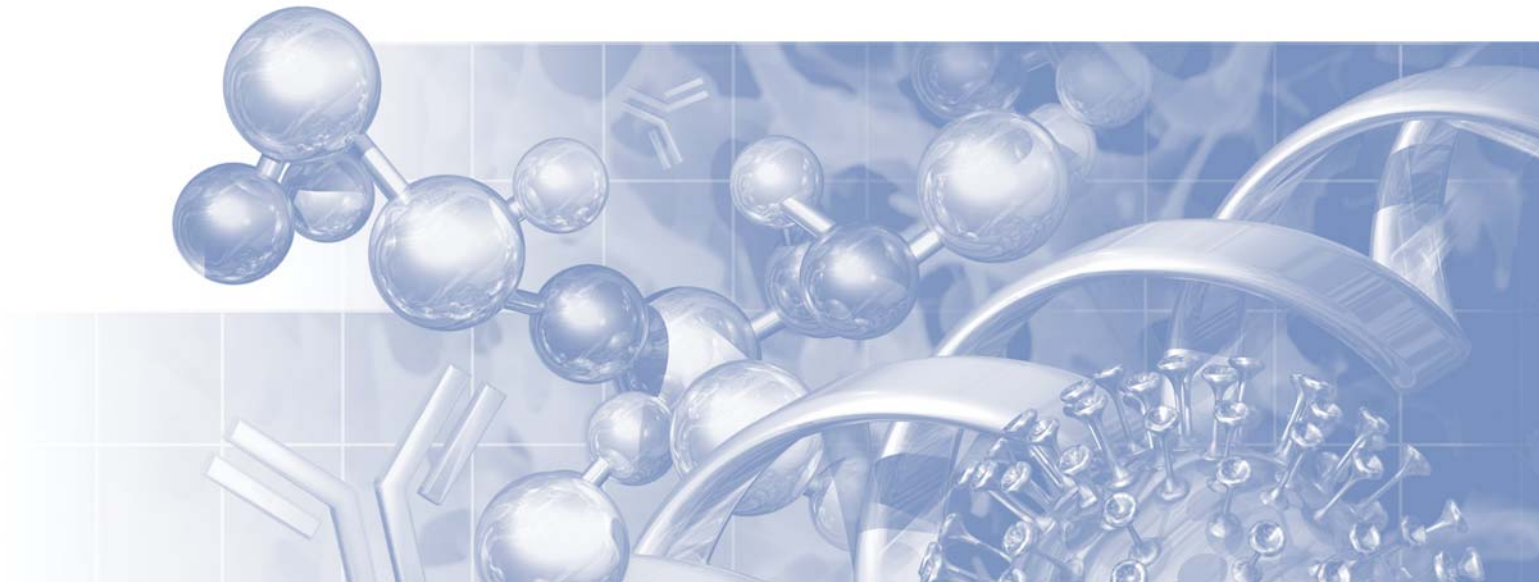
PALL Life Sciences

Application Note

USD 2916(1)

Purification of Influenza Virus by Ion Exchange Chromatography on Mustang® Q XT Membranes

Fast Purification Conditions Screening on AcroPrep™ Advance 96-Well Filter Plates



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Summary

Membrane chromatography is an effective alternative to conventional beaded sorbents for the purification of large molecules like viruses or viral-like particles (VLPs). Membranes have an open pore structure that allows fast kinetics and direct access to ion exchange binding sites, with flow rates orders of magnitude faster than conventional columns. They also provide, at these higher flow rates, much higher binding capacities for large molecules than beaded sorbents that are limited by mass transfer. Pall Mustang ion exchange membranes, used in flow through (FT) or bind/elute mode, are scalable membrane chromatography devices currently used in various therapeutic, vaccine and gene therapy products purification schemes. These ready-to-use devices (re-usable or single-use) allow users to significantly reduce hardware investments and validation costs typically associated with column chromatography.

This work shows how structured Design of Experiments (DoE) and High Throughput Screening (HTS) experiments performed on AcroPrep Advance 96-well filter plates with Mustang Q membranes can be applied for fast and effective selection of purification conditions of influenza virus from clarified cell culture feedstock. The HTS approach requires minimal sample consumption, and predicted results are in line with standard chromatography experiments directly obtained with Acrodisc® ion exchange chromatography units with Mustang Q XT membrane.

This study was performed in collaboration with the National Research Council Canada (NRC), Montreal, Canada.

1. Material and Methods

1.1. Production of Virus Particles and Feedstock

Influenza virus A/Puerto Rico/8/1934 (H1N1) was produced by infection of human embryonic kidney (HEK)-293 cells as described by Le Ru A., 2010 [1]. After culture, the cells were filtered out of the viral feedstock using a sequence of Pall HDC® II polypropylene depth filters (4.5 µm > 1.2 µm > 0.6 µm) and a bioburden reduction filtration on Pall Fluorodyne® II DBL membrane filter cartridge (0.45 µm). Virus purification conditions were selected in bind/elute mode on AcroPrep Advance 96-well filter plate with Mustang Q membranes.

1.2. Selection of the Purification Conditions Using HTS on AcroPrep Advance Filter Plates

For a high throughput screening and minimal sample consumption, the experiments were performed on AcroPrep Advance 96-well filter plates with Mustang Q membrane (Part Number 8171), operated at constant flow rate with a multi-well plate vacuum manifold (Part Number 5017).

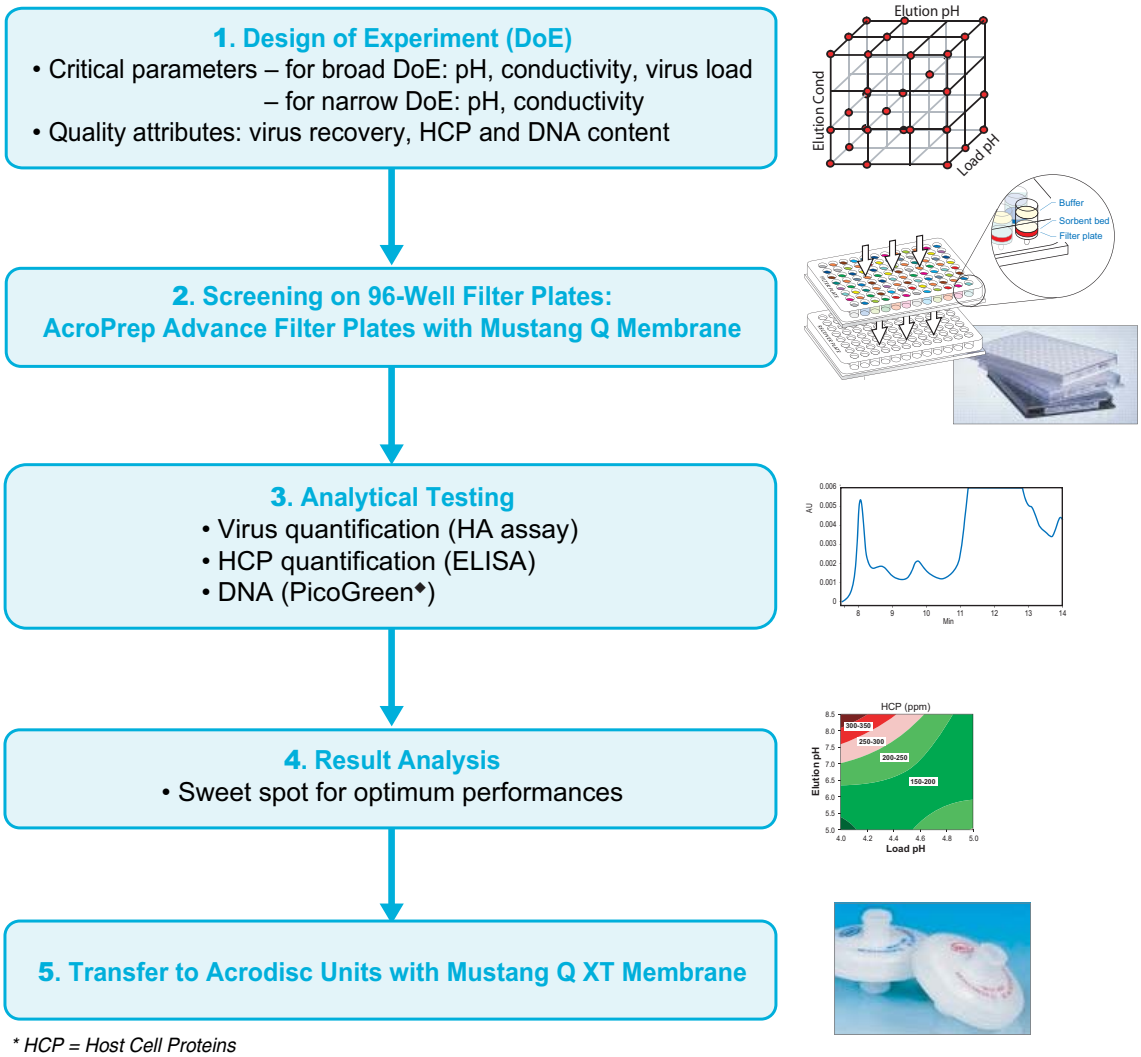
Design of Experiments (DoE) based on a response surface model and a fractioned factorial allowed evaluating the impact of multiple parameters on key product quality attributes using a limited number of experiments. The critical parameters for the Mustang Q XT membrane performance included pH, conductivity and virus content of the sample loaded onto the device. Eluted virus yield and contaminants (DNA, host cell proteins [HCP]) clearance are also major quality attributes monitored in the DoE.

The overall strategy (Figure 1) included a structured DoE approach. A first broad design space DoE was performed with three parameters to define axial point response design: pH ranged between 4.4 and 8.0, conductivity between 6.2 and 64.0 mS/cm, and virus load content was between 30 and 86 hemagglutinin (HA) units per well (Table 1). Results analysis using Minitab◆16 software (Minitab Inc.) allowed to determine the optimal purification conditions (Figure 2). The loading samples were prepared from the virus stock sample (156 HA units/mL; 8.4 µg/mL DNA; 40.6 µg/mL HCP), by dilution with 20 mM phosphate citrate buffer, at appropriate pH and conductivity. The same buffers were used for equilibration and post load wash. For elution 0.5 M NaCl and for strip 1 M NaCl was added to loading buffer.

To confirm the findings, a second sequence of narrower DoE was performed with only two variable parameters (pH and conductivity), load being kept constant. Response surface design was created and its result analyzed in Minitab 16 using the sweet spot from the initial DoE as the starting point. The conditions tested included pH of 4, 6 and 8, conductivity of 4, 10 and 16 mS/cm and constant load. Elution was carried out using 20 mM phosphate citrate enriched with 1 M NaCl. Process conditions and detailed protocol are shown in Table 2 and Table 3.

Figure 1

Overall Strategy for Virus Purification on Mustang Q Membrane



** HCP = Host Cell Proteins*

Table 1

Broad Range (3 Parameters) DoE for Virus Binding on AcroPrep Advance Filter Plates Wth Mustang Q Membrane

Run Number	pH	Conductivity (mS/cm)	Virus Load (HA units per well)
1	5.1	17.9	41.4
2	7.3	17.9	41.4
3	5.1	52.3	41.4
4	7.3	52.3	41.4
5	5.1	17.9	74.6
6	7.3	17.9	74.6
7	5.1	52.3	74.6
8	7.3	52.3	74.6
9	4.4	35.1	58.0
10	8.0	35.1	58.0
11	6.2	6.2	58.0
12	6.2	64.0	58.0
13	6.2	35.1	30.0
14	6.2	35.1	86.0
15	6.2	35.1	58.0

Table 2

Process Conditions Used in DoE Experiments on AcroPrep Advance Filter Plates With Mustang Q Membrane

Step	Volume Loaded per Well (mL)	Incubation Time (min)
Equilibration	2.4	2
Loading	1.6	5
Wash	1.6	0
Elution	1.6	5
Strip	1.6	0

1.3. Direct Selection in Bind/Elute Mode on Acrodisc Units With Mustang Q XT Membrane

Standard membrane chromatography for purification of influenza virus was carried out independently of the previous 96-well plate HTS experiments. Conditions were compared later on to evaluate the matching and predictive power of the HTS model (see Results and Discussion). The Acrodisc unit with Mustang Q XT membrane (Part Number MSTGXT25Q16) was connected to an ÄKTAprime◆ Plus System (GE Healthcare). Loading, washing and elution were run at a flow rate of 10 membrane volumes (MV)/min. PBS or 10 mM phosphate, pH 7.4, 4 mS/cm (equilibration buffers). The load of Acrodisc unit with Mustang Q XT membrane was followed by 10 MV wash with equilibration buffer and elution using a series of 10 MV incremental steps containing different amounts (from 10 to 100%) of 1 M NaCl. The device was finally cleaned with 1 M NaOH solution.

Table 3
Detailed Experimental Protocol for AcroPrep Advance Filter Plate With Mustang Q Membrane

1. Pre-wet AcroPrep Advance filter plate with Mustang Q membrane with a maximum of 800 µL/well of loading buffer of appropriate pH and conductivity.
2. Place filter plate on collection plate and incubate for 2 minutes on shaker. Place collection plate in vacuum manifold and filter plate on top of collection and apply vacuum of 20 mbar to remove loading buffer. All subsequent vacuum evacuation will occur at 20 mbar.
3. Repeat steps 1 and 2 until volume of loading buffer is processed over each well.
4. Load virus containing mixture to filter plate and incubate on shaker for 5 minutes.
5. Place filter plate on clean collection plate and evacuate liquid by vacuum. Retain flow through fraction containing unbound virus particles.
6. Repeat steps 4 and 5 until volume of sample is processed over each well. Combine samples from same well.
7. Wash with 800 µL/well of loading buffer, place filter plate on clean collection plate, vacuum and retain flow through (wash fraction).
8. Repeat step 7. Combine samples from same well.
9. Add 800 µL/well elution buffer, incubate on shaker for 5 to 10 minutes.
10. Place filter plate on clean collection plate and centrifuge. Retain eluate.
11. Repeat steps 9 and 10. Combine samples from same well.
12. Add 800 µL/well of strip buffer, incubate on shaker for 0 to 5 minutes.
13. Place filter plate on clean collection plate and vacuum. Retain eluate.
14. Repeat steps 12 and 13. Combine eluates.
15. Analyze collected fractions by HA, ELISA (Cygnus Technologies, Part Number F650) and PicoGreen (Life Technologies, Part Number P7589) analytical assays.

1.4. Transfer of Selected Conditions Defined by HTS to Acrodisc Units With Mustang Q XT Membrane

The selected pH and conductivity conditions for the bind and elution of influenza virus on AcroPrep Advance Filter Plates with Mustang Q Membrane were used on Acrodisc unit with Mustang Q XT membrane. The Acrodisc unit with 0.86 mL membrane has the same 16-layer membrane construction as larger capacity Mustang capsules and is the recommended starting point for accurate scale-up from process development to pilot or process scale, maintaining the flow/differential pressure ratio and dynamic binding capacity. The device was operated at 10 MV/min using ÄKTAexplorer◆ (GE Healthcare). Prior to load, sample pH was adjusted to 6.8 and conductivity to 6.2 mS/cm. Device was equilibrated with 20 MV of 10 mM phosphate pH 6.8 , 6.2 mS/cm and was followed by the virus load until the 10% virus breakthrough. Device was then washed with 30 MV of equilibration buffer and eluted with 50 MV of 10 mM phosphate, pH 5.8, 55.6 mS/cm. Finally, device was stripped with 50 MV of 2 M NaCl-enriched equilibration buffer and sanitized (30 MV of 1 M NaOH).

1.5. Analytical Assays

The influenza virus was quantified using both hemaglutination (HA) and virus infectivity assays (TCID50 - Tissue Culture Infective Dose – expressed in Infectious Viral Particle units IVPs (reference Le Ru 2010). A correlation was made between the HA titer (HA units/mL) and the $TCID_{50}$, using data points before and after processing over the Mustang Q membrane. It was found that the ratio remained constant at 4.0×10^5 virus particles per HA unit. The level of the major contaminants, host cell DNA and HCP, was determined using PicoGreen and HEK293 ELISA HCP analytical assays respectively.

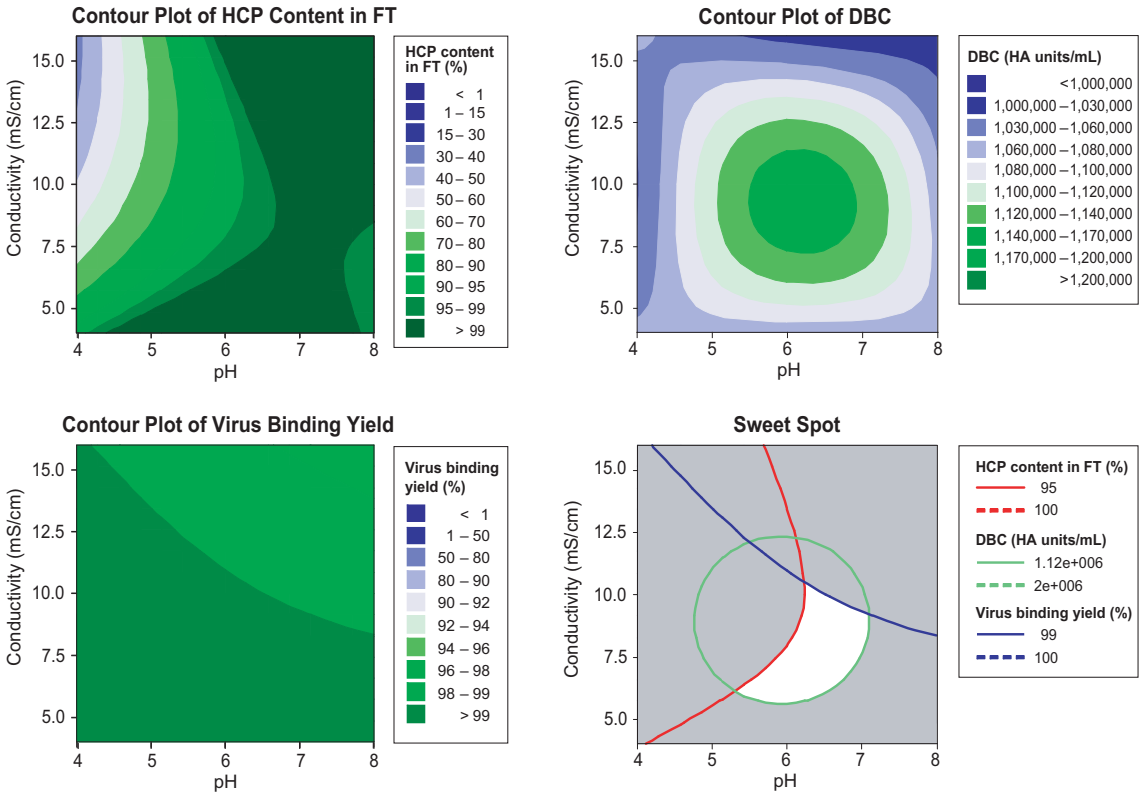
2. Results

2.1. Selection of Bind/Elute Conditions by HTS on AcroPrep Advance 96-Well Filter Plate With Mustang Q Membrane

Dynamic Binding Capacity (DBC) for Influenza Virus: The sweet spot of the initial DoE (Figure 2, down right) showed that a high DBC between 1.10 to 1.15×10^5 HA units/mL of membrane (4.1 to 4.5×10^{11} particles/mL of membrane) can be reached. Good HCP clearance was achieved (HCP remain mostly in the flow through), while no DNA clearance was observed during binding. The statistically fitted model of the contour plots was then taken through optimization tool of Minitab 16 software to determine optimal conditions (Table 3).

Figure 2

Selection of Binding Conditions of Virus on AcroPrep Filter Plates With Mustang Q Membrane



Contour plots from response surface modeling of two DoE parameters: pH and conductivity

Table 3

Selected Conditions Derived from DoE Analysis Results (Minitab Software) HTS on AcroPrep Advance Filter Plates With Mustang Q Membrane

	Binding	Elution
Conditions for bind/elute mode		
pH	6.8	5.8
Conductivity (mS/cm)	6.2	55.6
Number of HA units per well	85.9	NA
Results from Minitab analysis		
Virus yield /recovery (%)	NA	90.8
HCP removal (%)	>99.5	>99.8
DNA removal (%)	0	>99.7

NA = Not applicable

Data suggest that DBC and recovery are controlled by virus and DNA content in the load. HTS optimization of elution conditions enabled excellent DNA clearance, in addition to the very good HCP clearance already achieved during binding. Note that higher virus recovery is possible, but may jeopardize DNA clearance.

2.2. Direct Selection in Bind/Elute Mode on Acrodisc Units With Mustang Q XT Membrane

In parallel to the 96-well plate HTS approach, an independent standard membrane chromatography optimization was performed using the Acrodisc device with Mustang Q XT membrane. To study the impact of conductivity, both undiluted and 4-fold diluted virus feed (post depth filter) were loaded on the Acrodisc device with Mustang Q XT membrane until detection of virus breakthrough. Results are shown in Figures 3 and 4, and Table 4.

Figure 3

Membrane Chromatography on Acrodisc Unit With Mustang Q XT Membrane (Undiluted Sample)

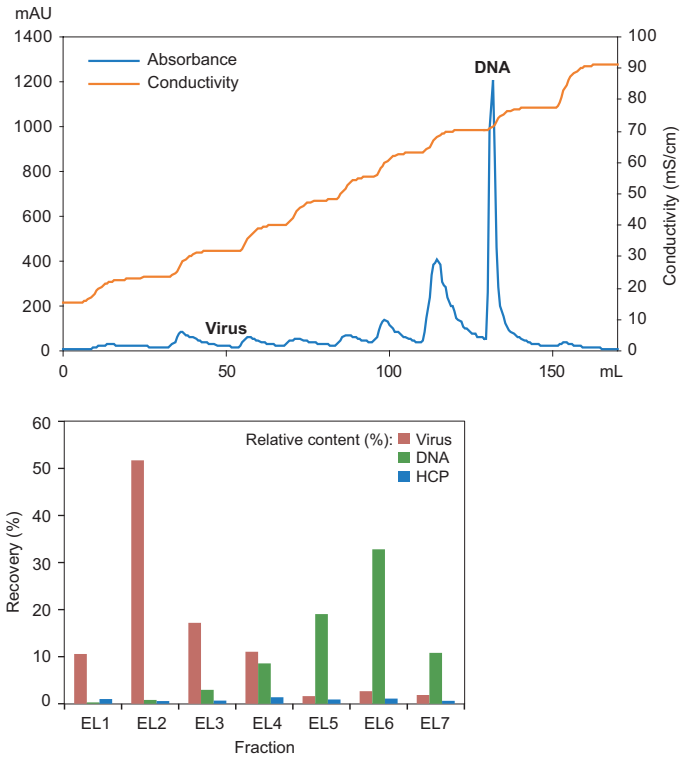


Figure 4

Membrane Chromatography on Acrodisc Unit With Mustang Q XT Membrane (Undiluted Sample)

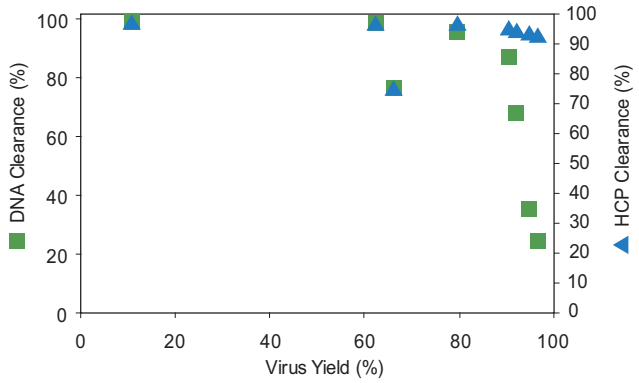


Table 4

Virus Recovery vs. Purity (Undiluted Sample) of Acrodisc Unit With Mustang Q XT Membrane

Conductivity at elution (mS/cm)	55.6	63.2
Virus yield/recovery (%)	82.3	93.7
DNA clearance (%)	95.9	87.0
HCP clearance (%)	97.7	96.3

The optimization work carried directly on Acrodisc devices with Mustang Q XT membrane confirmed that virus binding performance is determined by the DNA content and loading conductivity. The DBC was higher for 4-fold diluted sample versus undiluted feed due to lower conductivity (2.5×10^{11} vs. 9×10^{10} particles per mL of membrane respectively). Results show however that undiluted sample can be used, which would be beneficial for direct processing of viral feedstock only after clarification. Additionally, using undiluted sample will lead to better process economics (lower buffer consumption and process time). As found in the HTS study on AcroPrep Advance 96-well filter plates, the binding conditions provided excellent HCP clearance (>90%), while virus DNA were bound by the Mustang Q XT membrane.

Elution provided good resolution between top of DNA and virus peaks (Figure 5). However, the cross-over between bases of these peaks could not be completely resolved, limiting virus recovery or DNA clearance. Overall, a high elution conductivity of 56 to 63 mS/cm was required for good virus recovery. Data also showed good DNA and HCP (>95%) clearance with average virus recovery between 82 and 94% (Table 4)

2.3. Transfer of Selected Conditions Defined by HTS to Acrodisc Units With Mustang Q XT Membrane

The conditions for optimal bind/elute of influenza virus determined on the AcroPrep Advance 96-well filter plate with Mustang Q membrane in the HTS approach were successfully applied on an Acrodisc unit with Mustang Q XT membrane. Data summarized in Table 5 show virus DBC in the upper 10^{11} range. Also, elution yielded good recovery of ~75 % with excellent and constant DNA and HCP (>95%) clearance and process productivity (viral particles purified per hour).

Table 5
Data Obtained on Acrodisc Units With Mustang Q XT Membrane With the Conditions Defined by HTS Study

DBC (particle/mL membrane)	Virus Recovery (%)	DNA Clearance (%)	HCP Clearance (%)	Productivity (particle/hr/L)
~ 9×10^{11}	~75%	99.9	95	~ 2×10^{14}

3. Discussion and Conclusions

Membrane chromatography processes are currently being evaluated in process development for several new viral products both in capture and flow through mode. With viruses ranging in size from 20 nm to well over 150 nm, membrane chromatography processes have proven to outperform conventional chromatography sorbents in terms of capacity and processing time. The prepacked format Mustang devices typically are supplied in enables the hardware investment typically associated with chromatography to be reduced and allows for more flexible operation, including single-use, eliminating the need for cleaning validation.

Barriers to implementation of membrane chromatography include a general lack of experience with these methods linked to concerns about cost and yield. As with all chromatography, highest yields with best purities will result from optimization of the various parameters influencing the quality attributes but this requires time and resources. The presented data show how these barriers can partially be overcome by using a structured experimental design (DoE) and a high throughput screening (HTS) approach to define the optimal operating conditions for best quality of the virus product.

The conditions for optimal bind/elute of influenza virus on the Mustang Q membrane, determined on the AcroPrep Advance 96-well filter plate in the HTS approach, successfully matched data obtained on Acrodisc unit with Mustang Q XT membrane, confirming the validity of the conditions determined by the HTS approach.

In the context of the purification of influenza virus, the study demonstrates the predictive power of this methodology. The ratio between DNA and virus load is important for best recovery and purity. The HTS approach is fast, consumes only little sample, gives useful information on parameters such as the virus binding capacity and allows for a reliable transfer and scale-up to a chromatography capsule.

It needs to be emphasized that the purification was performed with a virus feedstock that was only processed through a depth filter for cell removal. There was no DNA reduction treatment with endo-nucleases prior to the load on the Mustang Q XT membrane. The positioning of the Mustang Q membrane capture/elute step directly post cell removal, allows to eliminate also the tangential flow filtration step, typically combined with endonuclease treatment (Figure 5).

In conclusion, the data confirm that Mustang Q XT capsule is a valuable alternative for the purification of influenza virus, and by extension for other viruses, from clarified cell culture feedstock, allowing for faster, simpler and economical processing.

Figure 5
Simplified Influenza Virus Purification Process Using Mustang Q XT Membrane

Reference

[1] *Le Ru, A., Jacob, D., Transfiguracion, J., Ansorge, S., Henry, O., Kamen, A.A. Scalable production of influenza virus in HEK-293 cells for efficient vaccine manufacturing. Vaccine 28 (2010) 3661-3671.*

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