SOP: Monoclonal Antibody Drug Substance Aggregate Testing by HPLC Analysis

Approvals:

Preparer: Dr. Matt Marshall	Date: 20MAR19
Reviewer: Robin Zuck	Date: 27MAR19
Reviewer: Dr. Maggie Bryans	Date: 04APR19

1. **Purpose:**
   1.1. To quantify the relative amount of aggregated and monomer molecules in solution of a monoclonal antibody (mAb) drug substance using size exclusion high performance liquid chromatography (HPLC) analysis.

2. **Scope:** Size Exclusion High performance liquid chromatography (HPLC) is an analytical chemistry technique for separating the components of a liquid sample based on molecular size. This SOP uses HPLC to quantify the amount of a monoclonal antibody drug substance that is present as monomers, dimers or higher order aggregates. HPLC is an FDA required test to adequately characterize the unconjugated mAb reagent of a drug product.

3. **Summary of Method:**
   3.1. Prepare the mobile phase solution
   3.2. Power up the HPLC system and equilibrate with mobile phase solution for 60 minutes [if system is in short-term storage] or 120 minutes [if system is in long-term storage].
   3.3. Prepare AdvanceBio SEC 300Å protein standards
   3.4. Prepare mAb drug substance for HPLC analysis
   3.5. Prepare buffer in which mAb drug substance is suspended.
   3.6. Power up the HPLC system and equilibrate with mobile phase solution
   3.7. Run an assay for using the prepared AdvanceBio SEC 300Å protein standards and mAb sample
   3.8. Prepare HPLC system of short-term or long-term storage.
   3.9. Compute the size of the conjugated and unconjugated mAb sample

4. **References:**
   4.1. SOP: Buck Scientific BLC-20P HPLC Operation, document QCB 7, revision 0
   4.2. SOP: Degassing a Solution by Helium Sparge, document number QCB 6, revision 0
   4.3. User Guide for Agilent AdvanceBio SEC Columns

5. **Definitions:**

CV	Column Volume; the volume (ml) of the column containing the stationary phase; CV= 2.91 ml for a standard size (4.6 X 250 mm) column
Equilibrium	Running the mobile phase solution through the column prior to injecting the sample in order to bring the system into equilibrium
Flow rate	The rate (ml/min) that solution is pumped through the column. The operating flow rate is determined by the assay protocol.

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Helium Sparge	Using a stream of helium bubbles to sweep dissolved air out of liquids (helium is virtually insoluble in most HPLC solvent solutions, so very little helium replaces the air)
HPLC	High Performance Liquid Chromatography
Isocratic	The composition of the mobile phase solution is constant; the system has only one pump.
Mobile phase	The solvent solution used to carry the sample through the column
PeakSimple	Software used to collect and display data
PSI	Pounds per Square Inch
Size exclusion chromatography	Separation based on molecule size. Molecules are separated on the basis of their exclusion from pores in the column packing material.
Stationary phase	The chromatography matrix through which the sample travels.

6. Precautions:

6.1. HPLC systems operate at high pressures. Personnel injury and equipment damage can result if maximum pressure is exceeded or the pump runs dry. Monitor pressure readings and solution level whenever the pump is running. If pressure exceeds 2500 psi or if the solution runs out, stop the pump immediately by pressing the RUN/STOP button. Do not set the flow rate higher than 1.5 ml/min with a 250 mm column.

6.2. Flow rate consistency is affected by the quality of the solutions. Use HPLC-grade solvents and filter solutions using a sub-micron filter (preferably 0.22 μm). Degas solutions prior to use.

6.3. To avoid microbial growth, do not leave the system in a high aqueous solution for a prolonged period. The system should be washed with a storage solution of 50% Methanol/H20 or 50% Acetonitrile/H20 if it is to be idle more than a few hours.

6.4. Methanol is flammable. Can cause blindness if swallowed. Vapor is harmful. Irritating to skin and eyes.

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. Buck Scientific BLC-20P HPLC system pre-configured with:

- 8.1.1. UV-Vis detector
- 8.1.2. PeakSimple Chromatography Data System
- 8.1.3. Computer system with PeakSimple software installed
- 8.1.4. AdvanceBIO 300Å- 4.6mm x 150mm HPLC column. Product # PL1580-3301

8.2. HPLC-grade methanol

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8.3. HPLC-grade water
8.4. monosodium phosphate monohydrate
8.5. disodium phosphate, heptahydrate
8.6. AdvanceBio SEC 300Å protein standards. Product # 5190-9417
8.7. Post protein A chromatography monoclonal antibody (mAb) sample to be analyzed
8.8. Sample overflow waste beaker
8.9. Analytic balance
8.10. 500ml graduated cylinder
8.11. 500ml volumetric flask
8.12. 100ml volumetric flask
8.13. 1.5ml Eppendorf Tube
8.14. Stirring plate
8.15. 2- 500ml laboratory bottles (for mobile phase solution and waste)
8.16. 125ml laboratory bottle (for mobile phase to rinse the sample syringe)
8.17. Nalgene Rapid-flow filtration unit
8.18. 0.22µm syringe filters
8.19. 5 ml Luer-Lok syringe
8.20. 100uL HPLC sample syringe (Hamilton syringe)
8.21. Parafilm
8.22. Timer

9. Procedure:

9.1. Prepare 500ml of mobile phase solution (150mM Sodium Phosphate, pH 7.0).
9.1.1. In a 500ml volumetric flask, add 3.976g monosodium phosphate, monohydrate and 12.379g disodium phosphate, heptahydrate and 480ml of MilliQ water.
9.1.2. Add magnetic stirring bar, and stir until completely dissolved.
9.1.3. Check the pH.
9.1.4. If required, adjust pH to 7.0±0.1 with 1N phosphoric acid. Bring the volume to 500ml.
9.1.5. Filter the mobile phase solution using a Nalgene Rapid-flow filtration unit.
9.1.6. Degas the mobile phase solution per the Degassing a Solution by Helium Sparge SOP QCB 6.
9.1.6.1. Transfer approximately 10ml of mobile phase solution into a labeled 125ml laboratory bottle. This will be used for rinsing the sample syringe. Transfer remaining solution to a labeled 500ml laboratory bottle.
9.1.6.2. Label an empty 500ml laboratory bottle as mobile phase solution waste and place waste line into it, cover with Parafilm.
9.2. Power up the HPLC system and equilibrate with mobile phase solution for 60 minutes [if system is in short-term storage] or 120 minutes [if system is in long-term storage] at a flow rate of 0.25ml/min.
9.2.1. Power up the HPLC system components and start the PeakSimple data collection software.
9.2.1.1. Switch the system to mobile phase solution.

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9.2.1.1.1. Verify that the pump is off. The Run LED should be off
9.2.1.1.2. Place the intake line into the mobile phase solution bottle and cover with Parafilm
9.2.1.1.3. Verify that the frit is submerged in the solution.
9.2.1.1.4. Place the outlet line into the mobile phase waste bottle.
9.2.1.1.5. Place the sample overflow line into a small waste beaker
9.2.1.2.Purge the intake line and prime the pump
9.2.1.2.1. Attach an empty 5ml Luer-Lok syringe to the purge valve
9.2.1.2.2. Open the prime/purge valve by turning it two full turns counter-clockwise.
9.2.1.2.3. Watching the intake line for bubbles, slowly draw the syringe plunger until it is fully drawn. Mobile phase solution and bubbles should fill the syringe.
9.2.1.2.4. Close the prime /purge valve by rotating it clockwise until it stops.
9.2.1.2.5. Remove the syringe from the purge valve and expel the contents into the waste bottle.
9.2.1.2.6. Repeat attaching the syringe to the purge valve, drawing bubbles and solution into the syringe, and expelling into the waste bottle until free of bubbles (generally 10-15 ml of mobile phase are needed).
9.2.1.3.Start the pump and gradually increase the flow rate to 0.25ml/min over 5min.
9.2.1.3.1. Set the initial flow rate to 0.1 ml/min:
9.2.1.3.1.1.Press the MODE button on the front panel repeatedly until the Flow LED turns on. The current flow rate (in ml/min) appears on the digital display.
9.2.1.3.1.2.Press the Flow up arrow button to increase the flow rate setting and press the down arrow button to decrease the flow rate setting.
9.2.1.3.1.3.Repeat pressing the Flow arrow buttons until 0.10 is displayed.
9.2.1.3.1.4.Start the pump by pressing the RUN/STOP button. The LED should turn on.
9.2.1.3.2. Monitor the pressure reading and mobile phase level.
9.2.1.3.2.1.Display the pressure reading by pressing the MODE button repeatedly until the Pressure LED turns on. The current pressure (in psi) appears on the digital display.
9.2.1.3.2.2.Verify that the waste is dripping into the waste bottle.
9.2.1.3.2.3.If the pressure exceeds 2500 psi or the mobile phase runs low stop the pump immediately by pressing the RUN/STOP button.
9.2.1.3.2.4.Gradually increase the flow rate to 0.25 ml/min over 5 minutes.
9.2.1.3.2.4.1. Increase the flow rate in 0.1 ml/min increments using the Flow up arrow button.
9.2.1.3.2.4.2. Monitor the pressure readings until a flow rate of 0.25ml/min is achieved and the pressure reading is stable.
9.2.2. Set the UV-Vis detector wavelength to 220 nm and autozero the detector.
9.2.2.1. Set the UV-Vis detector wavelength by pressing the λ up and down buttons until 220nm is displayed.
9.2.2.2. Press the Autozero button in the front panel.

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9.2.2.3.-If system was in short-term storage, equilibrate the system with mobile phase at the flow rate of 0.25 ml/min for 60 minutes.
-If system was in long-term storage, equilibrate the system with mobile phase at the flow rate of 0.25 ml/min for 120 minutes.

9.3. Reconstitute AdvanceBio SEC 300Å protein standards [Concentration=3.8mg/ml].
- 9.3.1. Reconstitute lypholized mixture vial contains 3.8mg total protein.
- 9.3.2. Add 1,000µL of freshly prepared mobile phase solution (150mM Sodium Phosphate, pH 7.0).
- 9.3.3. Invert the vial several times gently (do not vortex).
- 9.3.4. Place in a sonicating water bath. Pulse for several minutes in 30s intervals with 15s breaks to fully reconstitute the sample; avoid elevating temperature.
- 9.3.5. Prepare aliquots of this standard stock solution and store at 2-8°C for up to 1 year.

9.4. Prepare a 0.152mg/ml working stock of AdvanceBio SEC 300Å protein standards
- 9.4.1. To produce protein standards at [0.152mg/ml], place 40µL of reconstituted AdvanceBio SEC 300Å protein standards [Concentration=3.8mg/ml] into labeled 1.5ml Eppendorf tube.
- 9.4.2. Add 960µL of freshly prepared mobile phase solution, 150mM Sodium Phosphate, pH 7.0.
- 9.4.3. Invert the vial several times gently (do not vortex).
- 9.4.4. Gently pipette up and down several times to mix.
- 9.4.5. Transfer with pipette and filter with 0.22µm syringe filter into a sterile 1.5ml Eppendorf tube
- 9.4.6. Label as ‘S’ for AdvanceBio SEC 300Å protein standards

9.5. Prepare monoclonal antibody (mAb) sample for HPLC analysis.
- 9.5.1. The mAb is diluted in freshly prepared mobile phase solution (150mM Sodium Phosphate, pH 7.0) to a final concentration between 0.024 - 0.096mg/ml.
- 9.5.2. For a sample with 1.2mg/ml, place 40µL of post protein A chromatography mAb drug substance into labeled 1.5ml Eppendorf tube.
- 9.5.3. Add 960µL of freshly prepared mobile phase solvent containing 150mM Sodium Phosphate, pH 7.0.
- 9.5.4. Invert the vial several times gently (do not vortex).
- 9.5.5. Gently pipette up and down several times to mix.
- 9.5.6. Transfer with pipette and filter with 0.22µm syringe filter into a sterile 1.5ml Eppendorf tube
- 9.5.7. Label as ‘M’ for mAb sample

9.6. Prepare monoclonal antibody (mAb) buffer [1,000µl 0.1M Sodium Citrate, pH 3 + 200µl 1M Tris pH 9] for HPLC analysis
- 9.6.1. Dilute buffer that post protein A chromatography mAb is suspended in using the same dilution as used in step 9.4.
- 9.6.2. Invert the vial several times gently (do not vortex).
- 9.6.3. Gently pipette up and down several times to mix.
- 9.6.4. Transfer with pipette and filter with 0.22µm syringe filter into a sterile 1.5ml Eppendorf tube.

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9.6.5. Label as 'B' for buffer.

9.7. Prepare the system for running the samples.

9.7.1. Start the PeakSimple data collection software.

9.7.1.1. Verify that the pump is off. The Run LED should be off

9.7.1.2. Place the intake line into the mobile phase solution bottle and cover with Parafilm

9.7.1.3. Verify that the frit is submerged in the solution.

9.7.1.4. Place the outlet line into the mobile phase waste bottle.

9.7.1.5. Place the sample overflow line into a small waste beaker

9.7.1.6. Purge the intake line and prime the pump

9.7.1.6.1. Attach an empty 5ml Luer-Lok syringe to the purge valve

9.7.1.6.2. Open the prime/purge valve by turning it two full turns counter-clockwise.

9.7.1.6.3. Watching the intake line for bubbles, slowly draw the syringe plunger until it is fully drawn. Mobile phase solution and bubbles should fill the syringe.

9.7.1.6.4. Close the prime /purge valve by rotating it clockwise until it stops.

9.7.1.6.5. Remove the syringe from the purge valve and expel the contents into the waste bottle.

9.7.1.6.6. Repeat attaching the syringe to the purge valve, drawing bubbles and solution into the syringe, and expelling into the waste bottle until free of bubbles (generally 10-15 ml of mobile phase are needed).

9.7.1.7. Start the pump and gradually increase the flow rate to 0.25ml/min over 5min.

9.7.1.7.1. Set the initial flow rate to 0.1 ml/min:

9.7.1.7.1.1. Press the MODE button on the front panel repeatedly until the Flow LED turns on. The current flow rate (in ml/min) appears on the digital display.

9.7.1.7.1.2. Press the Flow up arrow button to increase the flow rate setting and press the down arrow button to decrease the flow rate setting.

9.7.1.7.1.3. Repeat pressing the Flow arrow buttons until 0.10 is displayed.

9.7.1.7.1.4. Start the pump by pressing the RUN/STOP button. The LED should turn on.

9.7.1.7.2. Monitor the pressure reading and mobile phase level.

9.7.1.7.2.1. Display the pressure reading by pressing the MODE button repeatedly until the Pressure LED turns on. The current pressure (in psi) appears on the digital display.

9.7.1.7.2.2. Verify that the waste is dripping into the waste bottle.

9.7.1.7.2.3. If the pressure exceeds 2500 psi or the mobile phase runs low stop the pump immediately by pressing the RUN/STOP button.

9.7.1.7.2.4. Gradually increase the flow rate to 0.25 ml/min over 5 minutes.

9.7.1.7.2.4.1. Increase the flow rate in 0.1 ml/min increments using the Flow up arrow button.

9.7.1.7.2.4.2. Monitor the pressure readings until a flow rate of 0.25ml/min is achieved and the pressure reading is stable.

9.7.2. Set the UV-Vis detector wavelength to 220 nm and autozero the detector.

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9.7.2.1. Set the UV-Vis detector wavelength by pressing the λ up and down buttons until 220nm is displayed.
9.7.2.2. Press the Autozero button in the front panel.
9.7.2.3.Equilibrate the system with mobile phase at the flow rate of 0.25 ml/min for 60 minutes.

9.8. The UV-Vis detector needs to warm up for 60 minutes prior to collecting data, monitor the UV-Vis signal for stability.For each sample (S, M, B) run an assay for 10 minutes at 0.25ml/min per the HPLC Operation SOP. See 10.1-10.3 for example chromatograms of S, M & B.
9.8.1. Use PeakSimple to start a new 10 minute run.
9.8.2. Autozero the UV-Vis detector.
9.8.3. Setup autosampler to record data for 10 minutes.
9.8.3.1.In PeakSimple software select EDIT – Channels.
9.8.3.2.Under Channel 1, select Details.
9.8.3.3.Change default display limits to 75mV MAX, -10mV MIN.
9.8.3.4.Change end time to 10 mins.
9.8.3.5.Leave other settings as default and click OK.
9.8.3.6.In PeakSimple software select EDIT – Overall
9.8.3.7.Change default display period Start: 1 min., End: 10 min.
9.8.3.8.Leave other settings as default and click OK.
9.8.4. Load and inject sample.
9.8.4.1.Fill Hamilton syringe with 100µl of sample.
9.8.4.2.Ensure the sample is free of air bubbles
9.8.4.3.Insert the syringe into sample injection port.
9.8.4.4.Inject sample into HPLC machine, ensuring air bubbles do not enter machine
9.8.4.5.Twist sample port injection port switch from LOAD position to INJECT position
9.8.4.6.Wait 10 seconds
9.8.4.7.Twist sample port back to LOAD position.
9.8.4.8.Remove syringe and empty contents into waste beaker.
9.8.4.9.Rinse syringe 3x with fresh mobile phase solution.
9.8.5. Note the time at the center of the sample peak on the chromatograph.
* The AdvanceBio SEC 300Å protein standards will contain 5 distinct peaks
9.8.6. Save the data to a separate chromatogram file.
9.8.7. View the results [VIEW – Results..] and copy the data to a separate sheet in an Excel workbook.
9.8.8. Save chromatogram file with new name, and print chromatogram.
9.8.9. Retention time and area associated with each peak will print with the chromatogram.

9.9. Prepare HPLC system for short-term (1 week or less) or long-term (longer than 1 week) storage.
9.9.1. Short-term storage (1 week or less): Wash the system with mobile phase solution for 30 minutes at a flow rate of 0.25ml/min.
9.9.2. Long-term storage (longer than 1 week):
9.9.2.1.Flush the column with a minimum of 10 column volumes with 0.2µm filtered MilliQ water to remove buffer salts.

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9.9.2.2. Flush the column with a minimum of 10 column volumes with 0.2µm filtered 20% Ethanol.

Flush the column with 20%

9.10. Graph the calibration curve using excel:

9.10.1.1. Add new sheet to Excel workbook with 2 columns. Label the left column "Retention Time (min)" and the right column "Protein Size (kDa)"

9.10.1.2. Fill in the Protein Size column with the values 670, 150, 45, 17, 1.

9.10.1.3. Fill in the Retention Time column with the retention time of each peak corresponding to the AdvanceBio SEC 300Å protein standards.

9.10.1.4. Highlight the data table and create a scatter chart.

9.10.1.4.1. Insert Tab > Scatter Chart.

9.10.1.5. Reformat the y-axis (Protein Size) to the log scale.

9.10.1.5.1. Right click on y-axis > Format Axis. Axis Options Tab > Check Logarithmic scale [Base 10]

9.10.1.6. Insert a Trendline.

9.10.1.6.1. Click on chart > + sign in upper right > Trendline ▶ More Options…> Trendline Options Tab > Select 'Exponential', Select 'Display Equation on Chart', Select 'Display R-squared value on chart'

9.10.2. Estimate size of mAb drug product by inserting retention time into equation of line derived from AdvanceBio SEC 300Å protein standards

9.10.2.1. Substitute 'x' in equation for retention time of mAb drug product peak.

9.10.2.2. Solve for 'y', protein size in kDa.

10. Attachments:

10.1. Chromatogram of AdvanceBio SEC 300Å protein standards run at pump speed of 0.25ml/min. x-axis: Retention time; y-axis: absorbance at 220nM

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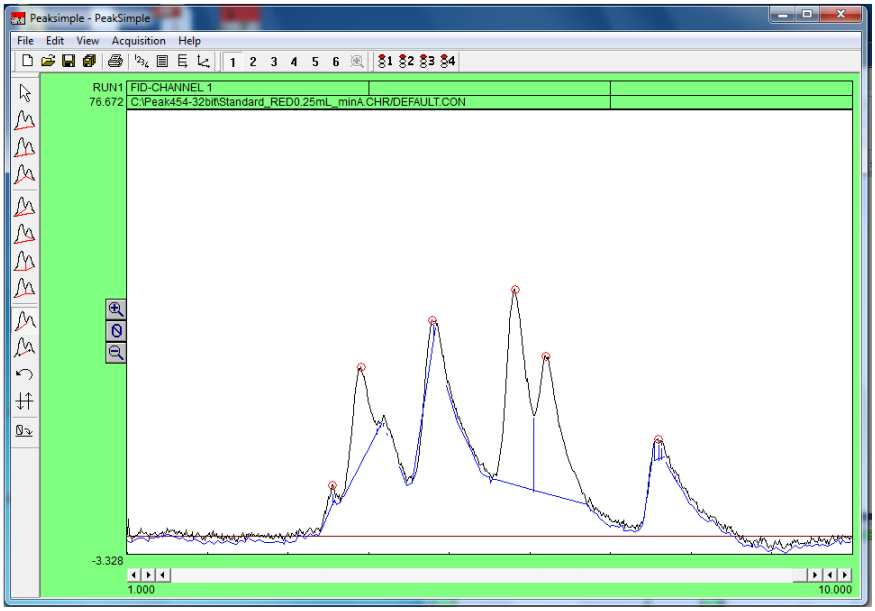

10.2. Chromatogram of anti-IL-8 mAb drug product run at pump speed of 0.25ml/min
x-axis: Retention time; y-axis: absorbance at 220nM

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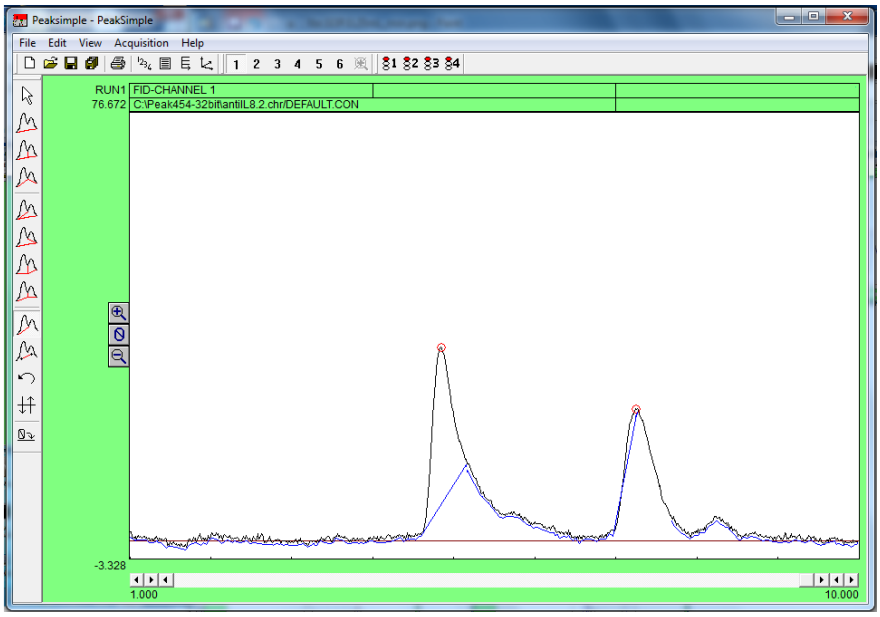
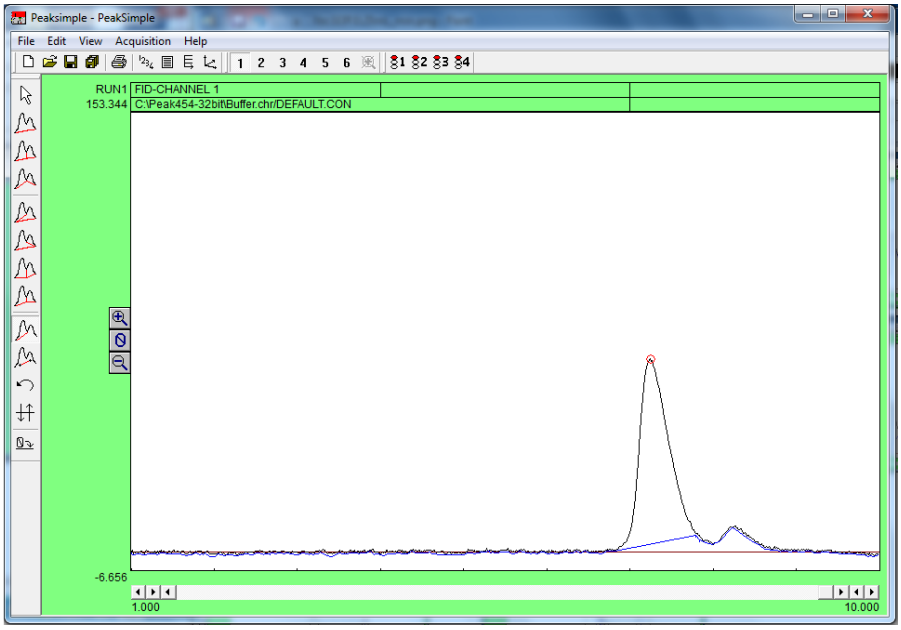


10.1. Chromatogram of mAb buffer run at pump speed of 0.25ml/min.



10.4 Standard curve

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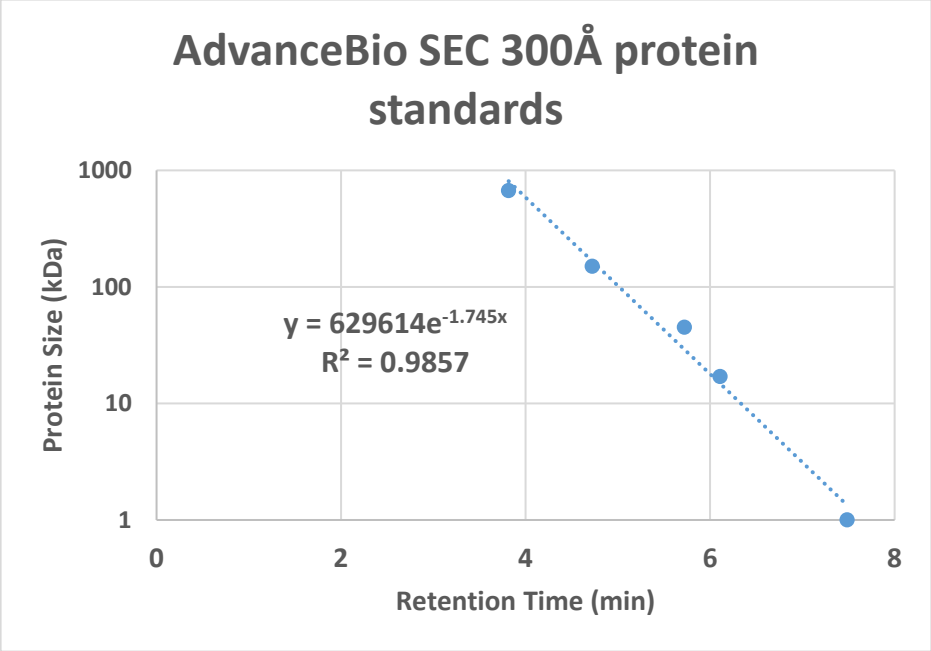
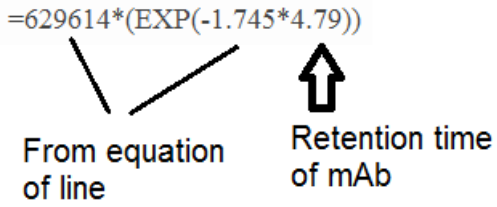


10.5 Example of equation typed into Excel to solve for the size of a mAb that exhibited a retention time of 4.79 seconds using the equation of the exponential line shown in 10.4.



11. History:

Name	Date	Amendment
Dr. Matt Marshall	04APR2019	Initial release