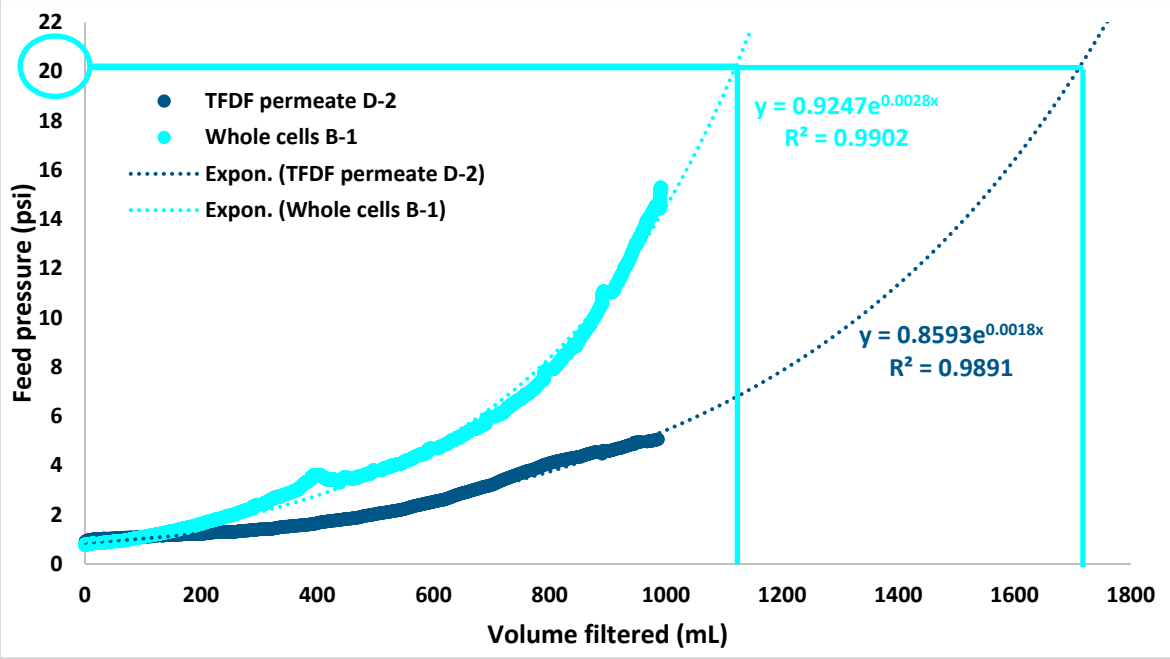

Figure 3. Pressure Curves: AAV8 TFDF Permeate vs AAV8 Whole Cells

From these results an estimation of the filtration area required at commercial manufacturing scale can be calculated. At 2000 L bioreactor scale, the AAV8 batch process will require ~4 m^2 surface area for the clarification process. Previous studies (Repligen internal data; Mendes et al., 2022) showed TFDF-based perfusion processes increase AAV production ~4 – 10-fold compared to batch cell culture. Considering a 4-fold increased virus production, and the data from this filtration study, a ~500 L bioreactor will provide the same quantity of virus as that of a 2000 L batch process. Such a process will use a 0.6 m^2 TFDF filter for the perfusion process and only 0.25 m^2 of secondary filtration to complete the clarification. This confirms that the implementation of perfusion processes using the TFDF technology not only significantly boosts the AAV manufacturing productivity, but also simplifies the clarification process after virus production.

AAV9 Lysate Clarification

Because approximately half of the AAV9 virus is intracellular, cell lysis is necessary for recovery. The TFDF filter has been previously shown (Mendes et al., 2022) to successfully clarify a HEK293 AAV8 lysate with throughput >500 L/m^2. However, the HEK293 AAV9 lysate throughput was only 30 L/m^2 for this study. A standard 2 stage filtration approach was, therefore, evaluated to filter the lysate at the end of the virus production.

In a standard batch manufacturing process, the excreted virus cannot be collected from the media and remains in the bioreactor for the duration of the virus production phase. The intracellular virus is then released by cell lysis, and the lysate is clarified through two filtration stages. Depth filtration, which can handle turbid feedstocks, is generally used for such clarification applications.
Two similar lysate feedstocks, with turbidities of ~1600 and 2000 NTU, were used to evaluate the performance of four primary clarification filters. The lysate is expected to contain both cells and cell debris, requiring filters with a broad pore size range. The

same filters as those previously used for crude AAV8 cell culture clarification without lysis were therefore used for the primary filtration of the lysate. For the secondary filtration step, tighter pore size filters were selected (Table 5). Throughput, turbidity reduction, and yield were measured following both the primary (Figure 4) secondary clarification (Figure 5).

Table 5. Clarification Filters for AAV9 Cell Lysate

Virus	Primary Clarification			Secondary Clarification		
	Feedstock	Filters	Pore Size Range (µm)	Feedstock	Filters	Nominal Pore Size Range (µm)
AAV9	HEK293 cell lysate	A-1	1.5 – 5	B-1 filtrate	A-2	0.2 – 0.8
		B-1	0.55 – 9		B-2	0.2 – 2
		C-1	2 – 20		C-2	0.2 – 0.4
		D-1	0.8 – 8		D-3	0.1 – 0.8

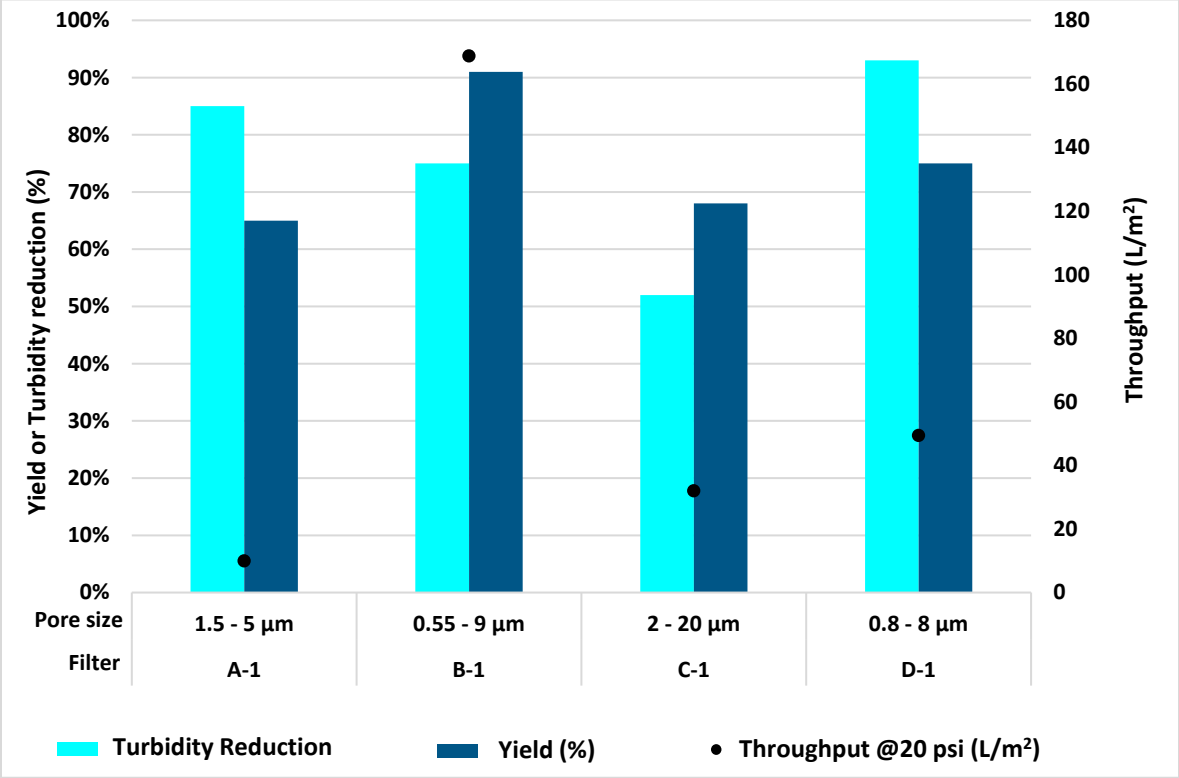

Figure 4. AAV9 Cell Lysate Primary Clarification: Filtration Throughput, Turbidity Reduction, and Yield

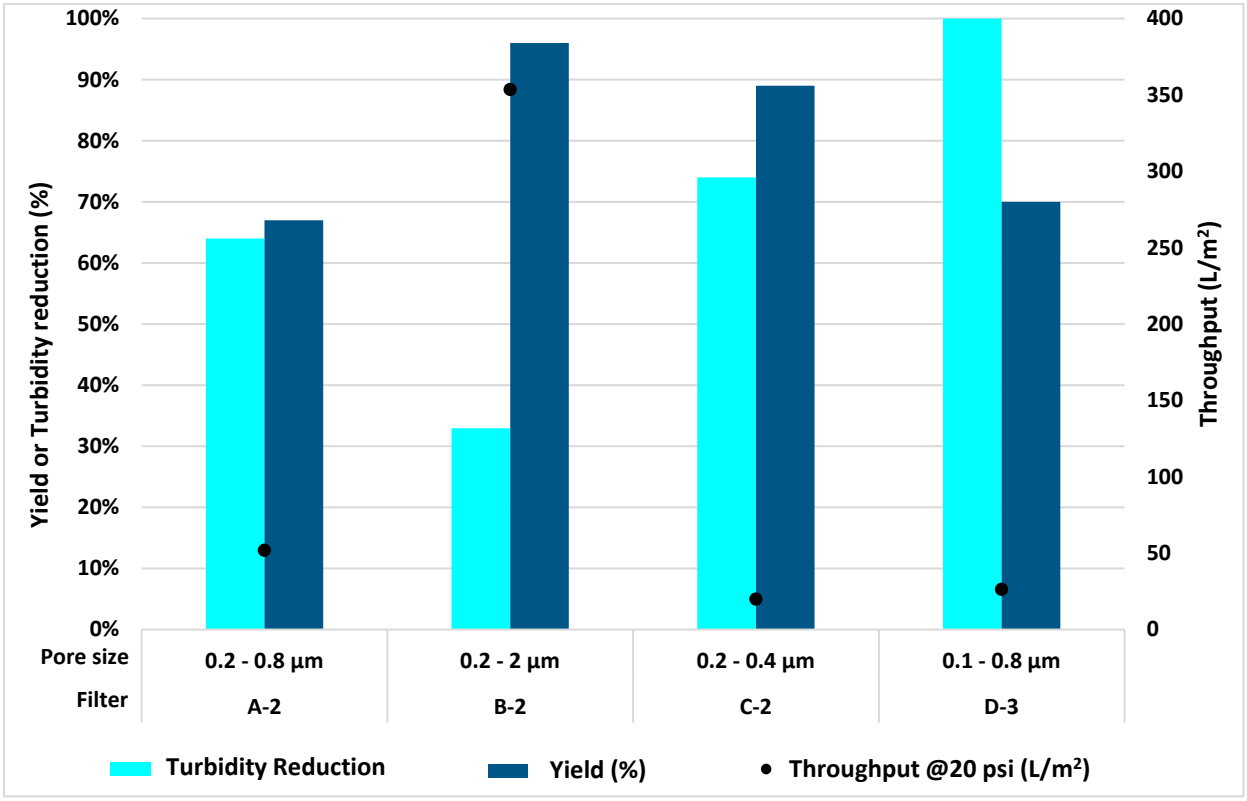

Figure 5. AAV9 Cell Lysate Secondary Clarification: Filtration Throughput, Turbidity Reduction, and Yield

For the primary clarification, only filter B-1 provided a high virus recovery yield (~90%). The yield was below 80% for the three other filters. Filter B-1 also achieved a significantly higher throughput (~170 L/m^2) compared to other filters (≤50 L/m^2), while achieving a good turbidity reduction (75%). Filter B-1 was, therefore, chosen as the primary filter to clarify the AAV9 lysate material. Additional feedstock was filtered through filter B-1 to obtain enough material to perform the secondary filtration testing. The starting material for the secondary filtration evaluation had a turbidity of 379 NTU.

None of the filters tested provided a good overall performance for the secondary filtration. Filter B-2 performed the best in terms of throughput (~350 L/m^2) and yield (96%) compared to the other filters (yield: 65 – 90%; throughput: ≤50 L/m^2), but the post-filtration turbidity was still very high (~250 NTU) and not suitable for loading onto a sterile or bioburden reduction membrane filter (0.2 – 0.45 µm). The post-filtration turbidity was also high for filters C-2 (100 NTU) and A-2 (136 NTU). Filter D-3 was the only filter achieving a post-filtration turbidity (<5 NTU) suitable for proceeding to a sterile filter or even directly to downstream purification, but this was at the cost of a moderate virus recovery yield (70%) and a low throughput (26 L/m^2). Such a low throughput would require the use of a filtration surface area well above 50 m^2 at 2000 L bioreactor scale. These data illustrate the difficulty of implementing an easy and cost-efficient clarification process for the manufacture of intracellular AAV where cell lysis is needed to recover the product, and justifying the development of processes where the virus is excreted and directly harvested from the cell culture media.

LV TFDF Permeate Clarification

Similar to AAV8, LV particles produced from the HEK293 cell culture are present outside the cells in the media. Cell lysis is, therefore, not required to harvest the virus. Previous studies (Repligen internal data; Tran M et al., 2022; Tona RM et al., 2023) have shown that process intensification using the TFDF filter in perfusion mode for LV production increases the virus production 5 to 80-fold compared to batch processes. Lentivirus particles are significantly larger (~100 nm) than AAV particles (~20 nm) but are, nonetheless, small enough to filter through the 2 – 5 µm pore size of the TFDF filter, while cells and most cell debris remain in the

bioreactor. The virus can be continuously harvested on the permeate side of the TFDF filter and stored immediately at 4°C to prevent virus inactivation. The larger size of the LV particles compared to AAV8 requires larger pore size filtration post-TFDF (Table 6). Throughput, turbidity reduction and yield were measured following secondary clarification (Figure 6).

Table 6. Clarification Filters for LV TFDF Permeate

Virus	Feedstock	Primary Clarification		Secondary Clarification	
		Filters	Pore Size Range (µm)	Filters	Nominal Pore Size Range (µm)
LV	TFDF permeate	TFDF	2 – 5	B-1	0.5 – 9
				C-3	0.2
				D-2	0.4 – 0.8

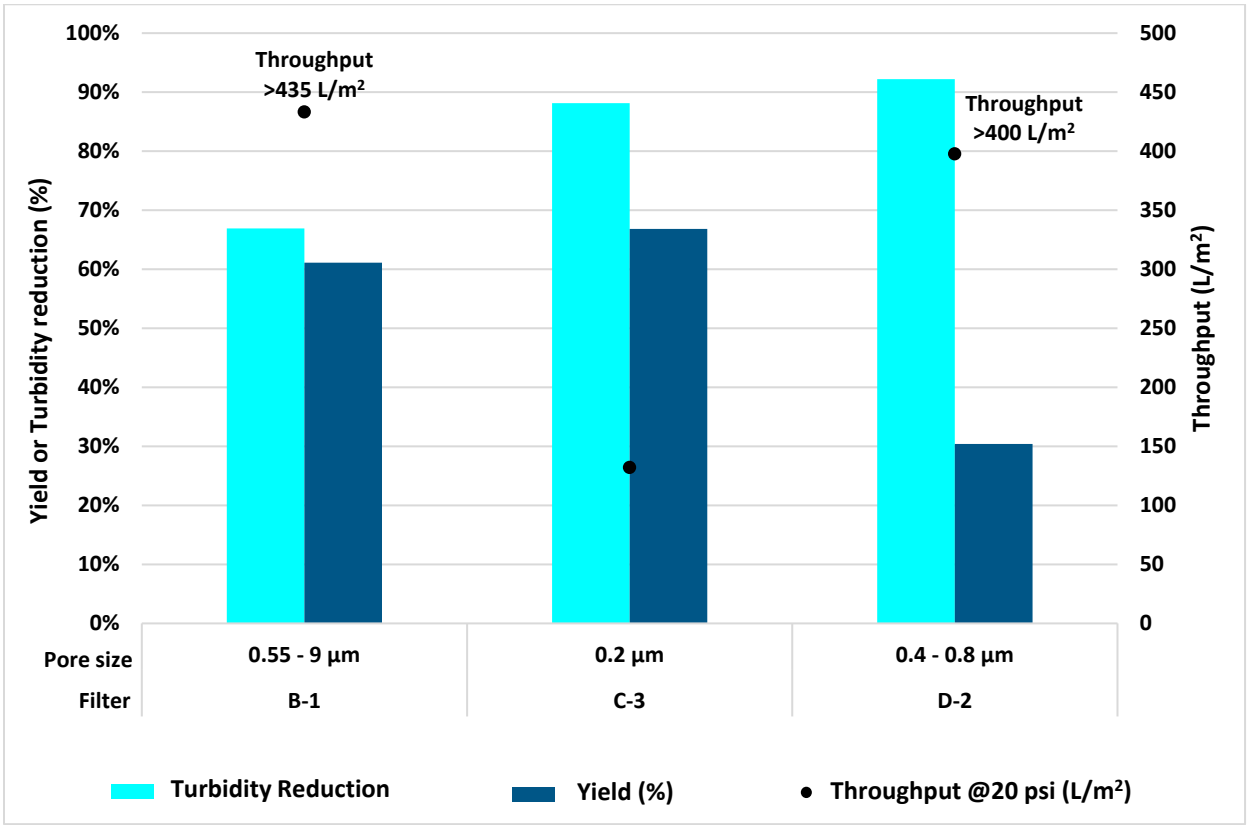

Figure 6. LV TFDF Permeate Secondary Clarification: Filtration Throughput, Turbidity Reduction, and Yield

Filter B-1 performed best with high throughput (>435 L/m^2; limited sample volume available), virus recovery yield (~61%) and turbidity reduction (~70%, from 24 to 7 NTU) that is suitable for the next process step. The low virus recovery yield obtained with filter D-2 (<30%) shows that the combination of tight pores (0.4 – 0.8 µm) and depth of filter (>1 cm) leads to virus retention. Even with significantly tighter pores (0.2 µm), but with much thinner membrane (<200 µm), the virus recovery yield is increased using filter C-3 (~67%). Overall, the lower virus yield measured for LV clarification compared to that of AAV is expected considering that LV particles are very unstable and much more prone to inactivation (Raghavan B et al., 2019; Chinnawar R and Marchand N, 2022). The lower throughput (~130 L/m^2) achieved with filter C-3 makes filter B-1 the preferable filter for the LV TFDF permeate secondary clarification.

The 20 psi pressure limit was not attained during the filtration of 1 L LV TFDF permeate feedstock on filter B-1. A nominal pressure increase indicates that throughput could be much higher than 435 L/m^2; however, turbidity increased continuously to 10 NTU at the end of filtration (Figure 7). The turbidity reduction was, therefore, considered the limiting parameter for defining the throughput so that the filtrate is suitable for direct processing to next purification step.

The filtration surface area needed for LV commercial manufacturing can be projected from this data. Using a conventional batch cell culture process, a bioreactor volume of 500 L is generally considered for large scale LV manufacturing. With a conservative scenario of 5-fold increased production of infective LV particles with a TFDF-based perfusion process, a 1500 cm^2 TFDF filter connected to a 100 L bioreactor and complemented with less than 0.25 m^2 of depth filtration would be sufficient to produce and clarify the required product quantity before further purification.

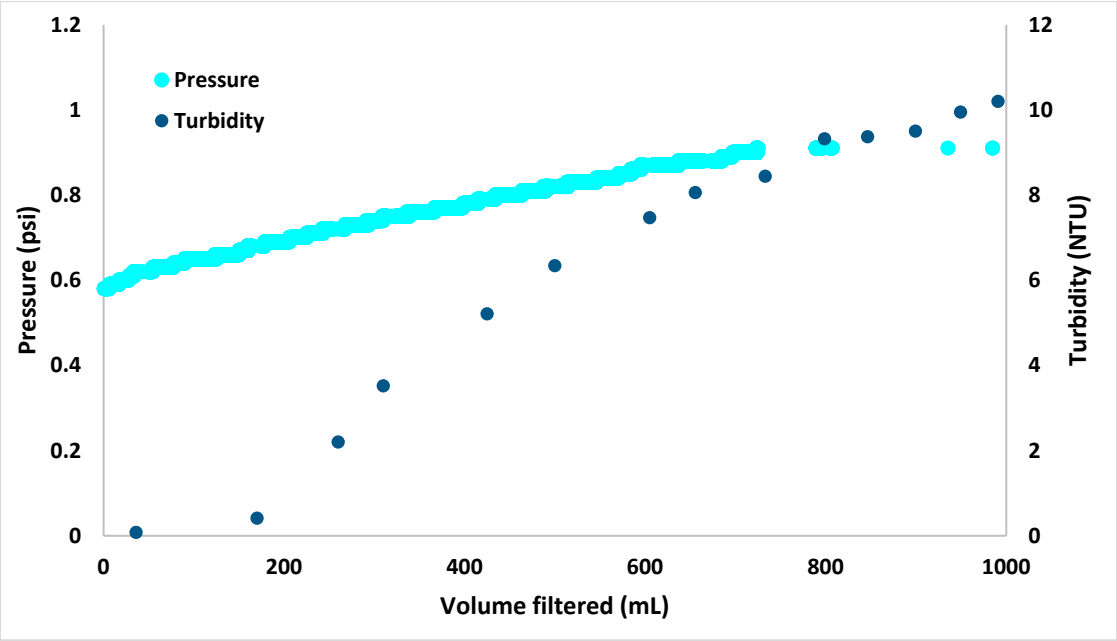

Figure 7. LV TFDF Permeate Filter B-1 Filtration: Pressure and Turbidity vs Volume

Conclusion

This study was designed to identify post-TFDF filters that ensure complete clarification with high virus yield, throughput, and turbidity reduction following LV, AAV8, and AAV9 viral vector production. The performance of several filters was analyzed to determine the most efficient filter that would permit directly proceeding to purification (Table 7). The data generated shows that the low turbidity (<60 NTU) of the TFDF permeate for all viral vectors feedstocks used (LV, AAV8 and AAV9) enables high virus recovery yield, high throughput, and high turbidity reduction during the secondary filtration. This performance translated into commercial scale manufacturing shows that the implementation of TFDF-based process intensification leads to high virus production combined with simplified clarification compared to batch cell culture.

Table 7. Performance of Filters Chosen For Each Clarification Process

Virus	Feedstock	Filter	Clarification step	Yield (%)	Throughput (L/m^2)	Turbidity Reduction (%)	Final Turbidity (NTU)
AAV8	Whole cells	B-1	Primary	60	>435	98	<5
AAV8	TFDF permeate	A-2	Secondary	82	>400	100	<
AAV9	Lysate	B-1	Primary	90	17	75	379
		D-3	Secondary	70	26	100	<5
AAV9	TFDF permeate	A-2	Secondary	97	>400	99	<5
LV	TFDF permeate	B-1	Secondary	61	>435	67	7

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