

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

Approvals:

Preparer: Jason McMillan & Dr. David Frank

Date: 15APR15

Reviewer: Dr. Maggie Bryans

Date: 15APR15

1. Purpose:

- 1.1. To harvest t-PA-containing conditioned medium and prepare for purification of t-PA by TFF concentration and pH adjustment; intermediate steps include centrifugation to remove cells prior to tangential flow filtration and to pellet precipitated protein following pH adjustment.

2. Scope and Applicability:

- 2.1. A biomanufacturing environment requires proper steps to recover and purify active pharmaceutical ingredient from a bioreactor or fermentor. This SOP provides bench scale procedures to accomplish that goal using conditioned medium from cells expressing recombinant tissue-type plasminogen activator. The method demonstrates the principles of tangential flow filtration, centrifugation, and pH dependent protein precipitation in preparation for downstream processing by column chromatography as may be employed in a typical process development, for later scale up to manufacturing.

3. Summary of Method:

- 3.1. Preparation of solutions:
 - 3.1.1. PBS/Tween 80 for preconditioning of the Pellicon cassette (for TFF)
 - 3.1.2. 0.1 N NaOH for cleaning the Pellicon cassette following use
 - 3.1.3. 0.05 N NaOH for storage of the Pellicon cassette
- 3.2. Flushing and preconditioning of TFF/Pellicon.
- 3.3. Transfer of culture from bioreactor to centrifuge bottles.
- 3.4. Centrifugation to pellet cells.
- 3.5. Transfer of conditioned medium (CM) from centrifuge bottle to storage vessel/bottle.
- 3.6. Addition of protease inhibitors and Tween 80.
- 3.7. Concentration of supplemented CM by tangential flow filtration.
- 3.8. Adjustment of pH of concentrated CM.
- 3.9. Centrifugation.

4. References:

- 4.1 Millipore Tangential Flow and Diafiltration Using Pellicon XL Device of tPA SOP
- 4.2 Oakton PC 700 Bench Series pH/Conductivity/°C/°F Meter SOP (Doc # 1.0).

5. Definitions:

- 5.1. Permeate- the material that passes through the membrane.
- 5.2. Retentate- the material that does not pass through the membrane.
- 5.3. TFF – tangential flow filtration
- 5.4. CM – conditioned medium, which contains the API of interest

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

6. Precautions:

- 6.1. 0.1N NaOH is very corrosive. It is extremely damaging to eyes and mucous membranes. It causes burns. Avoid contact with skin. It is harmful if swallowed or inhaled. The Millipore Pellicon XL Device is stored flat at 4-25°C with 10ml of 0.1N NaOH.
- 6.2. Acetic acid vapor is extremely irritating to the airways upon inhalation, and should therefore be used only in the fume hood.
- 6.3. NEVER tighten the clamp enough to completely restrict the flow in the Retentate tube. This could damage the filter and cause the tubing to disconnect.
- 6.4. Luer Lock fittings on the TFF device should be tightened with care not to exert too much force, to avoid stripping threads or damaging the fitting.

7. Responsibilities:

- 7.1 It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
- 7.2 It is the responsibility of the students/technician to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

8. Equipment and Materials:

- 8.1. 250 ml Nalgene centrifuge bottles (3)
- 8.2. 250 ml Corning bottles (3)
- 8.3. 0.1N NaOH (sodium hydroxide)
- 8.4. 0.05N NaOH (sodium hydroxide)
- 8.5. acetic acid, glacial
- 8.6. 10% (w/v) Tween 80
- 8.7. NaH_2PO_4 (sodium phosphate monobasic, anhydrous), or pre-made PBS (phosphate buffered saline)
- 8.8. $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (sodium phosphate dibasic, heptahydrate), or pre-made PBS
- 8.9. preconditioning buffer (PBS containing 0.1% Tween 80)- 50 ml
- 8.10. Stock solutions of protease inhibitors:
 - 8.10.1. 10 mg/ml PMSF (phenylmethylsulfonylfluoride); 250X
 - 8.10.2. Leupeptin, 2 mg/ml; 4000x
 - 8.10.3. Aprotinin, 10 mg/ml, 5000x
- 8.11. pH Meter and pH paper
- 8.12. 2 magnetic stir plate and stir bars
- 8.13. Millipore Tangential Flow Filtration System and Pellicon XL Device and Accessories
- 8.14. MilliQ Water
- 8.15. 10ml graduated cylinder
- 8.16. 25 ml beaker

9. Procedure:

9.1. Preparation and Set Up

- 9.2. Preparation of solutions (provided).
 - 9.2.1. Prepare 0.1N NaOH for cleaning.
 - 9.2.1.1. Using a 1L graduated cylinder, measure 1L of MilliQ water.
 - 9.2.1.2. Transfer water to a 1L flask.

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

- 9.2.1.3. Weigh 4.0 ± 0.05 g of NaOH.
- 9.2.1.4. Transfer NaOH to flask.
- 9.2.1.5. Add magnetic stir bar and stir to dissolve.
- 9.2.1.6. Sterile filter the solution and label container: 0.1N NaOH, [date], [initials], [group number], Storage: room temp, Disposal: adjust to pH 7 then drain.
- 9.2.2. Prepare 0.05N NaOH for Pellicon XL Device Storage
 - 9.1.2.1 Using a 10 ml graduated cylinder, measure 5 ml of MilliQ water
 - 9.1.2.2 Transfer MilliQ water to 25 ml beaker
 - 9.1.2.3 Using a 10 ml graduated cylinder, measure 5 ml of 0.1N NaOH
 - 9.1.2.4 Transfer 5 ml of 0.1N NaOH to 25 ml beaker
 - 9.1.2.5 Add magnetic stir bar and stir to dissolve.
 - 9.1.2.6 Sterile filter the solution and label container: 0.05N NaOH, [date], [initials], [group number], Storage: room temp
- 9.3. ***Labscale 500ML Reservoir Set Up***
- 9.4. Flushing and preconditioning of TFF/Pellicon. One should become familiar with the location of ports and tubing connection points as shown in the attachments at the end of this SOP prior to beginning setup.
 - 9.4.1. Set up the apparatus and confirm that all tubing connections are secure, according to the SOP (Millipore Tangential Flow and Diafiltration Using Pellicon XL Device of tPA SOP).
 - 9.4.2. Remove the 4 plugs on the Pellicon cassette and attach the Pellicon cassette to the Labscale apparatus.
 - 9.4.3. Add 500 ml MilliQ water to the reservoir and flush the cassette as described in section 9.4.4.
 - 9.4.4. ***Flushing the Pellicon cassette.***
 - 9.4.4.1. Disconnect retentate silicone (translucent) tubing from RET IN port and place end of retentate tubing in waste collection vessel.
 - 9.4.4.2. Place end of permeate silicone (translucent) tubing into waste collection vessel. Open retentate valve by turning the counterclockwise.
 - 9.4.4.3. Remove the reservoir cover and fill reservoir with 500 ml of MilliQ water. Remove the plug from VENT port and open tank outlet valve.
 - 9.4.4.4. Turn the pump on and increase the speed until the feed pressure gauge reads 1.38 Bar (20 psi).
 - 9.4.4.5. Continue pumping to the waste collection vessel until the level in the reservoir drops to 350 ml and then turn the pump off.
 - 9.4.4.6. Reconnect the retentate silicone (translucent) tubing to the RET IN port and turn the pump on. Slowly increase the pump speed until feed pressure gauge reads 1.38 Bar (20 psi). Check the system for leaks and tighten connections if leaks are found.
 - 9.4.4.7. Adjust retentate valve restriction by slowly turning retentate valve clockwise until the retentate pressure gauge reads 0.69 Bar (10 psi).

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

- 9.4.4.8. Adjust pump speed and retentate valve restriction to achieve 2.07 Bar (30 psi) feed pressure and 0.69 Bar (10 psi) retentate pressure.
- 9.4.4.9. Allow to run until 50 ml remains in the chamber.
- 9.4.4.10. Disconnect the pump outlet (Sta-pure, white) tubing from the pump outlet port and place in waste collection vessel.
- 9.4.4.11. Disconnect the retentate silicone (translucent) tubing from the RET IN port. Fluid should now drain by gravity. If additional drainage is required, a syringe can be placed on the end of the retentate tube and fluid can be blown down.
- 9.4.4.12. Remove the remainder of water in the chamber as follows: Replace retentate tubing (silicone, translucent) in retentate port. Reconnect pump outlet tubing (Sta-Pure, white).
- 9.4.4.13. Disconnect FEED IN tubing and place in collection vessel. Open tank outlet valve, turn pump speed up to drain reservoir.
- 9.4.4.14. Reconnect the pump outlet tubing (Sta-Pure, white) to the Feed In port.

9.5. Pre-conditioning

- 9.5.1. Place end of permeate tubing silicone (translucent) in the waste collection vessel.
- 9.5.2. Remove reservoir cover and fill the reservoir with 50 ml of PBS containing 0.1% Tween 80 (or other appropriate buffer) and then remove the Vent port plug.
- 9.5.3. Open the tank outlet valve. Turn the pump on and increase the pump speed until the feed pressure gauge reads 1.38 Bar (20 psi at its maximum; the needle will pulse as the pump turns). Check all system connections for leaks and tighten any connections as necessary.
- 9.5.4. Continue pumping to the waste collection vessel until the level in the reservoir drops to the bottom of the reservoir stir bar well making sure to stop the pump before air is pumped into the system. Turn the pump off.

9.6. Transfer of culture from bioreactor to centrifuge bottles.

- 9.6.1. Refer to the SOP: Applikon ez-Control Bioreactor Controller Operation for instructions on removing the headplate of the bioreactor, providing access to the cells and conditioned medium.
- 9.6.2. Transfer the culture to three 250 ml centrifuge bottles using a 50 ml pipet and PipetAid. Residual culture can be transferred to an Erlenmeyer flask for temporary storage.
- 9.6.3. Centrifuge cells in pre-chilled Sorvall centrifuge, fitted with a SLA1500 rotor, at 500 x g for 5 min, 4 degrees C.
- 9.6.4. Transfer conditioned medium (CM) from centrifuge bottle to storage vessel/bottle by carefully decanting the supernatant to appropriately labeled 250 ml Corning bottles.
- 9.6.5. Add protease inhibitors and Tween 80 as follows. To each 250 ml bottle of CM supernatant, add 1 ml 10mg/ml PMSF, 50 µl of 10 mg/ml Aprotinin stock and 62.5 µl 2 mg/ml Leupeptin stock. Also add 2.5 ml 10% Tween 80 (final concentration will be 0.1%).

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

9.7. Concentrate the Sample

- 9.7.1. Remove the reservoir cover and fill the reservoir with tPA sample (up to 500 ml) to be concentrated.
- 9.7.2. Ensure the vent port is open by removing the plug from the VENT port and leaving it open or installing a Millex Filter if required. Open the tank outlet valve.
- 9.7.3. Turn the pump on and increase the pump speed until the feed pressure gauge reads 1.38 Bar (20 psi). Check all system connections for leaks and tighten any connections as necessary.
- 9.7.4. Adjust the retentate valve restriction by slowly turning the retentate valve clockwise until the retentate pressure gauge reads 0.69 Bar (10 psi).
- 9.7.5. Adjust the pump speed and retentate valve restriction to achieve desired feed retentate pressures [2.07 Bar (30 psi feed / 0.69 Bar (10 psi) retentate)]. Do not exceed 4.14 Bar (60 psi) feed pressure.
- 9.7.6. Filter the solution until the desired volume is reduced 10 fold or greater, but ideally down to about 20 ml.
- 9.7.7. Turn off the pump and empty the permeate container into a large bottle with a cap and label as: tPA Permeate Waste, disposal; bleach then drain, [initials], [date].

9.8. Retrieve the Sample

- 9.8.1. Disconnect the pump outlet tubing (Sta-Pure, white) from pump outlet port and place in product recovery collection vessel (beaker with small stir bar is preferred; or 50 ml tube).
- 9.8.2. Disconnect the retentate tubing (silicone, translucent) from the retentate in port and open back pressure regulation valve (turn counterclockwise). Fluid should now drain by gravity.
- 9.8.3. When drainage ceases, rinse the Pellicon innards by injection of 5 ml PBS/0.1% Tween 80 from the retentate tube using a 10 ml syringe. Additional drainage is required; a syringe can be placed on the end of the retentate tube and fluid can be blown down.
- 9.8.4. Replace retentate tubing (silicone, translucent) in retentate port. Reconnect pump outlet tubing (Sta-Pure, white).
- 9.8.5. Disconnect FEED IN tubing and place in collection vessel. Open tank outlet valve, turn pump speed up to drain reservoir.
- 9.8.6. Stop the pump, close the outlet valve, and add 5 ml PBS/Tween80 to the chamber to rinse sides and effect collection of residual tPA. Pipet the solution along the walls repeatedly to rinse, then collect and transfer to the collection vessel.
- 9.8.7. Reconnect the pump outlet tubing (Sta-Pure, white) to the Feed In port.
- 9.8.8. Label the recovery collection vessel Concentrated tPA, [date], [initials].

9.9. Adjustment of pH of concentrated Conditioned Medium (CM).

Glacial acetic acid is irritating to the airways upon inhalation. Therefore perform the following operation in the fume hood with the sash as low as is comfortably possible to work.

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

- 9.9.1. Place the beaker containing concentrate on a stir plate, insert pH probe and monitor pH.
- 9.9.2. Add glacial acetic acid dropwise to the concentrated tPA to reduce the pH to 5.0 +/- 0.1.
- 9.9.3. Transfer the mixture to the minimum number of Oak Ridge centrifuge tubes necessary to contain it (capacity of each tube is about 40 ml).
- 9.9.4. Leave the mixture on ice for at least 30 min (overnight at 4°C is acceptable, and offers a convenient stopping point) to allow formation of precipitate.
- 9.10. ***Clarification of pH 5.0 Solution by Centrifugation***
 - 9.10.1. Centrifuge pH 5.0 mixture at 10,000 x g for 10 minutes, 4°C.
 - 9.10.2. Transfer the supernatant to appropriately sized tube(s) labeled tPA-TFF/pH 5.
 - 9.10.3. Store in 2°C-8°C refrigerator for use in further purification steps.
- 9.11. ***Flushing***
 - 9.11.1. To begin cleaning the Millipore TFF apparatus and Pellicon filter, repeat Flushing as described in 9.4.4
- 9.12. ***Cleaning the Labscale TFF/Pellicon cassette.***
 - 9.12.1. Disconnect the retentate tubing (silicone, translucent) from RET IN port and place in waste collection vessel. Place the end of the permeate tubing in the waste collection vessel.
 - 9.12.2. Open the retentate valve by turning it counterclockwise.
 - 9.12.3. Remove the reservoir cover and fill with 500 ml of 0.1N NaOH. Ensure the vent port is open by removing the plug from the VENT port and either leave open or install a Millex Filter.
 - 9.12.4. Open the tank outlet valve.
 - 9.12.5. Turn the pump on and increase the pump speed until the feed pressure gauge reads 1.38 Bar (20 psi). Check all system connections for leaks and tighten any connections as necessary.
 - 9.12.6. Continue pumping to the waste collection vessel until the level in the reservoir drops to 250 ml and then turn the pump off. Reconnect the retentate (silicone, translucent) tubing to the RET IN port.
 - 9.12.7. Connect the male luer end of the permeate tubing to the recirculation (DIA / RECIRC) port. Turn the pump on and increase the pump speed until the feed pressure gauge reads 1.38 Bar (20 psi). Check all system connections for leaks and tighten any connections as necessary.
 - 9.12.8. Adjust the retentate valve restriction by slowly turning the retentate valve clockwise until the retentate pressure gauge reads 0.69 Bar (10 psi). Adjust the pump speed and retentate valve restriction to achieve 2.07 Bar (30 psi) feed pressure and 0.69 Bar (10 psi) retentate pressure.
 - 9.12.9. Recirculate the cleaning solution for 30-60 minutes and then turn the pump off.
- 9.13. ***Drain the System***
 - 9.13.1. Disconnect the pump outlet (Sta-pure, white) tubing from the pump outlet port and place in waste collection vessel.

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

9.13.2. Disconnect the retentate silicone (translucent) tubing from the RET IN port. Fluid should now drain by gravity. If additional drainage is required, a syringe can be placed on the end of the retentate tube and fluid can be blown down.

9.14. *Flushing*

9.14.1. Repeat Flushing as described in 9.4.4.

9.15. *Pellicon XL Device Storage*

9.15.1. Turn all of the lock nuts until you are able to remove the Pellicon XL Device.

9.15.2. Fill a 10 ml syringe with 0.05N NaOH Storage solution.

9.15.3. Place the cassette in sink or tray that can contain any overflow. Attach the syringe to the retentate port and slowly push the solution into the device. Remove the syringe and replace all of the plugs on the ports and store flat at 4°C-25°C.

9.16. *Clean Base*

9.16.1. Disconnect the power cord.

9.16.2. Clean exterior surfaces, reservoir, and Labscale System Base with a mild soap and water solution.

10. Attachments:

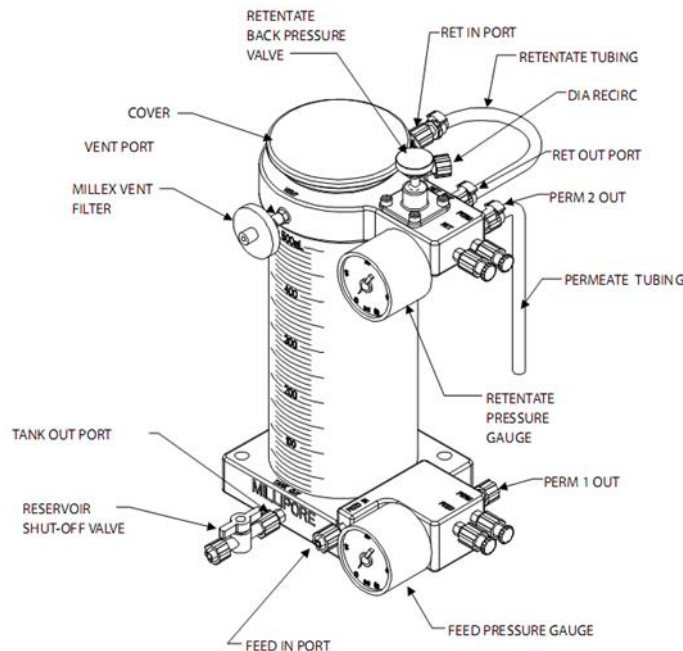


Figure 1: Reservoir Set Up

<http://www.millipore.com/userguides.nsf/docs/p60085>

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

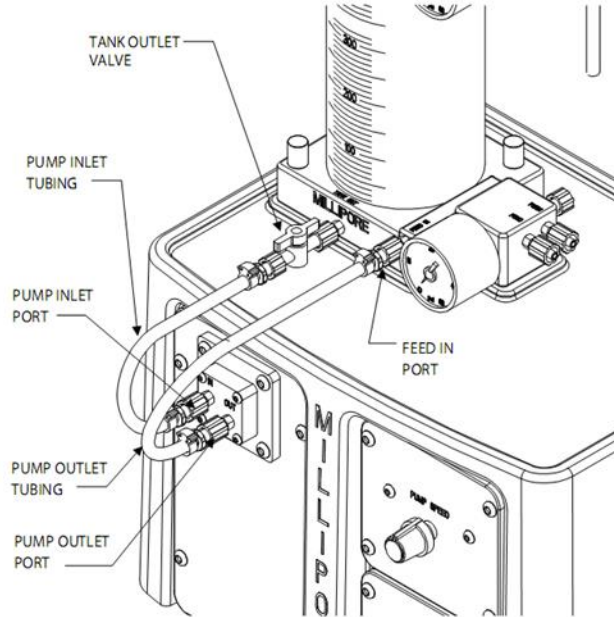


Figure 2: Pump Base Set Up

<http://www.millipore.com/userguides.nsf/docs/p60085>

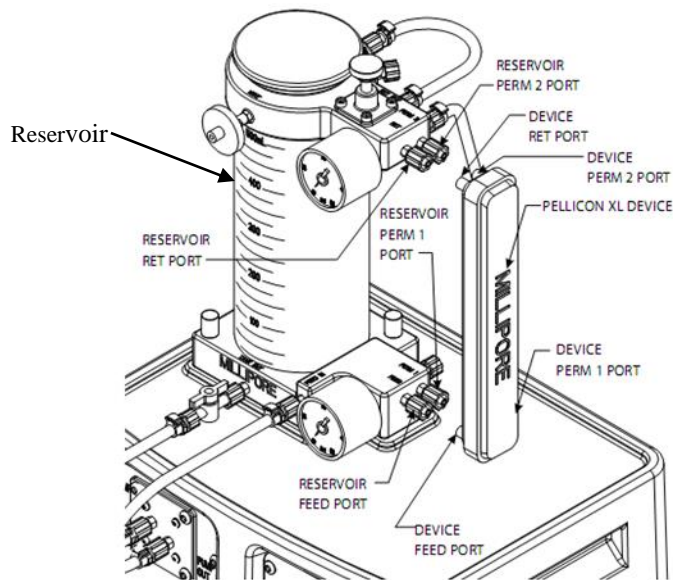


Figure 3: Installation of Pellicon XL Device

<http://www.millipore.com/userguides.nsf/docs/p60085>

SOP: End-of-Run tPA Process: Harvest, Centrifugation, Concentration, pH Adjustment

11. History

<i>Revision Number</i>	<i>Effective Date</i>	<i>Preparer</i>	<i>Description of Change</i>
0	07JAN14	Jason McMillan	Initial release