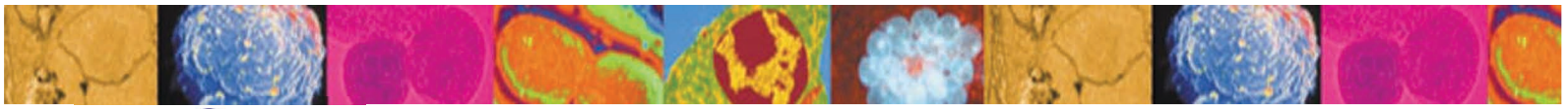


NCSRT

Works™ Optimization Procedure

Separation and clarification of inclusion bodies with *SmartFlow*™ TFF

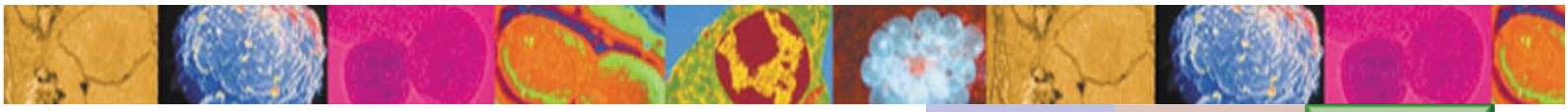
The *SmartFlow*™ filter *WORKS*™ *Separation and clarification of inclusion bodies* Optimization Procedure from NCSRT is intended for isolating inclusion bodies from the lyzated proteins and cell debris. NCSRT isolation techniques work best on inclusion bodies that are 1 micron in size and high quality.

NCSRT has considerable experience with developing *E. coli* processes including cell culture, inclusion body production and inclusion body (protein) isolation and purification. This *Separation and clarification of inclusion bodies* *WORKS* ™ Optimization Procedure is intended provide an efficient and economic alternative to centrifugation as an inclusion body recover procedure. To maximize the recovery of the inclusion bodies NCSRT can assist through its AT *WORKS* in developing the fermentation parameters to improve the inclusion body quality and size.

This TFF optimization procedure uses a microfiltration (MF) membrane that retains the inclusion body and permits the passage of the smaller cell debris. The passage characteristics of the cell components 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 thousands of NCSRT trials. 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.

Three factors are keys to isolating inclusion bodies. First, the size and quality of the inclusion body is very important. The best inclusion bodies are 1 micron and resistant to high pressure lysis and detergent treatments. Second, the homogenization of the cell debris is important. Finally, the concentration and viscosity of the lysate in addition to the operating parameters are very important. This final point is the one factor that NCSRT can control while the first two are part of the process and beyond NCSRT control.



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Each parameter of the TFF process: product, membrane type, shear, pore size, temperature, concentration factor, pH, anti-foam, 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 this shear that governs the separation performance and efficiency. Care must be taken in selecting and maintaining the shear at the membrane surface.
   c) The membrane area also affects the pump size required to achieve the necessary shear rates for a given separation.
2) Select the first membrane to test.
   Recommended starting membranes for isolation of inclusion bodies from E. Coli are the 0.65 µm, and the 0.45 µm modified polysulfone (MPS) membranes.
3) Select the channel height for the module:
   a) For the isolation of inclusion bodies, 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 a 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 inclusion body recovery, 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 inclusion bodies we have observed varies from 25 to 50 LM.
      i) The typical starting ratio for inclusion body isolation is 25 LM.
      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 hold up volume.
   d) The membrane area needed is the batch size divided by the LM ratio.
5) Determine the shear rate.
   a) The typical shear rate for the isolation of inclusion bodies is 10,000 sec^{-1}, with a range from 3,000 sec^{-1} to 16,000 sec^{-1}.

6) Calculate the flow rate needed to operate the selected module at the selected shear rate using the NCSRT 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 in 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 ft²	Optisep 11000	D	0.5 mm	1N-9065	MPS 0.65 µm
72	50 ft²	Optisep 11000	E	0.75 mm	1N-9045	MPS 0.45 µm
71	10 ft²	Optisep 11000	G	0.875 mm	1N-9020	MPS 0.2 µm
41	10ft²	Optisep 7000	H	1 mm		
40	5 ft²	Optisep 7000	J	1.5 mm		
52	2 ft²	Optisep 3000				
51	1 ft²	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) Perform the standardization of starting material steps:

a) This standardization takes into account that each type of cell fermentation is slightly different and thus the material may need to be diluted in some cases to permit operation at the optimal conditions.
Note: Do NOT use DI water for the dilution. Lysis or other suitable buffer should be used.

b) Add the starting volume calculated in step 4 above or determined from the Process Optimization procedure on page 5.

c) Start the pump and slowly increase the pump speed up to the desired recirculation rate, which was calculated in step 6 above. As the pump speed is increasing, note that the pressure does not exceed 12 PSIG (0.8 bar). If the pressure reaches 12 PSIG, add additional lysis buffer to the tank diluting the cell lysate and decreasing the pressure. Remember to record the total volume of buffer added to the solution.

d) For subsequent trials, use the same dilution factor as was determined above.

e) Calculation of Dilution factor:

i) Divide the quantity of buffer added to the starting volume of cells

ii) If you added 0.8 L of buffer to 1.5 L of lysate, your dilution is 0.53 parts buffer per 1 part lysate.

NOTE: The viscosity stabilization procedure needs to be performed quickly and concisely in order to continue the experiment with the inserted membrane. Any elongation of time for this procedure beyond two or three minutes will clog the membrane and render the protein passage values as non-representative.

4) Diafiltration of the inclusion body – the following describes the procedure for diafiltering the product 5x:

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a) Remove the permeate line from the retentate tank and start to monitor the permeate volume with a graduated cylinder or scale.
b) To start the diafiltration, add 5 to 15% of the starting 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. Record the permeate flow rate using a graduated cylinder, scale, or flow meter when each sample is taken.
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 5 times the total starting 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 to 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 5X diafiltration for a molecule with a 100% passage is 99%.
   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 an increased quantity of buffer will be needed or consumed.
   iii) Decreasing the diafiltration factor will decrease the yield. However, for molecules with high passage and/or 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) Spin the retentate samples to pellet the inclusion bodies and assay the protein levels.
2) Spin the permeate samples and check that there is no inclusion bodies present. Inclusion bodies will be a "white" pellet and debris will be a "yellowish" pellet. Assay the spun permeate samples for protein concentration.
3) Calculate the instantaneous protein percent passage by dividing the permeate protein content by the retentate protein content and multiplying by 100.
   a) The theoretical case will see the passage decrease as the experiment continues.
   b) Another common result is the passage increasing to a maximum between the second and third diafiltration volumes.
4) Divide the protein content of each permeate sample by the protein content of the permeate pool sample taken at the same time and multiply by 100. This is a comparison of the instantaneous protein passage versus the average protein passage. This will show you if protein passage is increasing with diafiltration or decreasing.
5) Use a protein gel analysis to determine the purity of the inclusion bodies and if any inclusion bodies are passing into the permeate.
6) Calculate the membrane flux rate or LMH ($L/m^2/h$) by dividing the measured permeate flow rate at each sample by the membrane area.
7) Record the data on the Membrane Test worksheet.

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Typical Results:

1) Good inclusion body performance is a membrane flux rate of 20 to 40 LMH with a 5X diafiltration to remove 90% of the contaminating proteins.
2) Excellent inclusion body performance is a membrane flux of 60 LMH or higher with a 4X diafiltration to remove 90% of the contaminating proteins.
3) A good LM ratio for viscosity adjusted material (i.e. diluted) is between 25 to 50.
4) An excellent LM ratio would be any ratio above 50.
   NOTE: The membrane flux rate can be increased by changing the volume to membrane area ratio.

Process Optimization:

The above steps should be repeating 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 effects the yield, passage, and flux rate for cell harvest is the membrane capacity or LM ratio.
   a) 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.
   b) Decreases the LM ratio increase 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.
   c) To find the optimal LM ratio:
      i) If the current trial was too fast with very high yield, increase the LM ratio by starting with a larger volume of starting material.
      ii) 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.
3) The module used is an important optimization parameter. By changing the membrane chemistry or membrane type, optimized flux rates and passage may be found. Typically the 0.2 µm MPS membrane is less cloudy than the 0.2 µm PVDF or 0.45 µm MPS membranes.
4) The optimal membrane will have a high flux, pass over 90% of the contaminating protein, and retain the inclusion body.

Inclusion Body Stability:

1) Normally inclusion bodies are extremely robust and are not damaged by shear rates above 40,000 sec^{-1}.
2) In the rare cases when there are problems with inclusion body stability, the shear rates can be lowered to 6,500 sec^{-1}.
3) In the cases of inclusion body instability, NCSRT recommends a development contract to optimize the process including E. coli cell culture, inclusion body production and inclusion body (protein) isolation and purification.

If the starting material at a shear rate of < 30,000 sec^{-1} had an inlet pressure between 4 and 8 PSIG (0.3 to 0.6 Bar) then several alternative processes can be optimized. Please consult with NCSRT directly concerning methods such as diafiltration, concentration, diafiltration and concentration, diafiltration, concentration after performing the initial screens described herein.

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Conclusion:

This *SmartFlow*™ filter *Separation and clarification of inclusion bodies* WORKS™ Optimization Procedure provides guideline for optimizing the application of NCSRT's *SmartFlow* filters. Additional documents to assist you in developing an optimized separations protocol include the *SmartFlow* worksheet and the NCSRT Scale Up calculator. To receive the complete application package, please request the *Separation and clarification of inclusion bodies* WORKbook.

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

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