SOP: Isolation of mAb (anti IL-8) from Conditioned Medium by Protein A Affinity Chromatography on the ÄKTA pure Chromatography System

Approvals

Preparer: Dr. David Frank	Date: 20APR16
Reviewer: Jason McMillan	Date: 21APR16
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1. **Purpose**
   1.1. This procedure describes the isolation of monoclonal antibody from conditioned medium (produced by CHO cells expressing recombinant anti-IL-8) using Protein A affinity chromatography with the ÄKTA pure Chromatography System, controlled by Unicorn 6.3 software.
2. **Scope and Applicability**
   2.1. Applies to purification of mAb from prepared conditioned medium, which has been concentrated and its buffer exchanged by ultrafiltration. The method employs a 1ml HiTrap Protein A-HP column installed on the GE ÄKTA pure 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 to affinity column
   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. AKTA pure 25 Users Guide (electronic)
   4.3. HiTrap Protein A HP 1ml column information booklet (GE Healthcare)
5. **Definitions**
   5.1. N/A
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 AKTA pure Cue Cards located near the instrument.
   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.

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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 AKTA pure instrument. Samples should be, at a minimum, centrifuged at 10000x*g* for 5 min before injection/introduction into the fluid path.
6.7. Equipment calibration check: The AKTA pure system calibration is automatic; baseline for measurements of A280 and conductivity are zeroed at the beginning of a chromatography run. Calibration of the pH detector is described below.

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. AKTA pure chromatography system
8.2. HiTrap Protein A HP (1ml) column; stored at 4ºC- bring to room temperature prior to installation (get it out now).
8.3. Additional Lab Equipment: pH meter, balance, table top centrifuge with swinging bucket rotor
8.4. Lab Utensils: Beakers (250, 500ml, 1200ml), 1 liter and 500ml graduated cylinders
8.5. Reagents:
8.5.1. 20mM sodium phosphate buffer, pH 7.0
8.5.2. 0.1M sodium citrate, pH 3.0
8.5.3. 1M Tris base pH9.0
8.5.4. Filtered deionized water (MilliQ or similar).
8.5.5. 20% Ethanol
8.5.6. 10N NaOH
8.5.7. pH Standard buffers: pH 7, pH 4.01
8.5.8. Stock solutions of protease inhibitors: 10 mg/ml PMSF in isopropanol, 2 mg/ml leupeptin, 10 mg/ml aprotinin.
8.6. Lab Supplies:
8.6.1. Filters (0.2µm); (3 bottle top; 2 syringe mounted)
8.6.2. Corning bottles for vacuum filtration, degassing of all chromatography buffers.
8.6.3. Syringe with leur lock (10ml) – (2).
8.6.4. Tubes for fraction collector – (30)
8.6.5. Graduated cylinders: 1L, 250ml, 100ml
8.6.6. Beakers: 1L, 400ml, 200ml

9. Procedure

9.1. ***Reagent Preparation***:

9.1.1. <u>Buffer A: Binding buffer</u>: 20mM sodium phosphate, pH 7.0

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9.1.1.1. Weigh 1.084± 0.02g NaH_2PO_4 and transfer to a 1200ml beaker with magnetic stir bar.
9.1.1.2. Weigh 3.2 ± 0.02g $Na_2HPO_4 \bullet 7H_2O$ and transfer to the same beaker.
9.1.1.3. Measure 980ml MilliQ water in a graduated cylinder and add the water to the solids in the flask.
9.1.1.4. Stir until the solids have dissolved, check the pH and adjust the pH to 7.0 if required with 1N phosphoric acid.
9.1.1.5. Transfer to a 1L graduated cylinder and adjust the final volume to 1L.
9.1.1.6. Sterile filter the solution, allowing it to degas for 15-20 minutes. Label appropriately.

9.1.2. Buffer B: Elution buffer: 0.1M sodium citrate, pH 3.0
9.1.2.1. Weigh 3.84g citric acid in a 400ml beaker with magnetic stir bar.
9.1.2.2. Dissolve in 180ml MilliQ water.
9.1.2.3. Adjust the pH dropwise with 10N NaOH, to a final pH of 3.0
9.1.2.4. Transfer the solution to a 250ml graduated cylinder. Adjust the final volume to 200ml.
9.1.2.5. Filter the solution, allowing it to degas for 15 – 20 minutes.
9.1.2.6. Label appropriately.

9.1.3. 1M Tris base pH9.00: added to fraction collector tubes to rapidly neutralize acid-eluted fractions from the protein A column.
9.1.3.1. Weigh 12.11 gm Tris base [tris(hydroxymethyl)aminomethane] into a plastic weigh boat and transfer to a 200ml beaker with a stir bar.
9.1.3.2. Measure 90ml MilliQ water in a graduated cylinder and transfer the water to the beaker containing Tris powder. Stir until dissolved, then adjust pH to 9.0
9.1.3.3. Transfer the Tris solution quantitatively to a 100ml graduated cylinder, rinsing the beaker with small aliquots of water, which are then added to the cylinder until a final volume of 100ml is obtained.
9.1.3.4. Filter the solution. Degassing is not necessary.

9.2. ***Calibration of the pH Electrode***

Calibration of the pH detector is performed daily, when the instrument is in use. The calibration procedure utilizes ordinary pH standards found in the lab. Calibration is dictated by a method built into Unicorn.

9.2.1. Obtain three small beakers and pH standards for pH 4.01 and pH 7.0, as well as a 10ml syringe and a bottle of MilliQ water.
9.2.2. In the Unicorn System Control window, choose 'Calibration' from the System menu. From the drop down menu under 'Monitor to calibrate', select 'pH'.
9.2.3. Click the 'Prepare for Calibration' button. You will hear the valve switch to the calibrate position.
9.2.4. Follow the on-screen instructions for both pH standards. Enter the pH of the first pH standard buffer in the ***pH for buffer 1*** field.
9.2.5. Fill a syringe with approximately 10ml of the first pH standard buffer (pH 7). Connect the syringe to the Luer connector of pH valve port **Cal**, and inject the buffer. When the ***Current value*** is stable, click the ***Calibrate*** button.

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9.2.6. Thoroughly rinse the syringe with 3-4 changes of MilliQ water. Wash the pH flow cell by injecting water into pH valve port **Cal**.

9.2.7. Enter the pH of the second pH standard buffer in the ***pH for buffer 2*** field. Fill a syringe with approximately 10ml of the second pH standard buffer. Connect the syringe to the Luer connector of pH valve port **Cal**, and inject the buffer. When the ***Current value*** is stable, click the ***Calibrate*** button.

9.2.8. The calibration date and time are displayed in the dialog, along with values for ***Calibrated electrode slope*** (should be ≥ 80%) and ***Asymmetry potential at pH 7*** (should be within the interval ± 60 mV. If the conditions are met, click the ***Close*** button to switch the pH valve back to the default position and to close the ***Calibration*** dialog.

9.2.9. If values for the slope and potential are not within acceptable values, clean the pH electrode and repeat the calibration procedure. If this does not help, replace the electrode.

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

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

9.3.1. Place the degassed buffers A and B on top of the AKTA pure instrument.

9.3.2. Locate Inlet tubing A1 and B1 (atop the instrument and resting in water or 20% ethanol). Each has a filter unit attached, which distinguishes them from A2 and B2; those end in a male threaded fitting and will not be used for this procedure.

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

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

9.3.5. 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 AKTA pure system is going through its brief initialization sequence.

9.3.6. 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.

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

9.3.8. 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.

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

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

9.3.11. Confirm that the correct column (HiTrap Protein A-HP 1ml) is attached to the system. If not, refer to Section 9.4 (Installing/Changing a Chromatography Column on the AKTA pure Chromatography System).

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9.3.12. Under the File menu, choose Open and select the method with file name "*HiTrap Protein A-HP 1ml Equilibration*".

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

9.3.14. While the equilibration method is running, prepare the fraction collector for later steps by filling the carousel with clean tubes. Add 200µl 1M Tris to the bottom of each tube – this serves to rapidly neutralize the acidic eluent (which destabilizes some antibodies).

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

9.4. ***Installing/Changing a Chromatography Column on the AKT Apure 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. Confirm that the Protein A column has reached room temperature.

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

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

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

9.4.5. 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.

9.4.6. 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.

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

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

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

9.5. ***Performing a chromatography run***:

9.5.1. 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.

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

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

9.5.4. Place the tube labeled Outlet in a 125ml Erlenmeyer flask.

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9.5.5. Fill a 10ml syringe with the mAb sample (concentrated, filtered conditioned medium), expel any bubbles and insert the loaded syringe into the injection port.
9.5.6. Inject the sample into the port to fill the 10ml Superloop.
9.5.7. Open the Unicorn software and navigate to the System Control window.
9.5.8. Under the File menu, choose Open and select the method with file name "*1ml Protein A Column*".
9.5.9. In the dialog box that opens, enter operator's name, sample notes.
9.5.10. Click Next; take note of the time and volume for the run; make sure there is excess buffer A and B.
9.5.11. Click Next. Enter the buffer composition of each buffer and the sample identity.
9.5.12. Click Next. Enter a filename composed of the method name, date, operator or group initials, for example **HiTrapProtA antiIL8 16May15 SDBiopharm**.
9.5.13. 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.
9.5.14. Observe that the fraction collector is receiving drops.
9.5.15. Monitor the computer screen for error messages or warnings.
9.5.16. Allow the method to run to completion, at which time the system will be re-equilibrated and ready for subsequent runs by repeating section 9.6
9.5.17. Remove tubes from the fraction collector and place in a rack for storage at 4ºC, awaiting further analysis. Cover the top of the tubes with lab film. The peak of absorbance at 280 nm which eluted with the low pH buffer B contains purified mAb, which will be examined by the QC Biochemistry Dept.

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

9.6.1. After completion of the final separation of the day, transfer Inlet tubing A1 and B1 to a flask of degassed Milli-Q water (250ml or greater).
9.6.2. In the Unicorn software, open the System Control window.
9.6.3. Under the File menu, choose Open, then select the method 'System Short Term Storage'.
9.6.4. Click Start.
9.6.5. Allow the method to run to completion, as indicated by an audible tone and onscreen window.
9.6.6. Turn off the instrument or perform the long term storage routine as needed (section 9.6).

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

9.7.1. For short term storage of the Superloop on the AKTA instrument, inject 2ml Milli-Q water into the sample chamber.
9.7.2. Pump it out to waste by temporarily disconnecting the outlet tubing that is connected to the injection valve at port 'loop F'.
9.7.3. Using manual control in the System Control window of Unicorn, set the flow rate to 2ml/min and the injection valve position to Inject. Allow pump A to run until the Superloop chamber is empty.
9.7.4. Reconnect to 'loop F'.

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9.7.5. Repeat steps 9.6.1 thru 9.6.4 three times.
9.7.6. Inject 10ml water into the sample chamber of the Superloop.

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 and B1 to a flask of degassed 20% ethanol (250ml 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 'Bypass' or 'Restrictor' position.
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. Referring to section 9.4 for guidance, remove the HiTrap Protein A-HP column from the instrument and cap both ends, taking care to avoid introduction of air into the column. Store the column in a refrigerator.
9.8.8. Turn off the AKTA pure 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.
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 specify 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 AKTA pure instrument, fraction collector and computer***

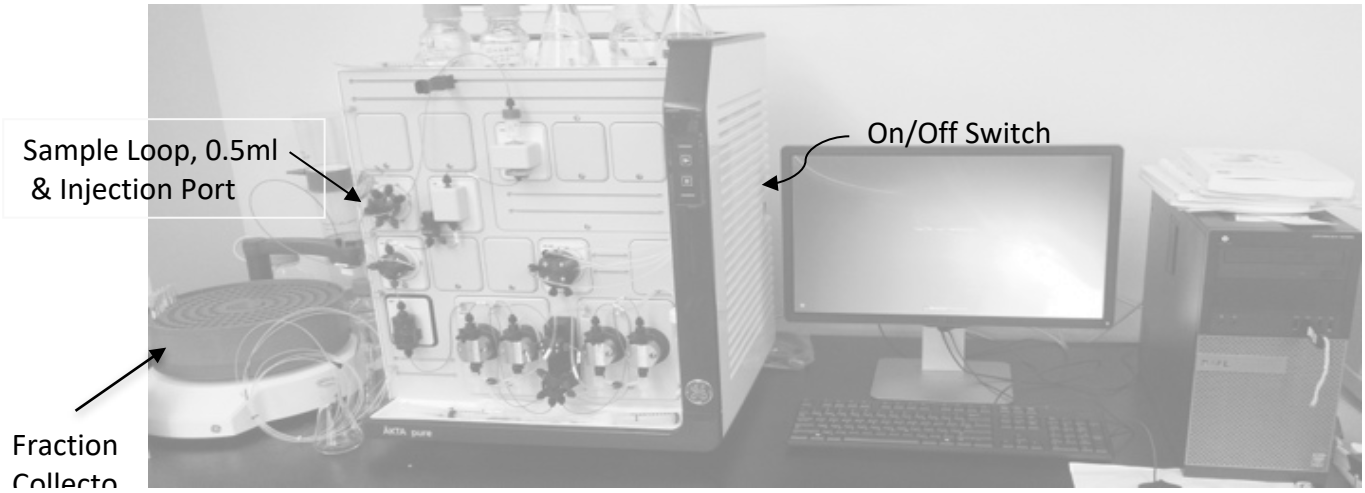

Fig. 2. ***AKTA pure Instrument Features***

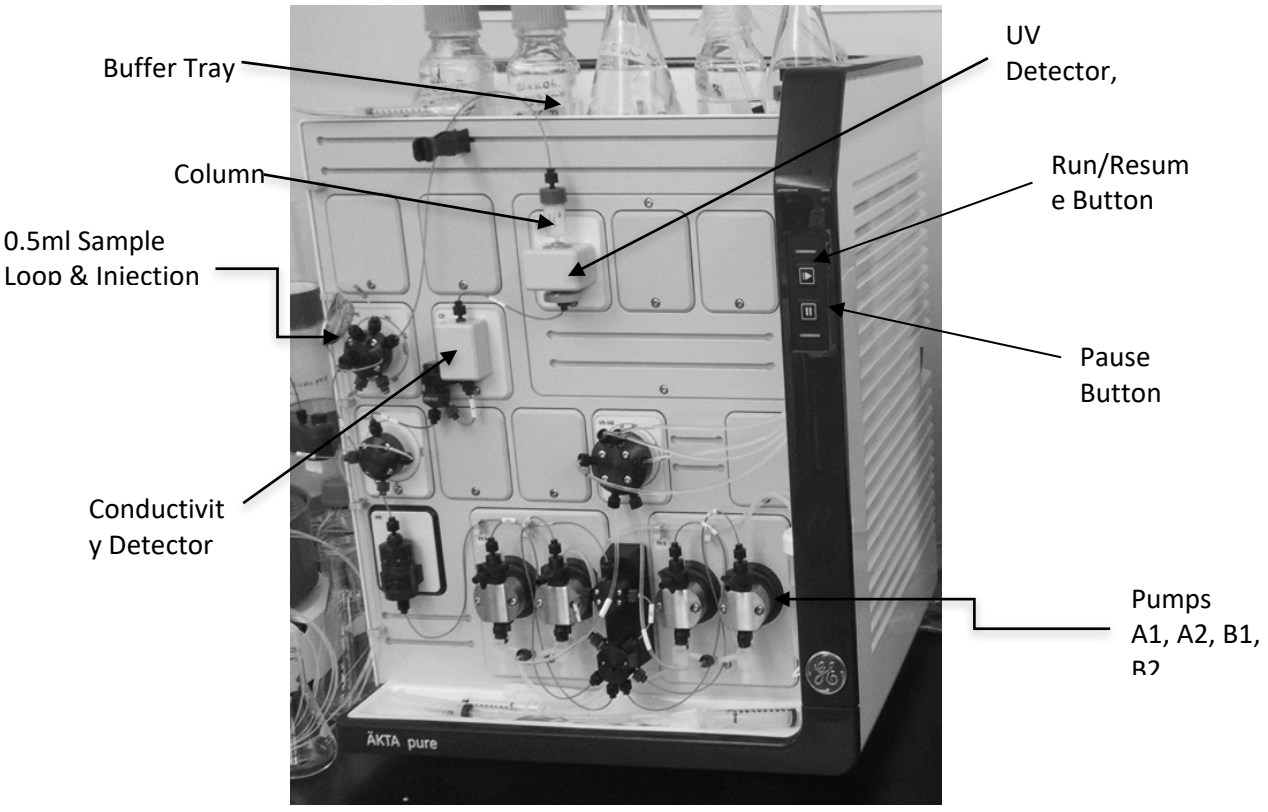

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Fig 3. ***System Control window within the Unicorn 6.3 software.***

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

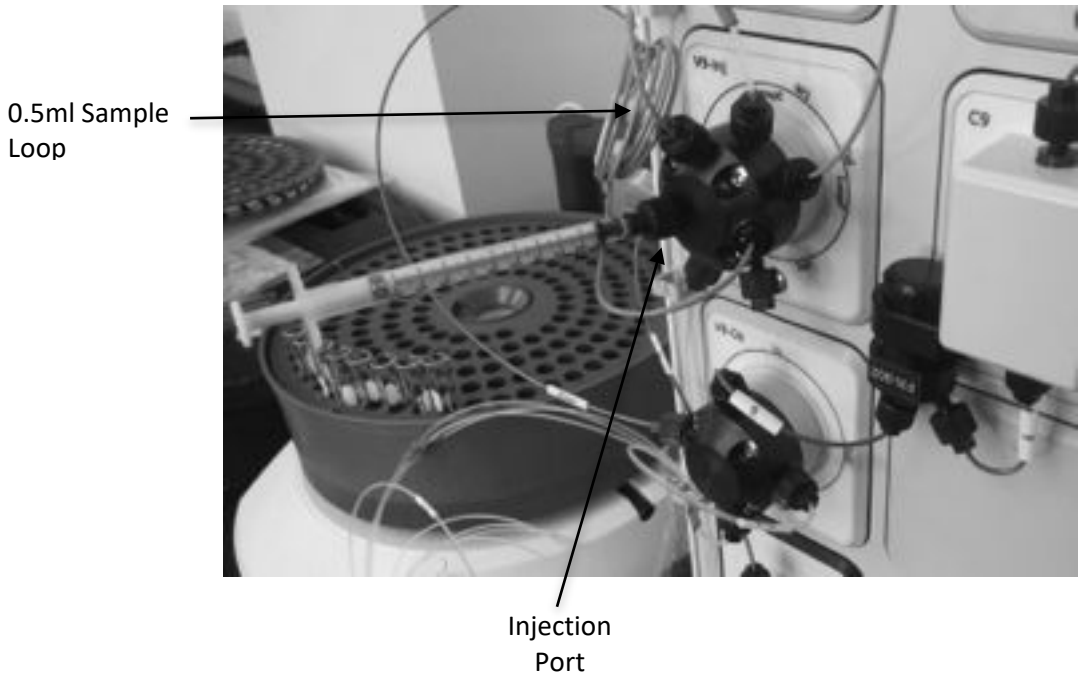

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Fig. 5. ***Fraction collector carousel rubber advancement roller/gear.***

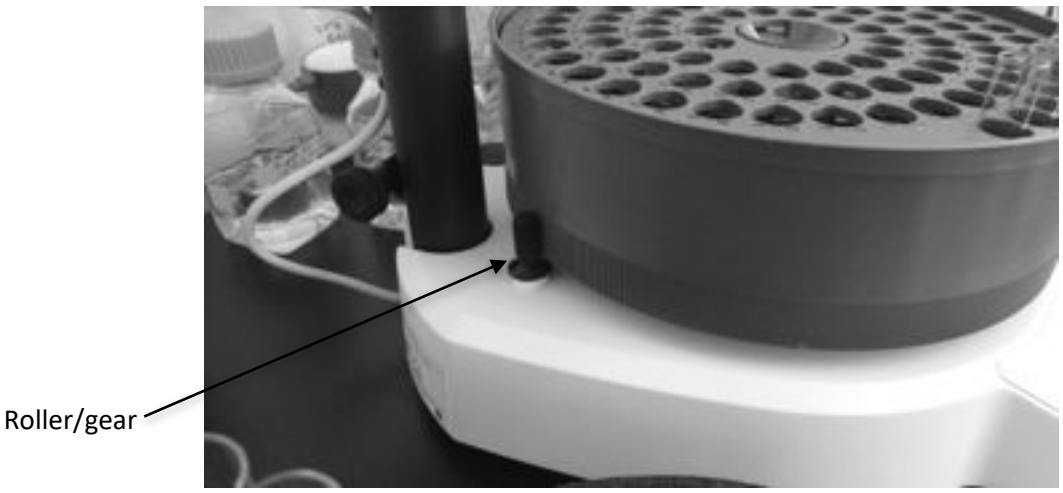

Fig. 6. ***Release of roller to allow free rotation of the carousel.***

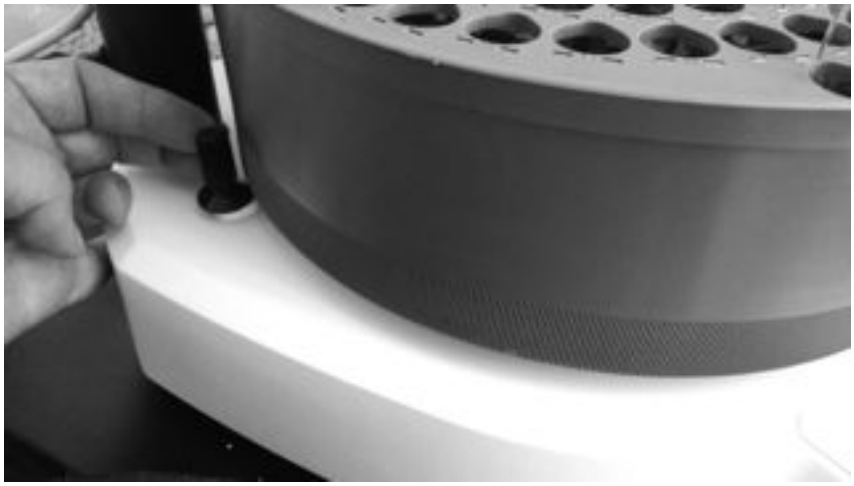

Fig. 7. ***Location of tube #1 under the fraction collector drip outlet.***

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10. History

Revision Number	*Effective Date*	*Preparer*	*Description of Change*
0	21APR16	D Frank, S Donnelly	Initial release
1	19FEB18	Hetal Doshi	Removed concentrating of the cell suspension step and Changed the neutralizing buffer pH to 9.0