SOP: Lysine Affinity Chromatography of t-PA on the ÄKTApure Chromatography System

Approvals

Preparer: David Frank Date: 17May16
Reviewer: Dr. Margaret Bryans Date: 20May16

1. Purpose

1.1. This procedure describes the operating steps necessary to purify recombinant tissue-type plasminogen activator from either CEX-purified enzyme or concentrated conditioned cell culture medium, using L-Lysine affinity chromatography on the AKTA pure.

2. Scope and Applicability

2.1. Applies to purification of t-PA from partially purified fractions from cation exchange chromatography contining t-PA, or from prepared conditioned medium, which has been concentrated by TFF. The method employs a L-Lysine-Ceramic HyperD 2.1 ml column installed on the GE ÄKTApure Chromatography System and controlled by Unicorn 6.3 software.

3. Summary of Method

3.1. Preparation of buffer(s)
3.2. Equilibration of system and column
3.3. Fraction collector setup
3.4. Application of sample
3.5. Washing and elution of column
3.6. Regeneration of system in preparation for subsequent run
3.7. Procedures for short or long term storage of the system

4. References

4.1. Unicorn 6.3 Users Guide (electronic)
4.2. AKTApure 25 Users Guide (electronic)
4.3. L-Lysine-Ceramic HyperD chromatography media information booklet
4.4. Batch Record for Downstream Processing of t-PA: TFF Operation

5. Definitions

N/A

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6. Precautions

6.1. Routine care should be exercised in handling of buffers and samples of biological materials, which may have harmful biological activity in the case of accidental ingestion, needle stick, etc.

6.2. User should read and be familiar with general good practice as outlined in the AKTApure Cue Cards

6.3. Avoid damaging the threads through the use of excessive force when connecting plastic fasteners.

6.4. It is imperative that pumps never be allowed to run dry. Care must be taken to avoid air in the fluid path, which could damage the pumps or give spurious and uninterpretable readout from the UV and/or conductivity detectors.

6.5. Gloves and protective eyewear should be worn when handling samples and reagents (buffers), however it is preferable that the user remove gloves prior to entering commands via the computer keyboard or mouse.

6.6. Buffers must be degassed and filtered prior to use with the AKTApure instrument. Samples displaying any turbidity should be centrifuged at 10000x*g* for 5 min; all samples must be passed through a 0.2µm filter before injection/introduction into the fluid path.

6.7. Equipment calibration check: The AKTApure system calibration is automatic; baseline for measurements of A280 and conductivity are zeroed at the beginning of a chromatography run. The pH meter requires calibration, however pH monitoring is not included in this SOP. If pH monitoring is desired, the calibration method is described in the AKTA pure Equipment SOP.

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. AKTApure chromatography system

8.2. Additional Lab Equipment: pH meter, balance

8.3. Lab Utensils: Beakers (250, 500ml, 1000 ml), 1 liter and 500 ml graduated cylinders

8.4. Reagents: Sodium phosphate monobasic monohydrate (137.99 gm /mol), sodium phosphate dibasic heptahydrate (268.07 gm /mol), sodium chloride, arginine hydrochloride, filtered deionized water (MilliQ or similar). 10 % w/v Tween 80, 20% ethanol, NaOH

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8.5. Lab Supplies: Filters (0.2 μm) and bottles for vacuum filtration and degassing of all chromatography buffers. Syringe (10 ml). Tubes for fraction collector.

9. Procedure

9.1. ***Sample Collection and Preparation*** Please refer to the batch record of a previous run of tPA on HiTrap SP column (Batch Process Record: tPA Production from CHO Cells: Chromatography Operation). The operator will require 10 ml of sample per sample injection (see below).

9.1.1. Obtain fractions containing t-PA, partially purified by cation exchange chromatography.[***Option***]: Alternatively, TFF-concentrated conditioned medium from t-PA expressing cells grown in low serum may be used. This is ready to load on the column without further concentration or buffer exchange; however it will require filtration. Skip to step 9.1.6

9.1.2. Dilute the t-PA fractions to a volume of 15 ml with buffer A.

9.1.3. Concentrate the t-PA using a Millipore Centricon 15 (30 kDa cutoff), centrifuging at 3500 x *g* until the concentrate volume is about 1 ml; about 30 min.

9.1.4. Dilute the concentrate to 15 ml with buffer A and repeat concentration.

9.1.5. Recover the concentrate, rinsing the membrane/concentrator with buffer A (combine rinses with concentrate and mix).

9.1.6. Filter the t-PA solution through a 0.2 μm syringe mounted filter.

9.2. ***Reagent Preparation***: Binding of t-PA to the Lysine affinity ligand is performed in sodium phosphate buffer. Nonspecifically bound protein is eluted with 0.8 M NaCl in the same buffer (80% buffer B1). Elution of t-PA from the column is effected with 0.2M arginine in sodium phosphate buffer.

9.2.1. Sodium Phosphate Buffer: 50mM Sodium Phosphate, pH 7.5, 0.01% Tween 80

9.2.1.1. Dissolve 6.90 gm sodium phosphate monobasic monohydrate (137.99 gm/mol), in 950 ml filtered deionized water in a one liter beaker, with stir bar.

9.2.1.2. Add 1.0 ml 10% Tween 80.

9.2.1.3. Titrate the pH to 7.5 with NaOH.

9.2.1.4. Adjust the final volume to 1000 ml. Set aside 500 ml of this solution and label ‘Buffer A’ along with its precise composition and date of preparation. Filter and degas the buffer by passage through a vacuum filter device attached to house vacuum, leaving the filtered solution under vacuum for 15-20 minutes.

9.2.1.5. Use the remaining 500 ml to dissolve 29.22 gm of NaCl in a 600 ml beaker. Following dissolution, filter and degas this solution and label the bottle ‘Buffer

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B1', along with the actual contents (50 mM Na-phosphate, 0.01% Tween80, 1 M NaCl).

9.2.1.6. If buffers are refrigerated between preparation and use, they should be warmed to room temperature and degassing repeated.

9.2.2. Arginine buffer for elution of tPA:

9.2.2.1. Dissolve 8.43 gm arginine-HCl (210.7 gm/mol) and 2.68 gm sodium phosphate dibasic heptahydrate (268.07 gm/mol) in 175 ml MilliQ water.

9.2.2.2. Add 0.2 ml 10% Tween 80.

9.2.2.3. Titrate the pH to 7.5

9.2.2.4. Adjust the final volume to 200 ml, filter and degas as above. Label as Buffer B2, along with composition.

9.3. ***Start-up and preparation of AKTApure Instrument and computer***:

Degassed buffers should be in place prior to turning on the AKTApure instrument. Equipment start-up requires turning on the instrument and, separately, the computer connected to it.

9.3.1. Place the degassed buffers A, B1 and B2 on top of the AKTApure instrument.

9.3.2. Locate Inlet tubing A1, B1 and B2 (atop the instrument and resting in water or 20% ethanol)..

9.3.3. Transfer tubing Inlet A1 to the buffer A bottle.

9.3.4. Transfer tubing Inlet B1 to the buffer B1 bottle.

9.3.5. Transfer tubing Inlet B2 to the buffer B2 bottle. Secure with a stopper, tape, lab film or foam to maintain the end of the tube on the bottom of the bottle.

9.3.6. The On/Off switch for the instrument is located on the right side toward the rear of the housing. Switch to the 'On' position. Audible emanations from within the instrument cabinet indicate that the AKTApure system is going through its brief initialization sequence.

9.3.7. The computer On/Off switch is located on the front of the Dell desktop computer unit, near the top of the case. Press in to turn on the computer.

9.3.8. Login to the computer using credentials provided by the College.

9.3.9. Double click the Unicorn 6.3 icon on the desktop to open the software which controls the instrument functions. Click OK in the "Log In – Unicorn" dialog box that appears.

9.3.10. Open the System Control window (under Tools menu, if not opened automatically on startup).

9.3.11. The top pane of the window will show the current state of the instrument, and the bottom pane shows the fluid path and manual controls. If the window is blank, go to

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the System menu and select Connect to Systems, check the box by AKTApure 25 and click OK.

9.3.12. Confirm that the correct column (Lysine-Ceramic HyperD) is attached to the system. If not, refer to Section 9.4 (Installing/Changing a Chromatography Column on the AKTApure Chromatography System).

9.3.13. Under the File menu, choose Open and select the method with file name "*Lysine Affinity 2.5ml Equilibration*".

9.3.14. A dialog box appears that allows the method to be run. Click Start to initiate flushing of the pumps and equilibration of the column.

9.3.15. While the equilibration method is running, prepare the fraction collector for later steps by filling the carousel with clean tubes.

9.3.16. Allow the program to run to completion, about 15 minutes.

9.4. ***Installing/Changing a Chromatography Column on the AKTApure Chromatography System.***

It is imperative that the following operations be performed in such a way as to prevent the introduction of air bubbles into the column, which is achieved by making liquid-to-liquid (drop-to-drop) contact prior to inserting the threaded fitting into its position.

9.4.1. Have on hand a few paper lab towels and a 250 ml beaker to catch waste.

9.4.2. Remove tube connector from the UV detector inlet by unscrewing the knurled fastener.

9.4.3. Initiate flow manually at 0.5 ml/min collecting waste in the beaker or towel.

9.4.4. Remove the plug from the column inlet and place a few drops of 20% ethanol in the inlet, filling it to insure the absence of air.

9.4.5. As a droplet emerges from the inlet tubing, touch it to the liquid in the column inlet and begin to thread the fitting in, leaving slight looseness of threads so that liquid escapes around the fitting and pressure buildup in the column is prevented.

9.4.6. Remove the column bottom plug and screw the column outlet directly into the UV detector inlet.

9.4.7. Tighten the column inlet fitting just enough to prevent leaking.

9.4.8. The column is now ready to equilibrate in buffer (step 9.3.12) prior to performing a chromatography run.

Performing a chromatography run:

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9.4.9. Place the fraction collector tube 1 near the outlet tubing from the instrument (refer to attachment Fig 1) so that it will touch the arrow on the white paddle of the fraction collector arm. Note: To rotate the carousel, reach around the left side of the collector to find a rubber roller pressing against the carousel (Fig 2). Pull the roller away from the carousel (Fig. 3); the carousel will rotate freely as long as the roller is held. When the first tube is in the correct position, release the roller.

9.4.10. Gently raise the arm and swing it into position against tube 1.

9.4.11. Place all 'Waste' tubing, labeled W, W1 & W2 in 500 ml Ehrlenmeyer flask.

9.4.12. Place the tube labeled Outlet in a 125 ml Ehrlenmeyer flask.

9.4.13. Fill a 10 ml syringe with the t-PA sample, expel any bubbles and insert the loaded syringe into the injection port.

9.4.14. Inject the sample into the port to fill the 10 ml Superloop.

9.4.15. Open the Unicorn software and navigate to the System Control window.

9.4.16. Under the File menu, choose Open and select the method with file name "*Lysine Affinity tPA Production*".

9.4.17. In the dialog box that opens, enter operator's name, sample notes.

9.4.18. Click Next; take note of the time and and volume for the run; make sure there is excess buffer A and B.

9.4.19. Click Next. Enter the buffer composition of each buffer and the sample identity.

9.4.20. Click Next. Enter a filename composed of the method name, date, operator or group initials, for example LysAffi t-PA 16May15 CertGroup.

9.4.21. Click Start. The instrument should begin to execute the method, as evidenced by a soothing hum from the pumps and drops of liquid falling into tube 1 from the fraction collector outlet.

9.4.22. Observe that the fraction collector is receiving drops.

9.4.23. Monitor the computer screen for error messages or warnings.

9.4.24. Allow the method to run to completion, at which time the system will be re-equilibrated and ready for subsequent runs by repeating section 9.4.

9.5. ***Equipment shut-down and short term (less than 3 days) storage***

9.5.1. After completion of the final separation of the day, transfer Inlet tubing A1 and B1 to a flask of degassed Milli-Q water (250 ml or greater).

9.5.2. In the Unicorn software, open the System Control window.

9.5.3. Under the File menu, choose Open, then select the method 'System Short Term Storage'.

9.5.4. Click Start.

9.5.5. Allow the method to run to completion, as indicated by an audible tone and onscreen window.

9.5.6. Turn off the instrument or perform the long term storage routine as needed (section 9.6).

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9.6. ***Cleaning of the Superloop 10 sample holder.***

9.6.1. For short term storage of the Superloop on the AKTA instrument, inject 2 ml Milli-Q water into the sample chamber.

9.6.2. Pump it out to waste by temporarily disconnecting the outlet tubing that is connected to the injection valve at port 'loop F'.

9.6.3. Using manual control in the System Control window of Unicorn, set the flow rate to 2 ml/min and the injection valve position to Inject. Allow pump A to run until the Superloop chamber is empty.

9.6.4. Reconnect to 'loop F'.

9.6.5. Repeat steps 9.6.1 thru 9.6.4 three times.

9.6.6. Inject 10 ml water into the sample chamber of the Superloop.

9.7. ***Storage of pH electrode***

9.7.1. In System Control, under the System menu, open the Calibration method for the pH detector. You should hear the electrode valve change to calibration position.

9.7.2. Wash the electrode 3 times with 10 ml water from a syringe.

9.7.3. Fill a syringe with pH electrode storage buffer and inject about 8 ml. Stop and leave the syringe attached.

9.7.4. Close the calibration dialog box in the System Control window of Unicorn.

9.8. ***Equipment shut-down and long term (3 days or more) storage***

9.8.1. After completion of the System Short Term Storage method, transfer Inlet tubing A1, B1 and B2 to a flask of degassed 20% ethanol (250 ml or greater).

9.8.2. In the Unicorn software, open the System Control window.

9.8.3. Confirm that the pH valve is in the 'Restrictor' position (to avoid bathing the electrode in EtOH, which may be harmful).

9.8.4. Under the File menu, choose Open, then select the method 'System Long Term Storage'.

9.8.5. Click Start.

9.8.6. Allow the method to run to completion, as indicated by an audible tone and onscreen window.

9.8.7. Turn off the AKTApure instrument.

9.9. ***Printing Your Chromatogram***

9.9.1. In the Unicorn software interface, open the Evaluation window.

9.9.2. In the Result Navigator pane, click the Results tab.

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9.9.3. Locate the file of interest and double click its name to display your chromatogram in the right pane.

9.9.4. *Optional:* Click the Customize button to open a dialog box that allows you to specifiy what curves display and the scale of each axis. Recommended are the UV Chrom curve, Conductivity, and Fraction Number.

9.9.5. Click the Report button, check the Default report in the selection window and click Preview.

9.9.6. Under File, choose to Print (or Save as PDF to use a different printer).

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Attachments/Figures

Fig. 1. ***Diagram of AKTApure instrument, fraction collector and computer***

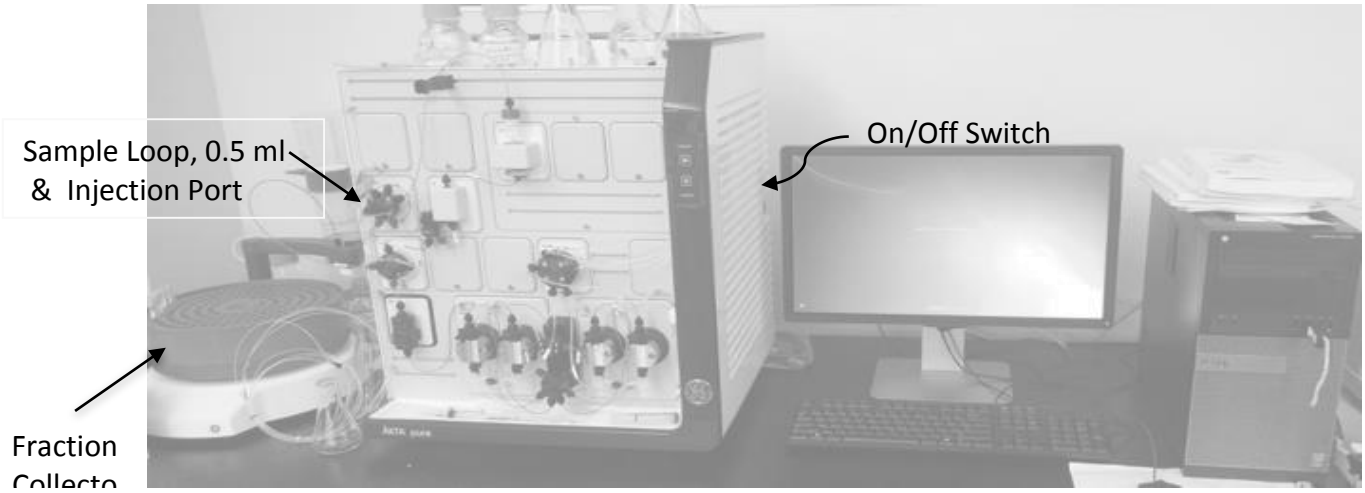

Fig. 2. ***AKTApure Instrument Features***

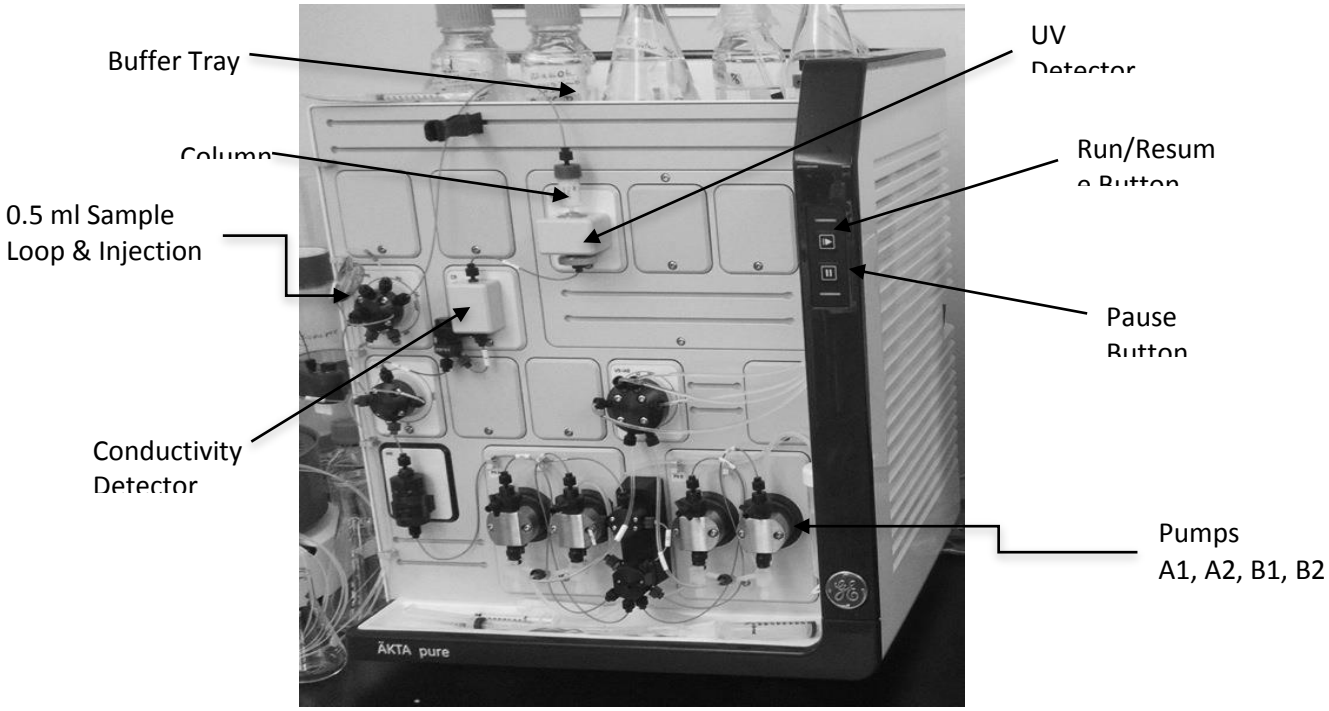

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Fig 3. ***System Control window within the Unicorn 6.3 software.***

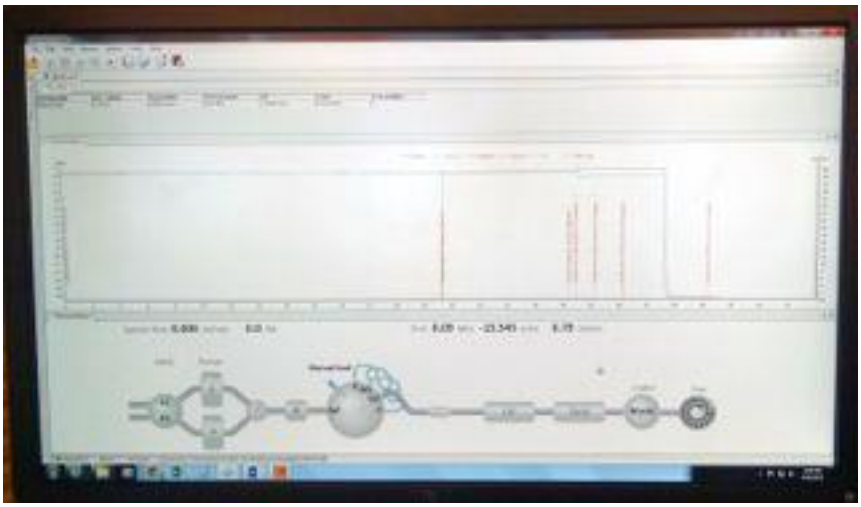

Fig. 4. ***Detail of Injection Port with Syringe in Place***.

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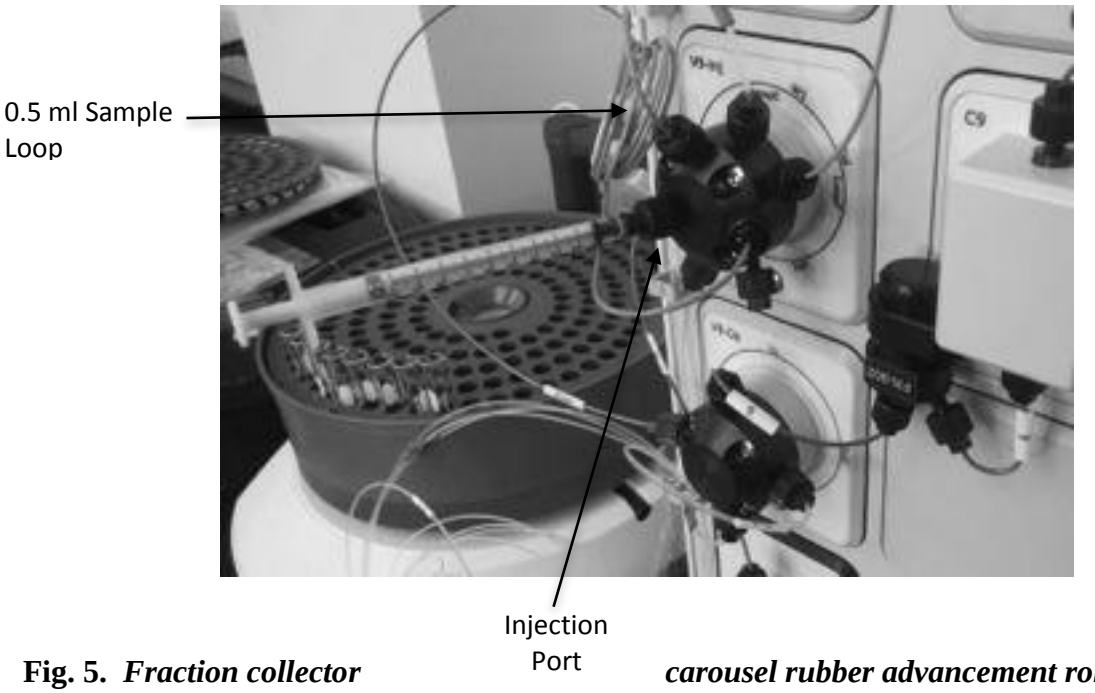

Fig. 5. ***Fraction collector carousel rubber advancement roller/gear.***

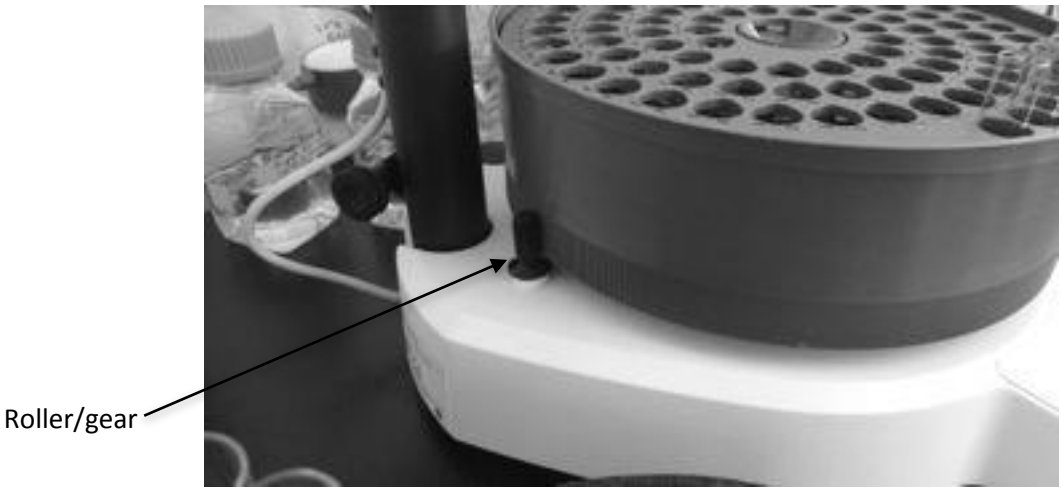

Fig. 6. ***Release of roller to allow free rotation of the carousel.***

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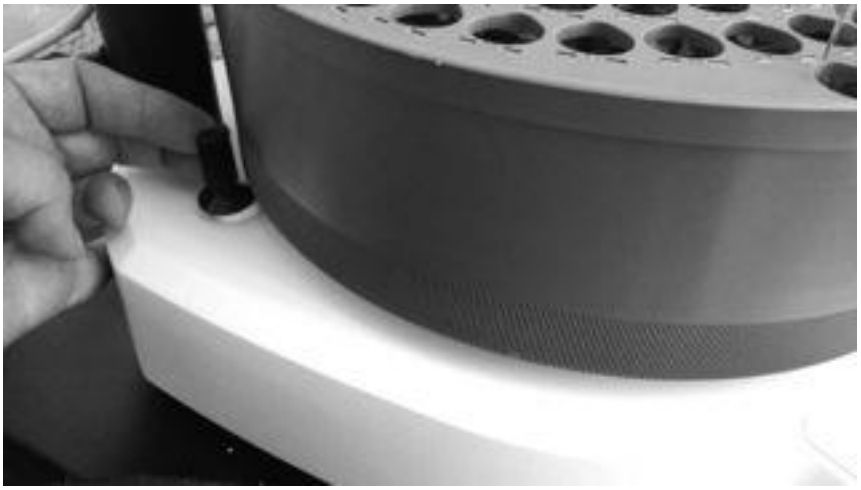

Fig. 7. ***Location of tube #1 under the fraction collector drip outlet.***

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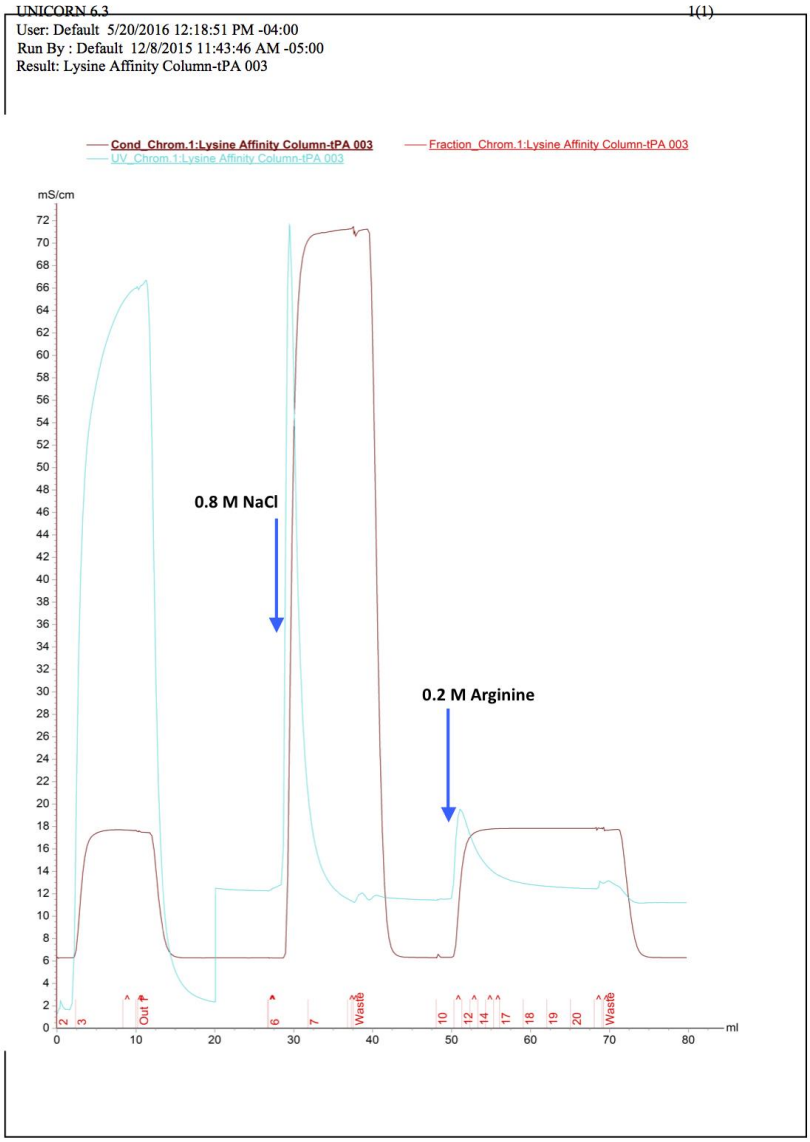
Fig 8. Sample Chromatogram.



10. History

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Revision Number	***Effective Date***	***Preparer***	***Description of Change***
0	20May16	David Frank	Initial release