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Application Note

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Centrifuge Free Clarification and Harvest of Adeno-associated Viruses (AAV)

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1 Abstract

This study aimed to evaluate the applicability of Sartoclear Dynamics® Lab Filter Aid, containing diatomaceous earth (DE), for clarifying crude lysates of adeno-associated virus (AAV) produced by HEK293 suspension cells. Filtration using Sartoclear Dynamics® Lab Filter Aid proved to be a viable clarification method for AAV2, AAV5 and AAV8. For standard AAV production processes, AAV particle recovery in the filtrate was >95%. Even difficult-to-handle serotypes such as AAV2 and lysates containing lower AAV particle titers could be clarified by Sartoclear Dynamics® Lab filtration with viral recoveries of >80%. This study outlines a successful optimization strategy that utilized a Design of Experiments (DoE) approach to increase the yield of AAV2 to over 95%. Moreover, the use of Sartoclear Dynamics® Lab Filter Aid reduced manual handling time 3-fold and increased the filter capacity 3.5-fold compared to the standard laboratory method of filtration combined with a prior centrifugation step. Therefore, we have demonstrated that filtration with Sartoclear Dynamics® Lab Filter Aid is a novel and efficient clarification method for multiple AAV serotypes.

2 Introduction

Viruses play a crucial role as vectors in gene therapy, and adeno-associated viruses (AAV) are among the most widely studied and promising vectors. At the time of writing, 263 clinical trials utilizing AAVs are underway [1], and three AAV-based gene therapies have received approval from the U.S. Food & Drug Administration (FDA).

The most common method for production of AAV is transient transfection of HEK293 suspension cells either with a two- or three-vector plasmid system [2]. The majority of AAV serotypes are expressed intracellularly [3] and therefore AAV harvest thus usually requires a cell lysis step prior to further purification.

The downstream processing of AAV poses a challenge due to variations in physicochemical characteristics between different serotypes, which complicates the development of standardized purification procedures. Clarification of cell culture lysates by filtration is commonly employed, however, due to the complexity of the AAV lysate resulting from cell lysis during harvest [4], filtration remains a difficult task. Clarification of AAV is therefore a critical step. In laboratory settings, AAV clarification is typically achieved through centrifugation followed by microfiltration [5]. Single-step filtration techniques that incorporate filter aids can simplify and optimize the clarification process in terms of yield, throughput, and handling. One such filter aid is the Sartoclear Dynamics® Lab Filter Aid, which is composed of diatomaceous earth (DE), a porous powder derived from the remains of diatoms [6, 7]. For clarification, the Sartoclear Dynamics® Lab Filter Aid is mixed with cell lysate and the suspension applied to a membrane filter, a process known as alluvial or body feed filtration. This approach creates a permeable filter cake that selectively retains the majority of the cellular debris, thus minimizing filter clogging [8]. Moreover, the need for centrifugation is eliminated and processing time is reduced.

3 Materials and Methods

AAV2, AAV5 and AAV8 were produced by transient double-plasmid transfection of HEK293 cells (Expi293F™ Inducible Cells, Thermo Fisher Scientific) in non-baffled glass shake flasks and in a 2 L single use Univessel® bioreactor (Sartorius) as described in detail by Meierrieks et al. [9].

Clarification of 50 mL AAV crude cell lysate was carried out by two different filtration methods using polyethersulfone (PES) Sartolab® RF 50 filters (0.22 µm; Sartorius) connected to a Microsart® e.jet vacuum pump (Sartorius). In the first clarification approach, the crude cell lysate was mixed with DE (Sartoclear Dynamics® Lab Filter Aid, Sartorius) at a concentration of half the wet-cell weight. The resulting suspension was then filtered under vacuum with a Sartolab® RF 50 unit. The second filtration method is based on a two-stage approach and is considered as the standard laboratory method. In this approach, the crude cell lysate was centrifuged at 800 g for 5 min first, then the supernatant was passed through the filter. AAV capsid titers were determined using the AAV Xpress ELISA kits (PROGEN Biotechnik GmbH) and calculated using a linear fitted standard curve. Viral genomic titers were determined externally at Sartorius Xell AG by droplet digital PCR (ddPCR).

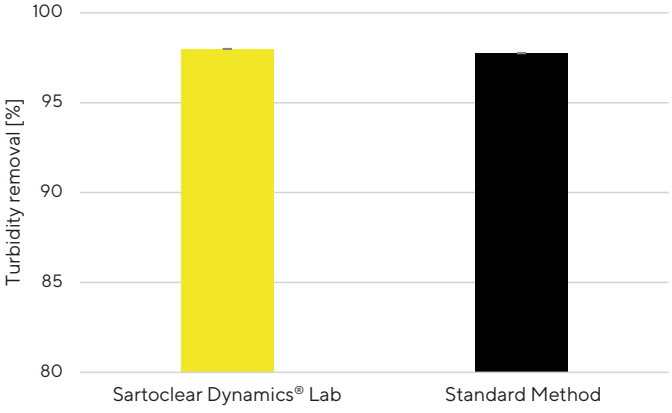
A design of experiment (DoE) approach was performed to examine the influence of the two factors DE concentration and incubation time on AAV2 clarification. The experimental setup was planned as a D-optimal design with 14 experiments in total and 3 center points using the MODDE® 13 software (Sartorius). DE concentration and incubation time were varied between 2-40 g/L and 0-20 min, respectively.

4 Results and Discussion

4.1 Evaluation of the impact of Sartoclear Dynamics® Lab on turbidity removal

A comparative analysis of clarification between filtration using the Sartoclear Dynamics® Lab Filter Aid and the conventional two-step centrifugation/filtration method, revealed a similar degree of turbidity reduction from AAV cell lysates (**Figure 1**). Prior to cell lysis, the AAV5 cell lysate had a cell count of 3.42 x 10^6 cells/mL and after cell lysis a turbidity of 385 NTU. The amount of DE used in our experiments corresponded to half the wet-cell weight. Clarification with DE led to a 98% reduction in turbidity, and the standard method resulted in a 97.75% reduction. We observed similar levels of turbidity reduction from AAV2 and AAV8-containing cell lysates (data not shown), regardless of the clarification method used

Figure 1: *Effects of body feed and conventional clarification methods on turbidity removal.*



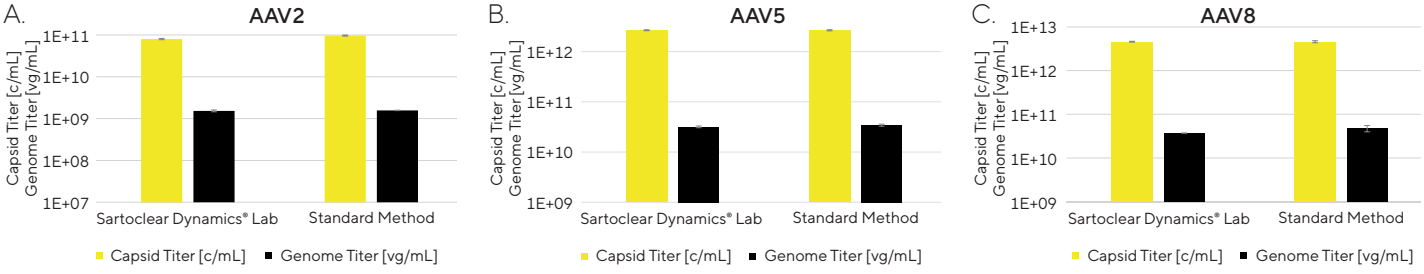
Note: Clarification of AAV5 cell lysate was performed either with Sartoclear Dynamics® Lab Filter Aid using DE at a concentration of half the wet-cell weigh (6.25 g/L DE) or by centrifugation followed by 0.2 µm filtration. Before cell lysis, the lysate had a cell count of 3.42 x 10^6 cells/mL and after cell lysis a turbidity of 385 NTU. Mean ± standard deviation; N = 3.

4.2 Evaluation of the impact of Sartoclear Dynamics® Lab on AAV yield

We evaluated whether clarification by Sartoclear Dynamics® Lab filtration had any influence on viral recoveries. Viral particle titers as well as genomic titers of the Sartoclear Dynamics® Lab filtration and the standard laboratory method were determined and are shown in **Figure 2**.

We examined three different serotypes (AAV2, AAV5, and AAV8) as these are known to exhibit variability in terms of adsorption, aggregation, achieved titers, and empty/full ratios [10]. Prior to cell lysis, the AAV-containing lysates had cell counts of 3.72 x 10^6, 3.42 x 10^6, 3.44 x 10^6cells/mL and after cell lysis turbidities of 439, 385 and 391 NTU for AAV2, AAV5 and AAV8, respectively.

Figure 2: *Effects of clarification methods on AAV yield.*



Note: Clarification of AAV2 (A), AAV5 (B) and AAV8 (C) cell lysate was performed either with Sartoclear Dynamics® Lab Filter Aid using DE in a concentration of half the wet-cell weight (AAV2: 6.25 g/L, AAV5: 6.25 g/L, AAV8: 6.64 g/L DE) or by 0.2 µm filtration with prior centrifugation. Before cell lysis, the lysate had a cell count of 3.72 x 10^6, 3.42 x 10^6, 3.44 x 10^6cells/mL and after cell lysis a turbidity of 439, 385 and 391 NTU for AAV2, AAV5 and AAV8, respectively. Mean ± standard deviation; N = 3; Viral titers were analyzed with unpaired t-test with a significance level of α=0.05.

For AAV5 (**Figure 2B**), viral recoveries were 2.66 x 10^{12} c/mL and 3.17 x 10^{10} vg/mL when using Sartoclear Dynamics® Lab filtration, while the standard laboratory method resulted in recoveries of 2.65 x 10^{12} c/mL and 3.43 x 10^{10} vg/mL. The implementation of Sartoclear Dynamics® Lab filtration for the clarification of AAV8 (**Figure 2C**) resulted in viral yields of 4.60 x 10^{12} c/mL and 3.71 x 10^{10} vg/mL, while the standard laboratory method yielded 4.59 x 10^{12} c/mL and 4.75 x 10^{10} vg/mL. Regarding AAV2 (**Figure 2A**), Sartoclear Dynamics® Lab filtration led to viral titers of 7.99 x 10^{10} c/mL and 1.53 x 10^{9} vg/mL, whereas the standard laboratory method yielded viral titers of 9.67 x 10^{10} c/mL and 1.57 x 10^{9} vg/mL.

In comparison to the standard laboratory method, clarification with Sartoclear Dynamics® Lab, using DE at a concentration of half the wet-cell weight, did not result in significant AAV5 and AAV8 particle losses (α=0.05, unpaired t-test), indicating its suitability as a viable alternative to the conventional clarification method. For AAV2, a significant particle loss (α=0.05, unpaired t-test) of 18% was observed when DE was used at a concentration of half the wet-cell weight. This can be attributed to AAV2 exhibiting a lower expression level and initial titer (about 10 times lower) than AAV5 and AAV8, resulting in a higher DE/AAV ratio used for AAV2 lysates. To address this issue, we performed a DoE to optimize the DE/AAV ratio.

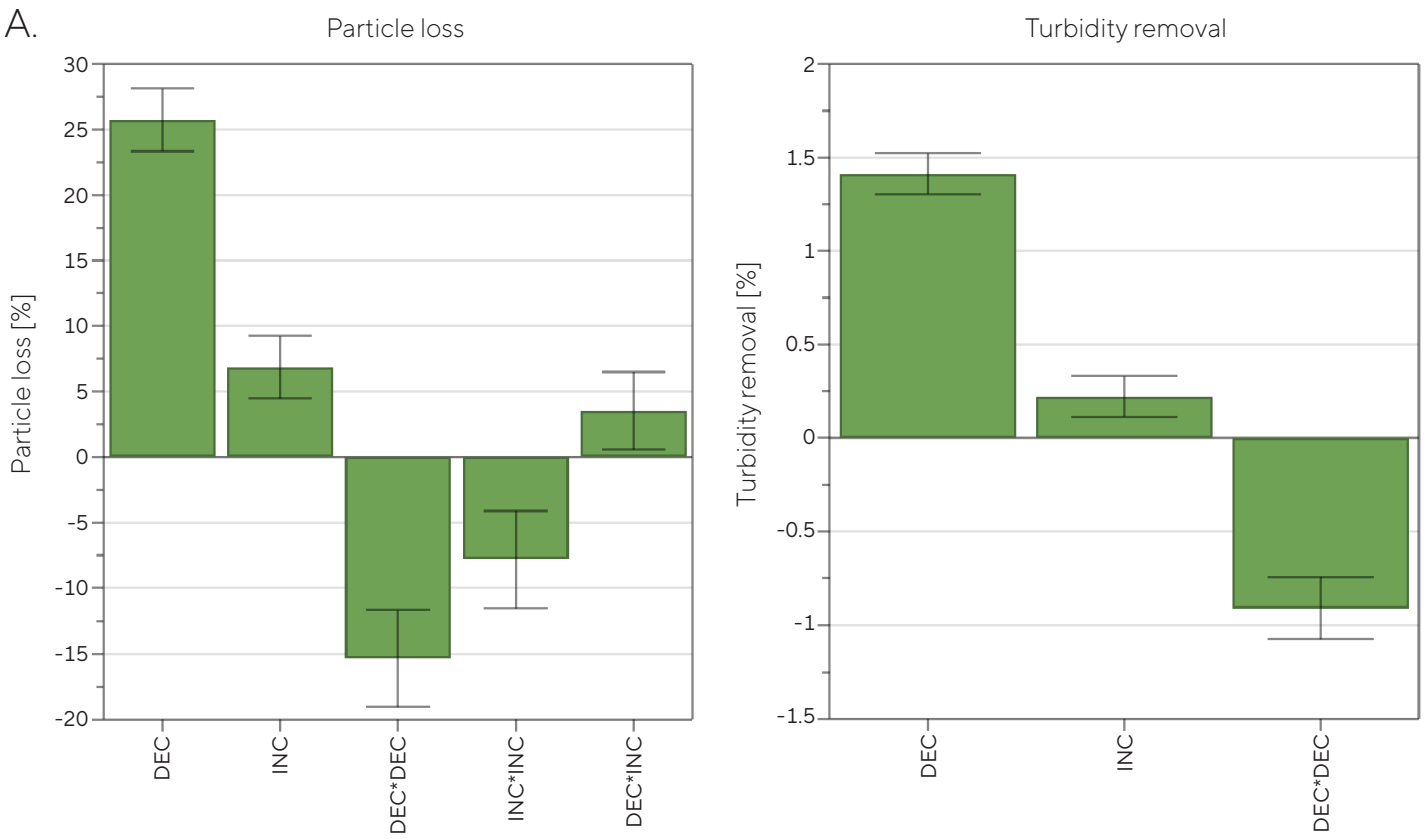
4.3 Evaluation of the impact of DE concentration and DE incubation time on AAV2 retention and turbidity removal in a DoE study using MODDE®

Compared with the standard laboratory method, which consists of a centrifugation step with subsequent microfiltration, an AAV2 particle loss of 18% was observed when Sartoclear Dynamics® Lab filtration was used with DE at a concentration of half the wet-cell weight. We assume that AAV adsorbs to DE and therefore performed a DoE approach to study the influence of the two process parameters DE concentration and incubation time with DE on AAV2 particle loss during filtration with DE (**Figure 3**). For both responses, the DoE approach generated a robust model, with R2 values of 0.99 and Q2 ≥0.94. **Figure 3A** shows that DE concentration has a stronger influence on both responses than did the incubation time.

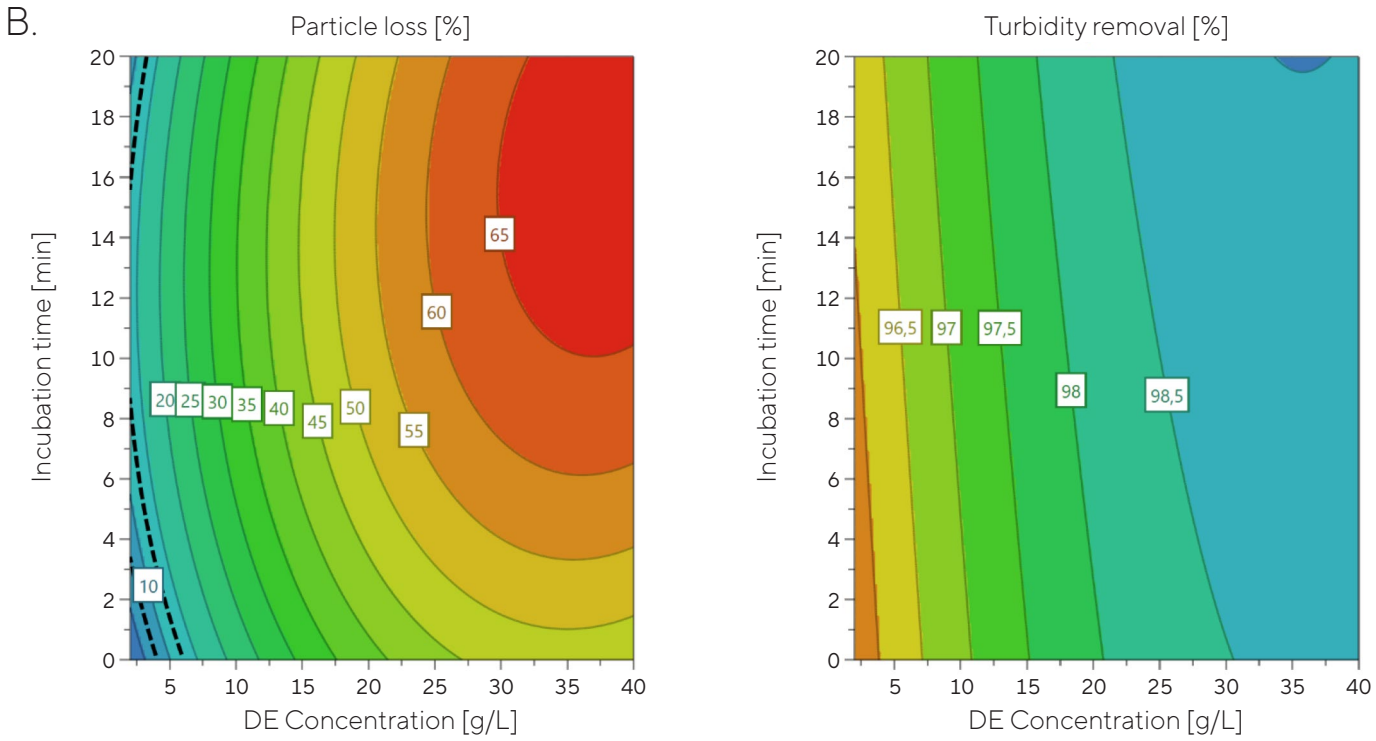
The contour plot in **Figure 3B** visualizes the relationships between the factors and the responses. The optimizer function of the MODDE® software resulted in a robust set point of about 2 g/L DE (equivalent to ~1/8 of the wet-cell weight) and 0 min incubation time. When the DE concentration was decreased from 1/2 of the wet-cell weight (as in **Figure 2**) to 1/8 (equivalent to 0.181 mg DE/10^{10} AAV), the loss of AAV2 particles during filtration with DE is reduced from 18% to <2%. Based on AAV8 experiments (data not shown) with DE concentrations correlating to the DE/AAV particle amount used for the AAV2 studies, it was proven that the particle loss due to high DE concentrations is not a serotype dependent but a general adsorptive effect.

Based on the results presented in **Figure 2** and the findings of our DoE experiment (**Figure 3**), we conclude that Sartoclear Dynamics® Lab filtration is a viable method for clarification of AAV crude lysates. When using standard AAV production processes by HEK293 suspension cell cultures, which results in a total cell density of 3-4 x 10^{6} cells/mL, a wet-cell weight of 12-14 g/L, and a particle titer of ≥10^{12} c/mL, DE filtration at a concentration of half the wet-cell weight is an effective approach. Furthermore, DE body feed filtration can also be employed for the clarification of AAV processes that yield particle titers lower than 10^{12} c/mL, as long as the amount of DE used is adjusted to avoid exceeding a ratio of 0.181 mg DE/10^{10} AAV particles.

Figure 3: *Effects of the factors DE concentration and incubation time on AAV2 crude lysate clarification performance.*



Particle loss (N = 14; DF = 8; R2 = 0.99); Turbidity removal (N = 14; DF = 10; R2 = 0.99); Confidence = 0.95



Note: Regression coefficient plot (A) and response contour plot (B) of the responses particle loss and turbidity removal. The starting cell broth had a cell density of 2.78 x 10^6 cells/mL, an AAV particle titer of 1.11 x 10^{11} c/mL, a wet-cell weight of 17.56 g/L and following cell lysis, a turbidity of 366 NTU. Abbreviations: DE, diatomaceous earth; DEC, DE concentration; Inc, incubation time; N, number of experiments; DF, degrees of freedom; R2, percent of the variation of the response explained by the model; c, capsids; NTU, nephelometric turbidity units.

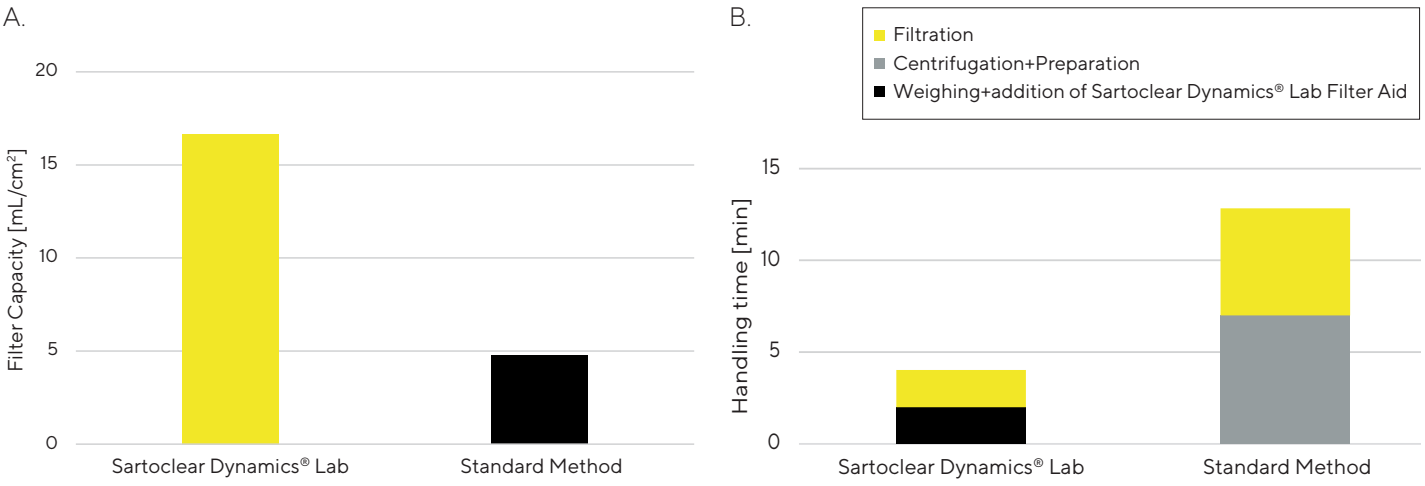
4.4 Evaluation of the impact of Sartoclear Dynamics® Lab on filter capacity and manual handling times

In a further experiment, the impact of Sartoclear Dynamics® Lab filtration on filter capacity and manual handling time was investigated, as depicted in **Figure 4**. In the two-step standard laboratory method, the filter clogged after processing 100 mL of crude AAV lysate, while the Sartoclear Dynamics® Lab filtration could handle 350 mL before filter blockage. Total throughput relative to the filter area was 4.76 mL/cm^2 and 16.67 mL/cm^2, for the standard or Sartoclear Dynamics® Lab methods, respectively.

Figure 4 presents the breakdown of manual handling time for clarifying 350 mL of AAV2 cell lysate using Sartoclear Dynamics® Lab filtration and the standard laboratory method. The standard method involved a total manual handling time of approximately 12.8 min, consisting of a 7-minute centrifugation step (including preparation) and a 5.8-minute filtration step. Sartoclear Dynamics® Lab filtration reduced manual handling time to approximately 4 min, representing a three-fold reduction. This handling time could be divided into two steps: a 2-minute step for weighing of Sartoclear Dynamics® Lab Filter Aid and connecting the Sartolab® RF 50 unit to a vacuum pump, followed by a further 2-minute filtration step.

In terms of both economy and ecology, Sartoclear Dynamics® Lab filtration was found to be more effective than the two-step standard laboratory method. It resulted in a 3-fold reduction in manual handling time and a 3.5-fold increase in filter capacity. In addition, Sartoclear Dynamics® Lab filtration can be easily scaled up by using the Sartolab® Multistation Filtration Unit Stand (Sartorius), which enables clarification of up to 6 L of crude lysate through running 6x1 L filtration units in parallel.

Figure 4: *Effects of clarification methods on filter capacity (A) and handling time (B).*



Note: Clarification of 350 mL of AAV2 cell lysate was performed either with Sartoclear Dynamics® Lab Filter Aid using DE in a concentration of half the wet-cell weight (6.3 g/L DE) or by the standard method of 0.2 µm filtration with prior centrifugation.

5 Conclusions

Sartoclear Dynamics® Lab filtration facilitates clarification of AAV-containing crude cell lysates without the need for a centrifugation step and was tested for the three serotypes AAV2, AAV5 and AAV8. Our results show that the Sartoclear Dynamics® Lab filtration system resulted in greater reduction of turbidity, increased filter capacity, and decreased manual handling time compared to the standard laboratory method, while maintaining high viral yields. Sartoclear Dynamics® Lab filtration is therefore a viable approach for clarification of AAV crude cell lysates. The amount of DE used for clarification depends on the AAV production process. Standard AAV production processes by HEK293 suspension cells can be effectively clarified using DE at a concentration of half the wet-cell weight. However, for processes with lower AAV yields per cell, the quantity of DE used should be adjusted to avoid exceeding a ratio of 0.181 mg DE/10^{10} AAV particles.

This application note was adapted from the publication by Meierrieks et al. [9].

6 Abbreviations

c	capsid
vg	viral genome
DE	diatomaceous earth
AAV	adeno-associated virus
FDA	Food & Drug Administration
HEK293	human embryonic kidney 293 cells
DoE	design of experiment
PES	polyethersulfone
ELISA	enzyme-linked immunosorbent assay
ddPCR	droplet digital polymerase chain reaction
NTU	nephelometric turbidity units
N	number of experiments

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