SOP Title:	**Procedure for Analytical Size - Exclusion Chromatography with Online UV-Visible, Refractive Index and Multi-Angle Light Scattering Detectors Using Agilent's HPLC in Conjunction with Wyatt's T-rEX (RI), TREOS II (MALS) and QELS Systems**
SOP Number:	**16134**
Revision:	**07**

TABLE OF CONTENTS

1. PURPOSE

Text This describes the basic operation of the Wyatt TREOS II (MALS) with embedded Quasi-Elastic Light Scattering (QELS) and the Optilab T-rEX Refractive Index (RI) detectors for data acquisition, processing and analysis using the ASTRA 7 (or current upgraded software version) software provided by Wyatt Technology. These systems are used online with a High-Pressure Liquid Chromatography (HPLC) 1100 from Agilent Technologies. From the MALS, QELS and RI signals and dn/dc of protein, the molecular parameters i.e., molecular weight (Mw) and its distribution, hydrodynamic radius (Rh) and the Polydispersity (Pd) can be estimated, and molecular aggregation detected.

2. SCOPE

This SOP applies to Process Analytics\Quality Control (PA/QC) Staff who use the MALS/RI detector system with online SEC-HPLC. The procedure also describes the maintenance routine followed after each operation.

3. RESPONSIBILITIES

3.1 Director / Technical Operations, Process Analytics\Quality Control (PA/QC)

- Defines procedure

3.2 PA/QC personnel

- Performs procedure

3.3 Biopharmaceutical Quality Assurance (BQA)

- Provides quality oversight

4. MATERIALS AND REAGENTS

NOTE: Form 16134-03 should be used to enter the details of the equipment and materials used in the assay.

Part Number	Description	BDP Approved Substitution Permitted?
N/A	Exclusion Column and Guard Column	☒ YES ☐ NO
10115	Methanol	☒ YES ☐ NO
20192	Filter Unit, 0.5 L, 0.2 um Cellulose Nitrate	☒ YES ☐ NO
20194	Filter Unit, 1.0 L, 0.2 um Cellulose Nitrate	☒ YES ☐ NO
20394	Eppendorf tube, 1.5 mL	☒ YES ☐ NO
20730	Autosampler vials	☒ YES ☐ NO
21449	Autosampler vial inserts	☒ YES ☐ NO
21470	Pipette tips – 200 µL (Rainin)	☒ YES ☐ NO
21471	Pipette tips – 1000 µL (Rainin)	☒ YES ☐ NO
21472	Pipette tips – 20 µL (Rainin)	☒ YES ☐ NO
30007	Phosphate Buffered Saline, Gibco (or suitable mobile phase as determined during method development of the product)	☒ YES ☐ NO
30060	Albumin Standard, Thermo Scientific, 2.0 mg/mL	☒ YES ☐ NO

Part Number	Description	BDP Approved Substitution Permitted?
30223	Gel Filtration Standards, Range 1,350 to 670,000Da, Bio-Rad	☒ YES ☐ NO
30622	Sodium Azide, Mallinckrodt	☒ YES ☐ NO

5. EQUIPMENT

- TREOS II- , Wyatt Technology, Corp. BDP MEF85780, or BDP-approved equivalent.
- Optilab T- rEX-RI Detector, Wyatt Technology, Corp. BDP MEF 85790, or BDP approved equivalent.
- Diode Array Detector, Agilent Catalog G1315A, BDP MEF 75001, or BDP approved equivalent.
- Column Compartment, Agilent Catalog G1316A, BDP MEF 75002, or BDP approved equivalent.
- Thermostated Autosampler, Agilent Catalog 1330A, BDP MEF 75000, or BDP approved equivalent.
- Quaternary Pump, Agilent Catalog G1311A, BDP MEF 75006, or BDP approved equivalent.
- Solvent Degasser, Agilent Catalog G1322A, BDP MEF 75004, or BDP approved equivalent.
- Computer System/Systems with Windows operating system and OpenLAB Chromatographic Data System [CDS] v 2.7 from Agilent and ASTRA 8.1 from Wyatt Technology, software programs.
- Sartorius Type I Water System MEF LWPS-009-B or equivalent.
- Calibrated Pipettes - 0.2, 10, 20, 100, 200 and 1000 µL (Rainin, Labsystems or equivalent).

6. SYSTEM STARTUP PROCEDURES FOR HPLC, TREOS II, AND T-REX

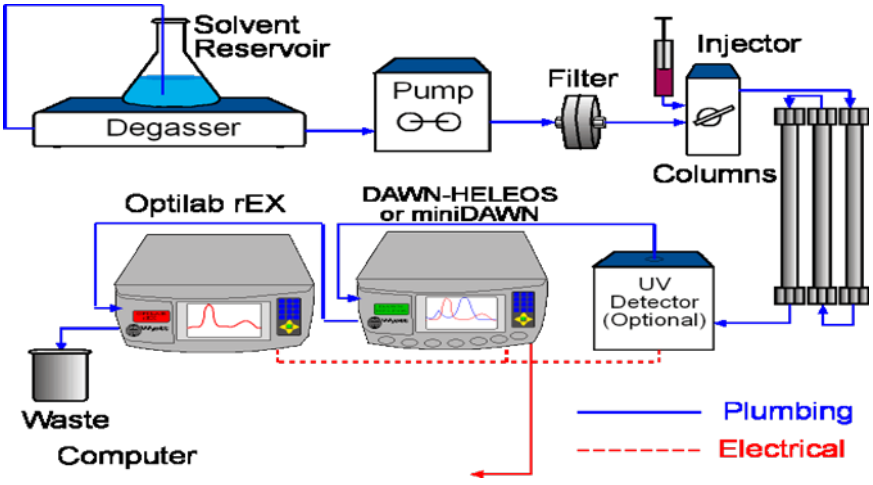
6.1 Refer to SOP 22178 Operation of the Agilent Technologies 1100 HPLC/1200 RRHPLC Using OpenLAB Chromatographic Data System (CDS) v 2.7.

6.1.1 Section 6.1 Hardware: Priming and Purging System for flushing the HPLC system when preparing the system for runs.

6.1.2 Section 6.2 Software: Familiarization with OpenLab CDS v 2.7 onwards which describes in detail how to log into the OpenLab CDS Control Panel, how to Create a Project, how to Launch the instrument, how to Create New Acquisition Method, how to Create a New Sequence and any other details pertaining to the SEC data.

6.1.3 Ensure that the appropriate size-exclusion column is connected to the HPLC. Flush water (0.2um filtered, 0.05% Azide water) through the system and ensure there are no leaks. To protect against residual particulates always use a suitable guard column with the analytical column. The order in which the systems are connected is shown in **Figure 1**.

Figure 1 Typical Setup of UV-MALS-RI Detectors



NOTE: All mobile phase should be 0.2 um filter. Run water through the system for 0.5 hour to expel the 20% methanol retained in the system from the last wash. This prevents the possibility of precipitation of salt with organic solvent.

6.2 MALS [TREOS II] and the RI [T-rEX] Systems are turned ON simultaneously with the HPLC system and allowed to equilibrate in the required mobile phase for > 2 hours until the baselines displayed on their screens are steady. Ensure that the laser is turned ON in MALS and the PURGE is ON in the Optilab rEX so that both the reference and the sample chambers are continuously flushed in the running buffer. The PURGE should be OFF prior to starting the run sequence.

Figure 2 Front Panel Keypad

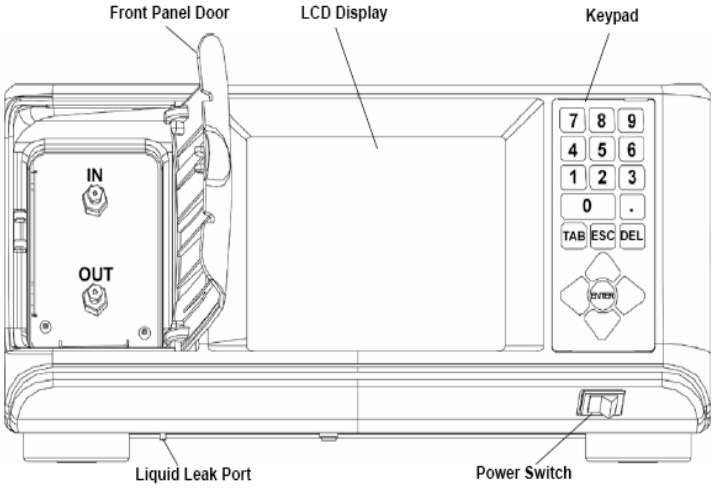


Figure 2-1: Front panel

6.2.1 The keypad (**Figure 2**) consists of a number, Tab, Esc, Del, Enter and arrow keys. You use these keys to move through the tabs on the LCD display on the instrument front panel and enter values where applicable.

- **TAB** key: Move forward to the next field within the tab.
- **ESC** key: Exit a pull-down list to cancel an entry.
- **DEL** key: Remove the previous character from a numeric entry field (similar to Backspace on a regular keyboard).
- **ENTER** key: Save an entry made using the number buttons, 'click' a button, select an option, or choose an item in a pull-down list.
- **Up** and **Down Arrow** keys: Move to a different selection within a pull-down list or radio field.

- **Right** and **Left Arrow** keys: Move to another tab or move the cursor within a numeric field.

6.3 Creating a Template on Astra 7

6.3.1 Under Programs > Wyatt Technology > Open Astra 7.1.3 (or upgraded Astra version)

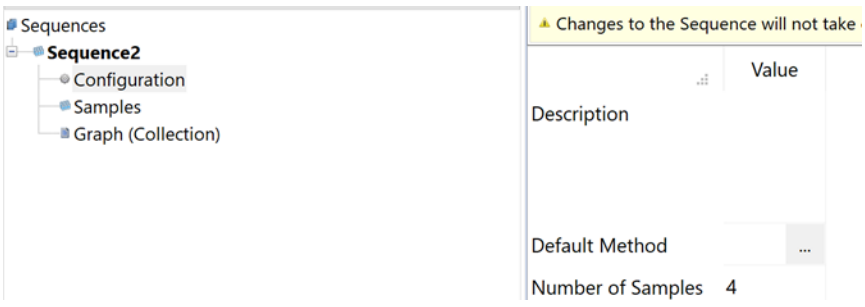
6.3.2 Go to File>New>Blank Sequence. This opens a blank sequence in the ASTRA workspace on the left-hand side (an appropriate method template for a standard run in PBS Aqueous was created and stored under an appropriately named folder at the time of system installation).

6.3.3 'Sequence 1' will appear on the workspace on the left side of the screen. The configuration, appropriate for this system set-up, i.e. UV+LS+QELS+RI has been configured during the instrument installation process.

6.3.4 Double click Configuration and a screen will open on the right. In the **Default Method** field on the right, click the 'browse' button and then navigate to and select the ASTRA method to be used for the sequence (**Figure 3**).

6.3.5 In the **Number of Samples** field, manually enter the number of samples to be run in the sequence.

Figure 3



6.3.6 Click the **Apply** button near the bottom of the window to save the changes.

6.3.7 Click the **Samples** tab on the left or bottom of the window to open the sample information page.

6.3.8 Set the following properties for the sample. (Any values entered will override the method settings).

Figure. 4

	Vial	Enable	Name	Descripti...	Inj	Method	Duration (min)	Inj Vol (µL)	Delay	dn/dc (mL/g)	A2 (mol mL/g^2)	UV Ext (mL/(mg cm))	Conc (mg/mL)
1	Vial 1	☑			1	...	1.000	0.00	0.000	0.0000	0.0000e+00	0.000e+00	0.000
2	Vial 1	☑			1	...	1.000	0.00	0.000	0.0000	0.0000e+00	0.000e+00	0.000
3	Vial 1	☑			1	...	1.000	0.00	0.000	0.0000	0.0000e+00	0.000e+00	0.000
4	Vial 1	☑			1	...	1.000	0.00	0.000	0.0000	0.0000e+00	0.000e+00	0.000

Vial: Can be left at 0 or can indicate the specific vial position in the autosampler.

Enable: Enable each box of the sample to be run with a check mark. Astra will not collect the data for the sample where this box is left unchecked. (The default is to have it checked).

Name: This is the file name that will be used when the file is saved on the hard drive. Do not use special characters.

Description: Sample description and relevant information.

Inj.: Number of injections to be made from the vial.

Method: This field defaults to the method selected in Sec.6.3.4. To use a different method, use the ‘browse’ button to navigate to the desired method.

Duration: Enter a value that is a minute less than that set in the chromatography run so that the software has time enough to complete data collection for this run and get ready for the next run.

Inj. Vol (µl): Injected volume (optional).

Delay: Optional. Typically recommended to leave it at 0.0

dn/dc (ml/g): Provide the dn/dc value of your sample (optional).

A2: Optional. Leave this as 0.

UV ext. coeff. (mL/(mg.cm): Enter this value for your sample if UV is your concentration detector. It is optional if RI is the concentration detector.

Concentration (mg/mL): Sample concentration (optional) or the concentration detector can calculate the value.

6.3.9 Click the **RUN** button near the top of the window to begin the sequence collection.

6.3.10 A 'Save' window should be displayed. Navigate to the location on the hard drive where you want to save the ASTRA data and enter a name for the Sequence in the 'File Name' field. Click '**SAVE**'. The data files generated by this Sequence will have the format "Name [SequenceName].afe 7 extension.

6.3.11 To create new sequences from this sequence, right click on the sequence name and select **Save as Template**.

6.3.12 A message '**Waiting for Auto Injection**' should be displayed.

6.4 System Suitability Test for HPLC

Gel Filtration Standards vial from Bio-Rad (Section 5.9.1) is rehydrated in 0.5 mL of WFI water. It is then diluted 1:10 in the mobile phase. The injection volume is 10µl.

A typical Gel Filtration Standard profile, for an injection volume of 10 µL, consists of five major peaks: Thyroglobulin (670 kDa), Gamma Globulin (158 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa), and Vitamin B12 (1.35 kDa). The criteria for system suitability are based on the measurement of column efficiency or the theoretical plate count N using the formula N = 5.54 (t/W)2 where t = retention time of peak and W=peak width at ½ peak height of the B12 peak. An N value of > 15,000 will be an acceptable column efficiency value.

6.5 System Validation of MALS (TREOS II)

Normalization coefficient, alignment, and band broadening corrections are determined before system validation. A mono-disperse; low molar mass protein such as bovine serum albumin is used for validation of the system. About 120 µL of Albumin Standard (Section 5.9.2), at 2 mg/mL concentration, is pipetted into an auto-sampler vial with an insert and 100 µL of the sample is injected into the system.

- Albumin Standard has a theoretical molar mass of 66.4kDa. The Astra software calculates the molecular parameters (molar mass, hydrodynamic radius and polydispersity) of the sample using a dn/dc of 0.185 mL/g, solvent RI and the instrument constants. A typical profile should show a monomer peak along with a well-separated dimer, trimer, and some higher molecular weight (Mw) component peaks. A calculated Mw range of 62.9-68.3kDa should indicate a properly working system.

6.6 Determination of Injection Volume for Unknown samples

The injection volume is determined by the light scattering signal to noise ratio, which is molar mass and concentration dependent and depends on a smooth stable baseline. The injection volume should be determined depending on the initial sample concentration. Sample dilution should be carried out only if necessary. These parameters should be calculated for individual proteins at the time of assay development.

6.7 Sample information, sample preparation and other assay details along with data file location should be completed in **Form 16134-01** at the time of assay performance.

NOTE: Sample injection amount depends on the molar mass of the sample protein, its concentration, and the signal/noise ratio. A minimum signal/noise ratio of 50 is recommended to enhance quality of measurements. The signal/noise ratio should be determined for individual proteins. The product – specific conditions determined at the time of assay development by the development scientist should be entered in **Form 16134-02** and completed by the analyst performing the assay.

7. SEC-MALS DATA PROCESSING

7.1 Data processing is preferably done at the end of data collection. Go to File>Open>Experiment and navigate to the respective ASTRA 7 experiment. This should open the experiment in the ASTRA workspace on the left hand of the ASTRA display. Expand the (+) under Procedure.

7.1.1 Highlight the name of the experiment in the workspace.

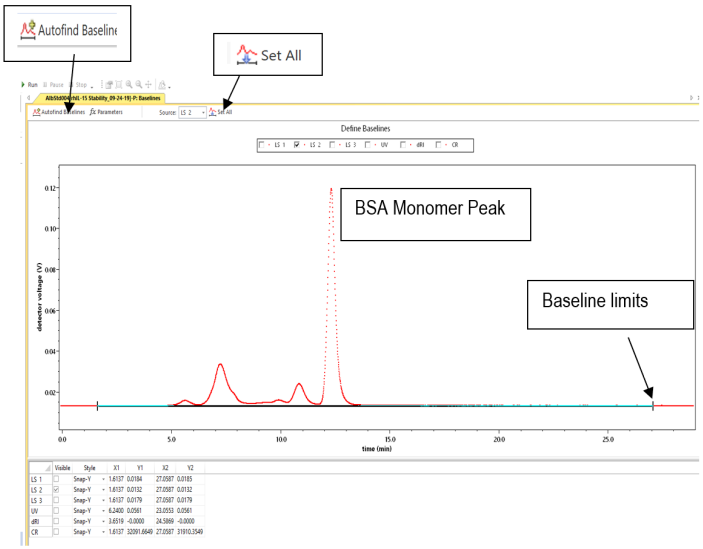
7.1.2 Next, click icon under 'Processing' at the top.

If baseline parameters were not defined in the method used, a message may appear saying 'you do not have any baseline defined – please specify the baseline for your data now.' If the message appears, click OK. The 'define baseline' window should appear. If not, double-click Baseline under Procedure and proceed to Step 7.2 and set the baselines for your data.

7.2 Setting Baselines

7.2.1 From the legend, select the 90° detector, i.e., detector 2 in this case. Now set the baseline for detector 2 by clicking and dragging a baseline with the left mouse button from a region well before any peaks to a region well after any peaks (**Figure 5**).

Figure 5



NOTE: It is recommended that the region of interest be viewed by 'Zooming IN' (hold down the CTRL key and select the region with the left mouse button). To return stepwise to the previous view, hold the CTRL key and right click on the chromatogram.

7.2.2 You can click on the '**Autofind Baselines**' button near the top of the window to automatically set the baselines for all detectors simultaneously. Alternatively, the baselines can be manually drawn for the source detector (LS 2=900). Left click and hold onto the ends of the baselines and drag them from well before any peaks to well after any peaks, i.e., set in a flat region where detector signal comes from pure solvent or mobile phase. Click on the '**Set All**' icon to apply the source baseline to all other detectors. If necessary, adjust baseline limits for any single detector.

7.2.3 Once the baselines for all the LS detectors, UV and RI detectors are set, close the Baselines window by clicking the **OK** button at the bottom.

7.2.4 A message may appear indicating that some of the necessary peak parameters are not set and to specify peaks covering the regions of your data you wish to have analyzed. If this message appears click **OK** and the '**Peaks**' window should open. Proceed to Step 7.3. If not, double click on Peaks under Procedure to define the peak limits for the normalization Step 7.3.

NOTE: Alignment and band broadening need to be performed when the system is installed using suitable samples like BSA for an aqueous mobile phase. They need to be redetermined if the tubing connections (length or inner diameter) between the detectors are altered.

Normalization is to be performed once in a given solvent for MALS analysis. It needs to be redetermined if a different mobile phase is used in an analysis.

Periodic checks of alignment, band broadening and normalization are recommended.

7.3 Normalization Using the BSA Monomer Peak

7.3.1 The BSA start and end times are set to include only the central portion of the monomer peak (maximum LS peak height) by clicking and dragging the peak limits to the desired position (**Figure 6**). Uncheck and turn off the UV and RI signals from the legend on top. Click OK in the lower left corner to close the window and accept the peak settings.

Figure 6

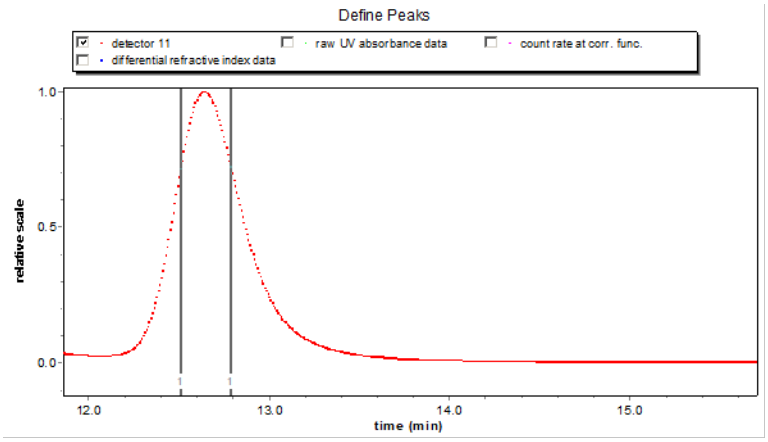
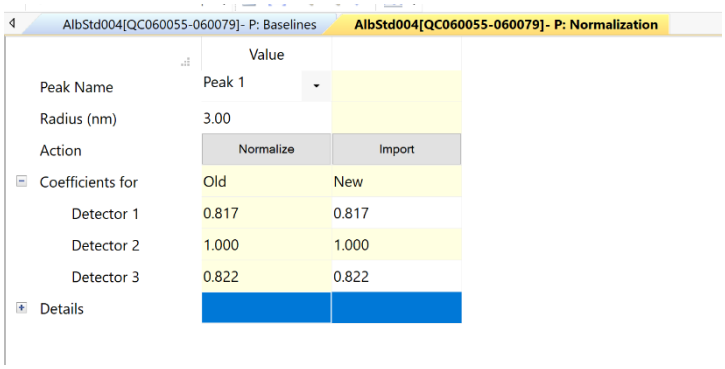


Figure 6a



7.3.2 To open the Normalize function, either right click on Configuration and select Normalize or select Experiment>Configuration>Normalize.

7.3.2.1 In the Normalize window, enter '1' for the peak name.

7.3.2.2 Click the Normalize button next to Action (**Figure 6a**) to execute the Normalizations. The old and new normalization values for the detectors will be displayed on the window.

7.3.2.3 Click OK in the lower left corner to close the window and accept the new normalization coefficients.

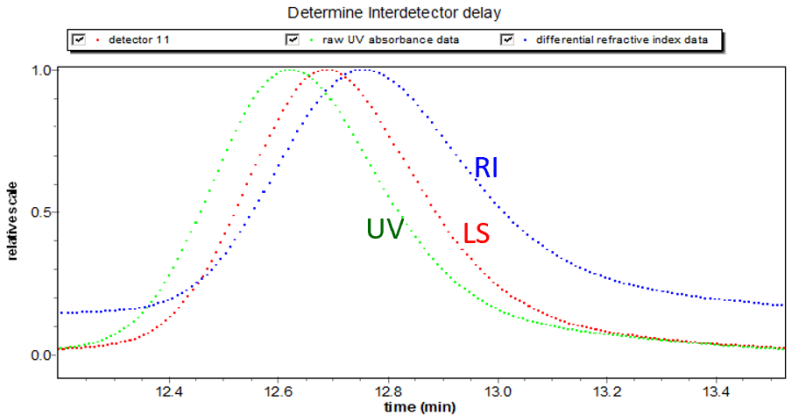
NOTE: The 90° detector (detector 2) should have a normalization coefficient of 1.00, whereas the rest of the TREOS 1, 3 detectors should have a coefficient value close to but not equal to 1.00.

7.4 Setting the Inter-detector Delay Volumes

NOTE: The baselines and the peaks must be set before the delay volumes can be set. This procedure needs to be performed only once when the instruments are connected or tubings between instruments are changed. The inter-detector delay volume remains the same until you change the length of tubing between the instruments or change the instrument sequence.

7.4.1 Right click on Configuration and select Alignment. Expand the peak region by holding down the CTRL key while clicking and dragging a zoom box around the apex of the peaks from all detectors (**Figure 7**).

Figure 7



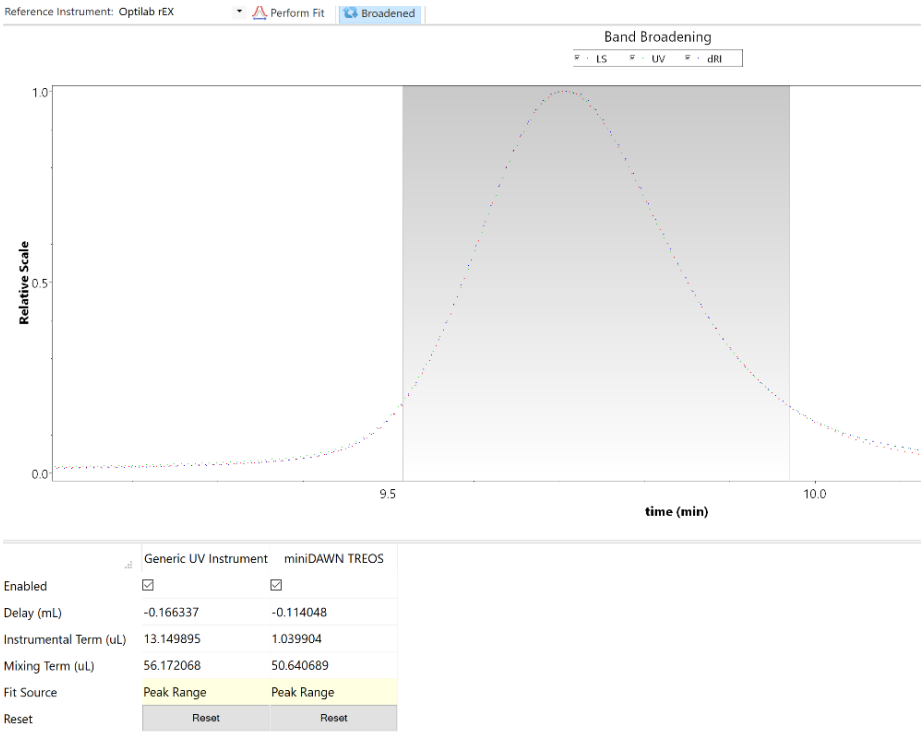
Click on **Align Signals** above the plot. The peaks from all the three detectors should now be overlaid. Click the 'OK' button near the bottom of the window to save these delay volumes.

7.4.2 Right click on **Configuration** and select **Band Broadening**. Select the instrument that has the broadest signal, i.e., RI detector here.

7.4.3 Make sure Enabled is checked for all instruments (**Figure 8**).

7.4.4 Click and drag to select a range that spans the major peak (**Figure 8**).

Figure 8



7.4.5 Click **Perform fit** icon at the top of the window. Typical correction terms for the three signals at the end of this function are approximately 1.0µl (instrument term) and approximately 50µl (mixing term).

7.4.6 Use the **Broadened** button to toggle the broadening on and off. If the match between the peaks is not good, repeat the fit.

7.4.7 Click **OK** to save the band broadening correction. Right click on the name of the experiment and select **Save** to save this experiment with the above changes.

7.5 Setting the Peak Regions for BSA Monomer.

7.5.1 Double click on Peaks procedure to open the Define Peaks window. After the earlier procedures, the peak region of the monomer requires a little adjustment. The region shown in **Figure 9** is selected.

NOTE: The peak limits may be adjusted by placing the cursor on the peak start or end vertical bars, clicking, and dragging to a new position.

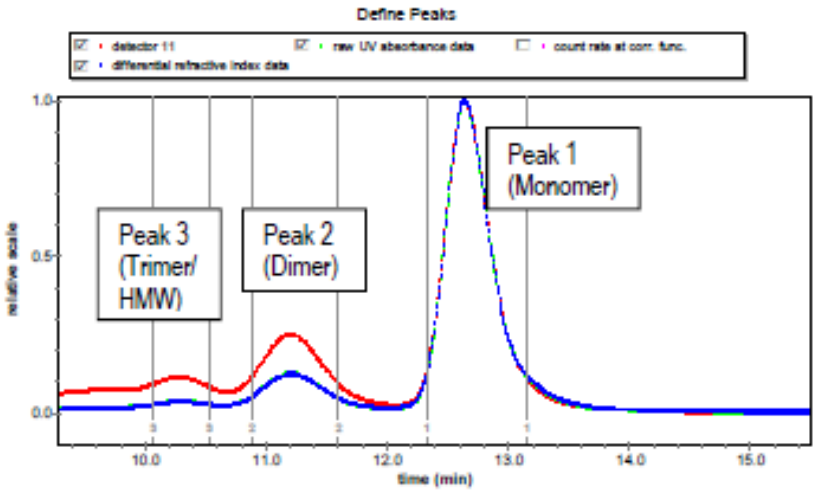
Multiple peaks may be set, and the Abscissa Units may be changed by double clicking on Configuration and selecting the desired unit, e.g., minutes or mL.

7.6 Computing the Molar Mass of BSA Monomer, Dimer and Trimer

7.6.1 Double click on Peaks procedure to open the Define Peaks window.

7.6.2 Set the three peak regions, one for the large suspected BSA monomer peak, one for the smaller suspected dimer peak and one for the suspected trimer peak by clicking and dragging from the desired peak start to desired peak end (**Figure 9**) and click OK at the bottom of the window.

Figure 9



7.6.3 Expand the Results folder in ASTRA workspace by clicking the '+' sign and double click Report Summary for results in report format.

7.6.4 The EASI Graph in the ASTRA'S workspace can be opened to display plots of molar mass vs time or volume. By changing the Distribution Type to Cumulative Weight Fraction, the cumulative weight fraction distribution can be determined from the peak ranges for the monomer, dimer, and trimer. Similarly, by clicking on Rh from QELS, under Procedure, the correlation function graph and the count rates are displayed. By left clicking with the cursor placed on a peak, the size distribution of the molecule at that region of that peak is displayed below. For more details, refer to the ASTRA software manual.

7.6.5 Processing Unknown Chromatograms

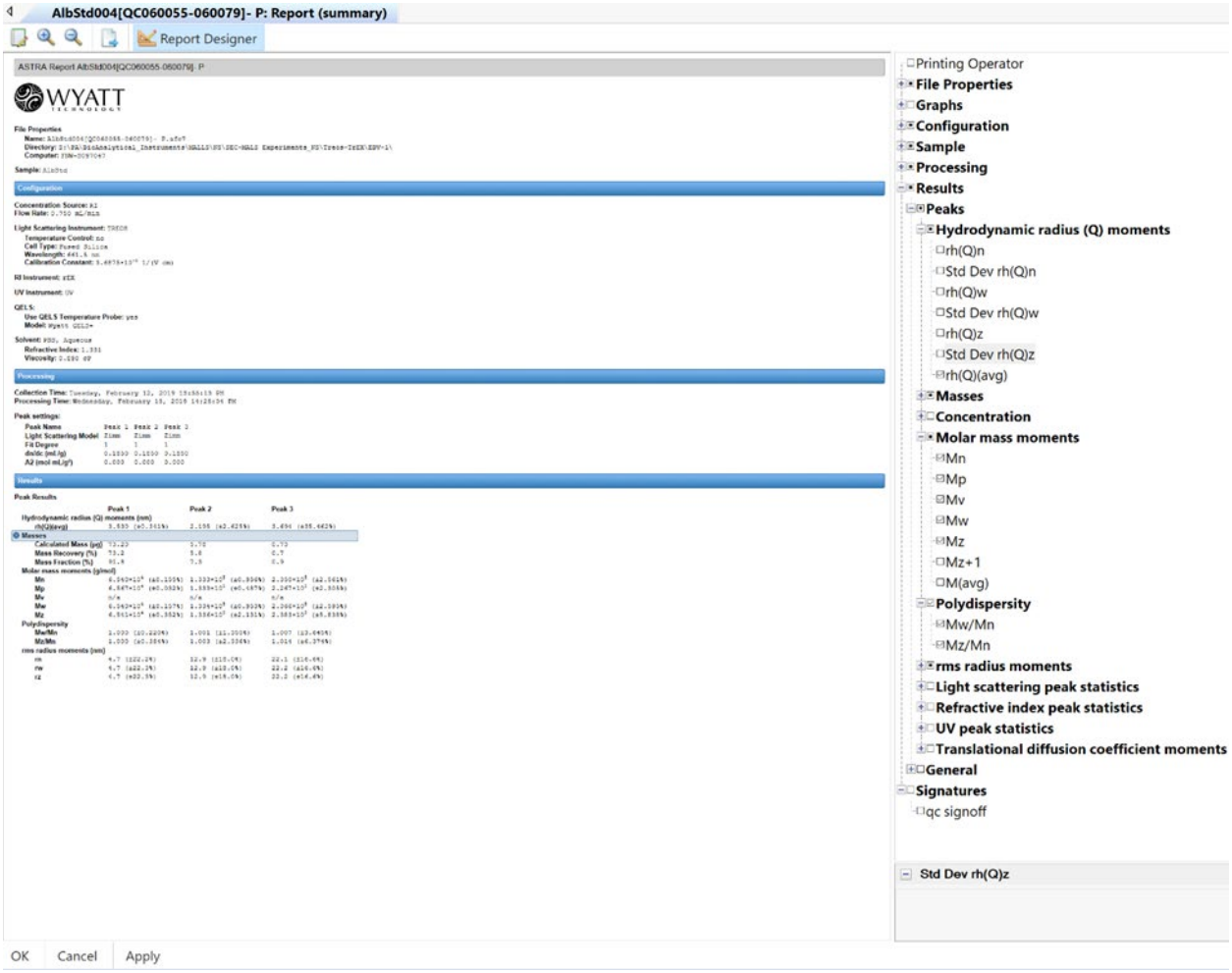
7.6.5.1 For analysis of an unknown sample go to File>Open>Experiment and navigate to the respective file and double click to open. Repeat steps under Sec. 7.1.2 to 7.2.3. Next, double click on Peaks under Procedures to open the **Define Peaks** window.

7.6.5.2 Select the product component peak regions (monomer, dimer, etc.) and set the limits by clicking and dragging from the desired peak start to the desired peak end (similar to the steps under Step 7.5 described for BSA) and click OK at the bottom of the window.

7.6.5.3 Proceed with Step 8.0 for results and report.

8. RESULTS AND REPORT

Figure 10



8.1 For a Custom Report of the results expand the Results tab on the left (summary or detailed). The window on **Figure 10** opens showing the results of the experiment.

8.1.1 Click on **Report Designer** icon at the top of the window to display the Report Designer pane on the right side. Expand each category and by checking or unchecking each item, within the category, the required item can be included or excluded from the custom report.

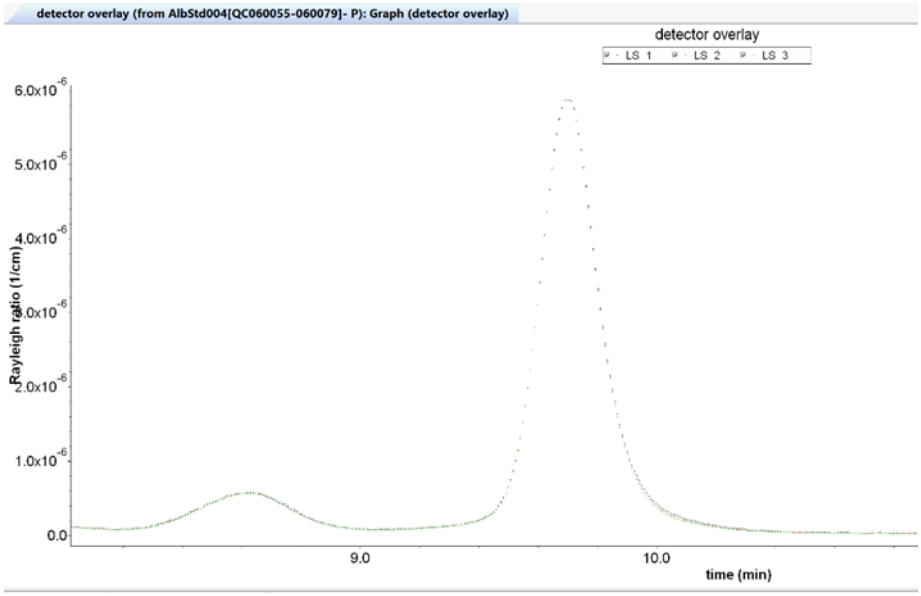
8.1.2 Click OK or Apply to save the report.

8.2 Assessing Quality of Data

8.2.1 Ensure that the baselines have been set, normalization performed, and the peak region has been set to include the entire BSA monomer peak.

8.2.2 Right click the file name in the workspace. Then Apply Method> Diagnostics>Detector Overlay. This opens a new experiment that has generated the Detector Overlay plot from the original experiment. Expand Results and select Graph for Detector Overlay from the workspace. If the flow cell is clean, then the peaks should overlay perfectly (**Figure 11**).

Figure 11



NOTE: For additional operational details refer to the help topics in **ASTRA 7** (or current upgraded Astra software version) User's Guide.

9. DOCUMENTATION AND RECORDS

9.1 All use of instruments, maintenance, and service must be documented in an issued equipment logbook as per **SOP 21531 - Equipment Logs**.

9.2 All solutions used for this procedure that are prepared must be documented as per **SOP 22702 - Solutions Used in Process Analytics**.

9.3 All experimental details and sample information must be documented in the issued laboratory notebook as per **SOP 21408 - Laboratory Notebooks Control and Use**, and **SOP 21409 - Good Documentation Practices**.

10. REFERENCES AND RELATED DOCUMENTS

Document Number	Title
16134-01	SEC-HPLC-MALS Assay Template
16134-02	Product Specific Details for SEC/MALS
16134-03	Equipment and Materials
21408	Laboratory Notebooks Control and Use
21409	Good Documentation Practices
21531	Equipment Logs
22178	Operation of the Agilent Technologies 1100 HPLC/1200 RRHPLC Using OpenLAB Chromatographic Data System (CDS) ChemStation Edition
22702	Solutions Used in Process Analytics
N/A	Wyatt Technology Astra 7 (or upgraded ASTRA version) User's guide

11. ATTACHMENTS

Attachment 1 Sample Custom Report

Attachment 1 Sample Custom Report