

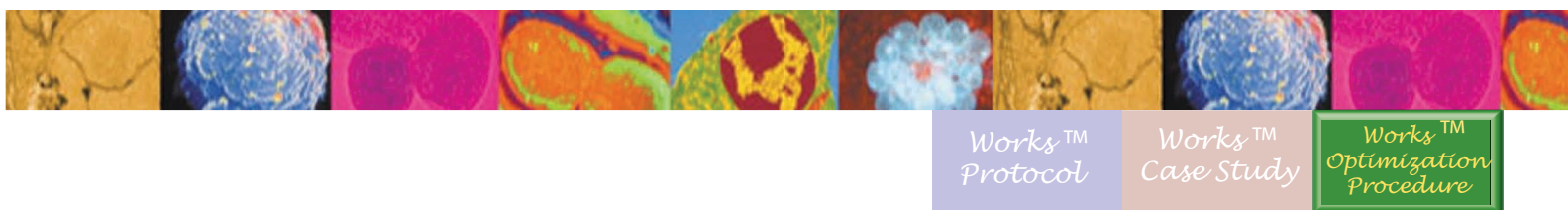
Purification of secreted viral antigens from mammalian cells with SmartFlow™ TFF

This *Purification of secreted viral antigens from mammalian cells WORKS™* Optimization Procedure is intended to isolate secreted viral antigens or secreted whole viruses from cell culture media. This process has been repeatedly implemented with consistent success for isolating viral antigens excluding the smallpox, rotavirus, parvo, and hepatitis viruses.

This optimization procedure uses a microfiltration (MF) membrane that retains the cells and cell debris and allows the viral antigens or whole viruses to pass freely through the membrane. The passage characteristics of viral antigens or whole viruses from mammalian cells change with different buffers, temperatures, concentrations, and membranes. By examining the passage characteristics of the different MF membranes available in the appropriate process conditions, a well defined and executed process development study can identify the most efficient membrane and process conditions to achieve the required performance.

This optimization procedure starts with selecting the membrane module, membrane polymer, and pore size most likely to work based upon numerous NCSRT evaluations. Once this module is selected, ranges in which to begin optimizing parameters such as membrane capacity, recirculation rate, and pressure are presented. Because of the variability in the products and processes using NCSRT's *SmartFlow™* technology, we do not make specific process recommendations on parameters of temperature, pH, buffers, or other variables that may affect the separation process and the target product activity.

The *SmartFlow* filter *WORKS Purification of secreted viral antigens from mammalian cells* protocol provides a detailed instructions on how to execute the process under conditions that should provide >90% yield without a comprehensive optimization program. For many customers, this provides the opportunity to rapidly implement the *SmartFlow* technology and realize significant yield improvements over current operations and exceed their internal targets for the process step. Subsequent optimizations studies that use this *SmartFlow* filter *WORKS Purification of secreted viral antigens from mammalian cells* optimization procedure will result in further yield improvements and process efficiencies.

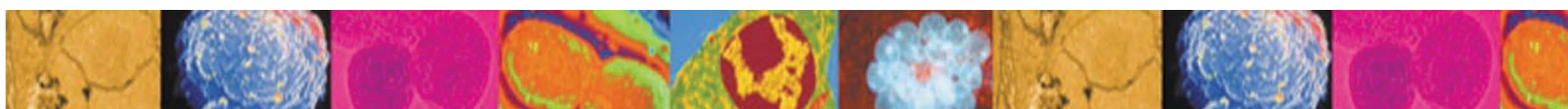


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Each parameter of the TFF process: product, membrane type, shear, pore size, temperature, concentration factor, pH, etc. may impact the viral antigens passage through the membrane. This is why a systematic experimental plan must be developed and executed to optimize a concentration and diafiltration process.

Module and System Selection:

- 1) Select the *SmartFlow*™ filter module to evaluate. The selection requires specifying a combination of membrane type, channel height, and membrane area for a given module that will be tested.
 - a) NCSRT has filtered thousands of solutions and therefore can provide several membrane chemistries and pore sizes that will likely work in the majority of cases. In general the pore size should be 5 to 10 times the size of the molecule to be passed through the membrane and one half to one third the size of a molecule to be retained.
 - b) The combination of the channel height and the fluid velocity through the flow channel created by the recirculation pump produce a shear at the membrane surface. It is the velocity that governs the antigen passage and efficiency. Care must be taken in selecting and maintaining the velocity at the membrane surface.
 - c) The membrane area also affects the pump size required to achieve the necessary velocity for a given separation.
- 2) Select the first membrane to test.
 - a) Recommended starting membranes for isolation of protein from mammalian cells are the 0.45 µm, 0.2 µm, and 0.65 µm modified polysulfone (MPS) membranes or the 0.65 µm polyethersulfone (PES) membranes.
- 3) Select the channel height for the module.
 - a) For the isolation of viral antigens or whole viruses from mammalian cells, a channel height between 0.75 and 0.875 mm is recommended.
 - b) In most cases a channel height of 0.75 mm is recommended because it will require the lowest recirculation rate (and thus the smallest pump) and produce the highest flux rate.
 - c) Cases to use a higher channel height include:
 - i) If cell aggregation is occurring, the lower height channels may clog. If the channel is clogged by aggregates or process particles, the inlet pressure will increase dramatically and the permeate rate will decrease over a short period of time. This will occur usually in the first five minutes.
 - ii) In cases where high solids (greater than 60% WCW) are desired, a channel height of 0.875 mm or above will be necessary.
- 4) Select the membrane area.
 - a) The membrane area depends upon the batch size to be processed. For filtration process development trials, usually the smallest size membrane and thus the smallest batch size is desired.
 - b) For cell harvests an important parameter is the membrane capacity or LM ratio. The LM ratio is defined as the volume of starting material divided by the membrane area.
 - c) The range of LM ratios for the isolation of the secreted viral antigens we have observed varies from 30 to 120 LM.
 - i) The typical starting ratio for mammalian cell harvest is 60 LM. See below for additional information on optimizing the LM ratio.



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- ii) The minimum batch size is the system hold up volume times the concentration factor. For a continuous diafiltration, the minimum batch size is simply the concentration factor.
- d) The membrane area needed is the batch size divided by the LM ratio.
- 5) Select the velocity.
 - a) The typical velocity for the isolation of secreted viral antigens from mammalian cells ranges from 50 cm/sec to 150 cm/sec.
 - b) The typical starting shear rate for a process development run is 100 cm/sec.
 - c) The benefit of increasing the velocity is an increased in antigen passage.
 - d) The disadvantages of increasing the velocity beyond the optimum are:
 - i) Higher pump costs due to higher recirculation flow rates.
 - ii) Higher velocity and higher transmembrane pressures (TMPs) which may decrease the passage of the viral antigens or whole viruses.
- 6) Calculate the flow rate needed to operate the selected module at the selected velocity using the WORKS™ Scale-UP LPM GPM spreadsheet. Ensure that a pump is available that can produce this flow rate at the needed pressure. If a suitable size pump is not available, consider either running a smaller trial or calling NCSRT to determine if a suitable size pump is available.
- 7) Use Table 1 to determine the module(s) part numbers for ordering.

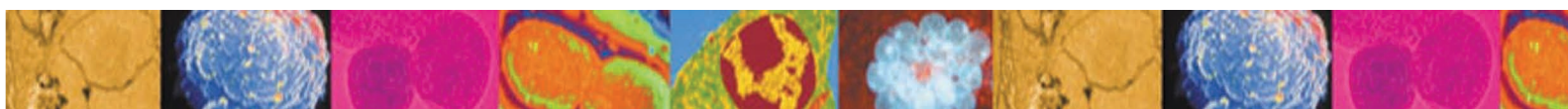
Table 1: SmartFlow™ filter module part numbers

Module Size	Channel Height	Membrane polymer and pore size
74 100 ft ² Optisep® 11000	D 0.5 mm	1N-9045 MPS 0.45 µm
72 50 ft ² Optisep 11000	E 0.75 mm	1N-9020 MPS 0.2 µm
71 10 ft ² Optisep 11000	G 0.875 mm	1N-9065 MPS 0.65 µm
41 10ft ² Optisep 7000	H 1 mm	24-9065 PES 0.65 µm
40 5 ft ² Optisep 7000	J 1.5 mm	
52 2 ft ² Optisep 3000		
51 1 ft ² Optisep 3000		

Filter Operation:

- 1) After loading the filter modules and making all the connections, the first step is to perform a water and/or buffer rinse of the system directing the permeate to the waste.
- 2) After the rinse, direct the permeate line back to the retentate tank so no concentration occurs prior to establishing the desired shear rate.

IMPORTANT: Do not permit the permeate line to come in contact with the retentate fluid. This can contaminate the permeate pool in later samples.
- 3) Slowly increase the flow rate of the recirculation pump to the calculated rate from step 6 above.
- 4) Start with the backpressure at zero. The inlet pressure should be at least 4 psig (0.3 bar) and remain below 10 psig (0.7 bar). If the inlet pressure is above 10 psig (0.7 bar), the recirculation flow rate should be reduced such that the inlet pressure remains below 10 psig (0.7 bar). If the inlet pressure is less than 4 psig, then slight backpressure can be added until the inlet pressure increases to 4 psig. Table 2 provides typical TMP values for the different membranes used in SmartFlow™ TFF filter modules.
- 5) Begin Concentrating.
 - a) Remove the permeate lines from the retentate tank and place them in the permeate vessel.



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- b) Wait until about 5% of the starting retentate volume has passed through the membrane to the permeate to take the initial samples of the retentate and permeate.
- c) Take permeate and retentate samples when each additional concentration factor is reached.
- d) When each sample is taken, record the permeate flow rate using a graduated cylinder, scale, or flow meter.
- 6) Once the desired concentration factor is reached, record the volume remaining in the retentate.
 - a) The remaining volume in the retentate can be calculated by subtracting the permeate volume and retentate volume samples from the starting volumes.

$$\text{Retentate Volume} = \text{Starting Volume} - \text{Permeate Volume} - \text{Retentate Sample Volume}$$

- 7) Diafiltration – the following describes the procedure for diafiltering the product 3x:
 - a) Start to monitor the permeate volume with a graduated cylinder or scale.
 - b) To start the diafiltration, add 5 to 15% of the retentate volume to the retentate tank.
 - c) When the permeate volume has increased by the volume added in step b, take a retentate sample from the retentate tank and a permeate sample directly from the permeate hose simultaneously. When each sample is taken, record the permeate flow rate using a graduated cylinder, scale, or flow meter.
 - d) Continue to add buffer at a rate equal to the permeate rate in aliquots equal to between 5 and 15% of starting retentate value. Continue until 3 times the total retentate volume has been added to the system.
 - e) Take samples from the permeate hose and retentate tank when each diafiltration factor is reached. (i.e. take a sample when the permeate volume is equal to a multiple of the retentate volume such as 1X, 2X, etc.)
 - f) For other diafiltration factors, continue the process until the amount of diafiltration buffer equals number of desired diafiltration factor times the system volume recorded in step 6.
 - i) The theoretical recovery from a 3X diafiltration for a molecule with a 100% passage is 95%.
 - ii) Increasing the diafiltration factor will increase the yield especially when the target molecule has low passage. However, the cost of increasing the diafiltration volume is that the process time will be greater and a larger supply of buffer will be needed.
 - iii) Decreasing the diafiltration factor will decrease the yield. However, for molecules with high passage and low value, the small decrease in the yield may be worth the faster processing time and the saving on buffer.

Data Analysis:

Sample Analysis:

- 1) Check the permeate samples for cells.
- 2) Calculate the membrane flux rate or LMH (L/m²/h) by dividing the measured permeate flow rate at each sample by the membrane area.

$$\text{LMH} = \text{Permeate Flow Rate (mL/min)} * \frac{1\text{L}}{1000\text{mL}} * \frac{60\text{min}}{1\text{hr}} \div \text{Membrane Area (m}^2\text{)}$$

- 3) Calculate the instantaneous viral antigen percent passage by dividing the permeate viral antigen content by the retentate viral antigen content and multiplying by 100.
- 4) Record the data on the Membrane Test Worksheet.



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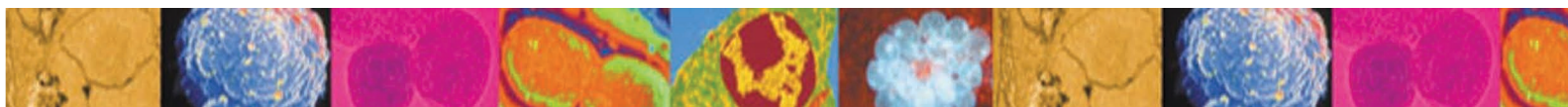
Process Optimization:

The procedure should be repeated under different process conditions to ensure that the optimized conditions are reached.

- 1) The important variables to optimize are the yield, membrane passage, and membrane flux rate.
- 2) An important parameter that affects the yield, passage, and flux rate for cell harvest is the membrane capacity or LM ratio.
- 3) Increasing the LM ratio decreases membrane performance, which increases processing time and decreases membrane costs. If membrane performance suffers greatly, then saving a little bit on membrane will not offset the costs in higher processing time.
- 4) Decreasing the LM ratio increases the membrane performance and increases membrane costs. Increasing membrane performance may decrease the processing time at a small incremental membrane cost, therefore decreasing total cost.
- 5) To find the optimal LM ratio:
 - a) If the current trial was too fast with very high yield, increase the LM ratio by starting with a larger volume of starting material.
 - b) If the current trial was too slow or had a low yield, decrease the LM ratio by starting with a smaller volume of starting material.

Table 2 Typical transmembrane pressure values for SmartFlow modules

Membrane Pore Size	Transmembrane Pressure Starting Value PSIG (Bar)	Transmembrane Pressure Ranges PSIG (Bar)	Cell Harvest Inlet PSIG (Bar) Starting Value	Cell Harvest Outlet PSIG (Bar)
Ultrafiltration Membranes				
1 kDa	75 (5)	90 to 150 (6 to 10)		
5 kDa	45 (3)	60 to 90		
10 kDa	30 (2)	45 to 90 (3 to 6)		
30 kDa	15 (1)	30 to 75 (2 to 5)		
100 kDa	15 (1)	20 to 60 (1.37 to 4)	20 (1.37)	12 (0.83)
300 kDa	10 (0.69)	15 to 45 (1 to 3)	20 (1.37)	10 (0.69)
500 kDa	7.5 (0.5)	10 to 30 (0.7 to 2)	7.5 (0.5)	0
Microfiltration Membranes				
0.1μ	2 (0.13)	4 to 15 (0.27 to 1.0)	4 (0.275)	0
0.2μ	2 (0.13)	4 to 15 (0.27 to 1.0)	4 (0.275)	0
0.45μ	2 (0.13)	4 to 10 (0.27 to 0.69)	4 (0.275)	0
0.8μ	1 (0.07)	1 to 6 (0.07 to 0.41)	2 (0.13)	0
1.0μ	1 (0.07)	1 to 6 (0.07 to 0.41)	2 (0.13)	0
2.0μ	1 (0.07)	1 to 6 (0.07 to 0.41)	2 (0.13)	0
3.0μ	1 (0.07)	1 to 6 (0.07 to 0.41)	2 (0.13)	0



Works™
Protocol

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Procedure

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- 6) The module used is an important optimization parameter. By changing the membrane chemistry or membrane type, optimized flux rates and passage may be found.
- 7) Using the same membrane, the velocity can be optimized by increasing and decreasing the flow rate and measuring the effects on the membrane flux rate and passage. If an increase in the velocity results in a relatively large increase in the passage rate, then the savings in membrane cost will offset the increased energy consumption.
 - a) The concentration factor before starting diafiltration should also be optimized.
 - b) The goal is to begin the diafiltration with the instantaneous passage of at least 50% to increase diafiltration efficiency.

After analysis of the data, select the best performing membrane. The best performing membrane will retain the cells, permit the desired media component to pass into the permeate, and have a high permeate flux.

Conclusion:

This *Purification of secreted viral antigens from mammalian cells with SmartFlow™ TFF WORKS™* Optimization Procedure provides guideline for optimizing the application of NCSRT's *SmartFlow* filters. NCSRT has developed a simple step-by-step protocol that has been proven to deliver >90% product yield in the applications to which it has been applied. Along with the *Purification of secreted viral antigens from mammalian cells with SmartFlow™ TFF WORKS* Protocol, NCSRT has developed a Scale-Up Component listing providing the part numbers and ordering information for the *SmartFlow* filter modules to execute the protocol at all process stages and volumes. To receive the complete application package, please request the *Purification of secreted viral antigens from mammalian cells with SmartFlow™ TFF WORKbook*.

NCSRT's *SmartFlow* filter technology...It *WORKS*.



NCSRT, Inc.
1000 Goodworth Dr.
Apex, NC 27539
USA

Phone: +1 919 387 8460
Fax: +1 919 387 8540
email: info@ncsrt.com
Web: <http://www.ncsrt.com>