

TN6006: Eclipse and VISION Handbook

Summary

This handbook describes typical procedures, workflows, and best practices for operating Wyatt Technology's Eclipse™ (NEON™) with VISION™ 3 for field-flow fractionation (FFF), a technique for the separation of macromolecules. Although more information may be found in respective VISION™, ASTRA®, Eclipse™, Separation Channel, and Eclipse Mobility™ User's Guides, the aim of this technical note is to illustrate the key strategies for utilizing the combined hardware and software. Topics will include a discussion of the technical and theoretical aspects of asymmetric flow field-flow fractionation (AF4), how AF4 can be further enhanced by applying an electrical field (EAF4), and best practices when coupling these techniques with multi-angle light scattering (MALS) and other analytical detections (FFF-MALS).

The protocols outlined in this technical note will differentiate as best as possible between the Eclipse base model, the Eclipse with Dilution Control Module™ (DCM), the Eclipse with SEC switching option, and the Eclipse with both DCM and SEC switching option. Certain configurations, flushing, method attributes, and maintenance procedures will differ between models. Visit the Wyatt Support Center at www.wyatt.com/support for the latest resources for your system.

Related References

VISION User's Guide	M1103
Eclipse User's Guide	M3410
Channel User's Guide	M1061
Mobility User's Guide	M3420



VISION DESIGN



VISION RUN

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How to Use this Guide

This guide is organized by workflow—beginning with hardware and software installation and configuration before moving on to solvent exchange and channel membrane installation protocols. If your system is already solvent equilibrated and a new membrane is installed, you can skip ahead to procedures for an initial conditioning run and setting up methods and sequences in VISION. For running samples, some general method development considerations will be shared and how to simulate and develop methods *in silico* will be explained. This handbook will conclude with some maintenance, troubleshooting, and cleaning recommendations, as well as short and long-term storage recommendations.

Quick Reference Sheets

Although this guide will go into some detail for each procedure, one-page quick reference sheets are included that can be printed and used as a resource once the general workflow is understood. These are compiled in [Appendix A – Quick Reference Sheets](#) and are also included in relevant sections:

- Basic AF4 Theory ([Page 220](#))
- Overview of Fluid Connections ([Page 221](#))
- Channel Membrane Installation ([Page 222](#))
- VISION: Running a Sequence ([Page 225](#))
- Eclipse System Best Practices ([Page 224](#))

Notes (Record method names and file locations here for easy reference):

Software	Item	Name(s) & File Location Information
 VISION RUN	VISION RUN Standard Method	
 VISION RUN	VISION RUN “Template” Method	
 ASTRA	ASTRA FFF Method	
 VISION RUN	Solvent Exchange Method	
 VISION RUN	Sequence File or “Template”	
	Other:	

Eclipse and VISON Handbook FAQ Shortcuts

Search the table below for common questions and the relevant sections. You can search (Ctrl + F) this document for relevant keywords. If you can't find what you are looking for, please reach out to Wyatt Technology Support at support@wyatt.com. If you need urgent assistance, please call us at +1 (805) 681-9009 option 4.

FFF-MALS System Questions

Objective	Relevant Section
What is FFF or AF4? How does it work?	Pages 10, 11, 226
How do I reconnect my system after preventative maintenance or service?	Pages 12, 29
What is the dilution control module (DCM)?	Pages 94, 235
What are the recommended HPLC settings?	Pages 19, 36
How do I perform a solvent exchange for my HPLC and Eclipse?	Pages 53, 113
What's the difference between my legacy AF4 or DualTec and the Eclipse?	Page 207

VISION Software Questions

Objective	Relevant Section
How do I reconnect my Agilent HPLC in VISION?	Page 31
What are the recommended HPLC and Eclipse settings in VISION?	Page 36
What are the different flows in FFF-MALS?	Page 93
How do I analyze mobility data?	Page 174
What are some conditioning methods?	Page 118
How do I run a sequence?	Page 104
How do I configure and view 3D spectral data?	Page 103, 162
How do I run mobility experiments?	Page 174

Eclipse Hardware Questions

Objective	Relevant Section
How do I replace the channel membrane?	Page 59
How do I use the Eclipse manual control for applying flows and checking the system?	Page 42
How do I calibrate the Mobility Module?	Page 191
Do you have a flow diagram for understanding the fluidics?	Page 243

Troubleshooting & Maintenance Questions

Objective	Relevant Section
What are some best practices for maintaining my system?	Page 224
How do I replace the channel membrane and/or spacer?	Pages 59, 222
How do I select a membrane material for my experiments?	Page 56
How do I clean my channel?	Page 202
How do I clean my Eclipse system?	Page 198
What can I do to resolve high pressure?	Page 210
What can I do to resolve poor flow regulation?	Page 210

Introduction

Field-flow fractionation (FFF) is a separation technique where a field is applied to a solution through a channel to separate macromolecules, typically by exploiting some physical property like diffusion. In flow FFF, the separation is achieved based on hydrodynamic size. When coupled with analytical detectors like multi-angle light scattering (MALS), the absolute characterization of macromolecule molar mass distributions and sizes can be acquired in a single fluid pathway downstream of the FFF separation device.

A variation of this technique, referred to as asymmetric flow field-flow fractionation (AF4), utilizes a single semi-permeable membrane on the bottom of the channel (referred to as the accumulation wall) and is capable of separating species from the 1 nm radius range to the 10 μm radius range. This technique can be enhanced by siphoning off pure mobile phase to concentrate the sample (using the dilution control module, DCM) entering downstream analytical detectors or by simultaneously applying an electrical field (with the Eclipse Mobility) for electrical AF4 (EAF4).



Figure 1. This guide is written for the Eclipse (NEON) with VISION 3 software.

Asymmetric Flow Field Flow Fraction (AF4) Overview

AF4 separation depends primarily on the equilibrium between an applied cross flow, which is achieved via mobile phase flowing through a semi-permeable membrane, and the diffusion of a sample due to Brownian motion against that force. An overview graphic of the separation process for AF4 is provided in Figure 2.

This equilibrium results in statistically different heights above the membrane as a function of the diffusion coefficient, and therefore hydrodynamic size. A longitudinal channel flow, parallel to the membrane, is applied. Due to its parabolic flow profile (laminar flow), smaller macromolecules that have an equilibrium state higher in the channel will elute first, followed by larger macromolecules that experience slower flow velocities being closer to the bottom of the channel (the accumulation wall).

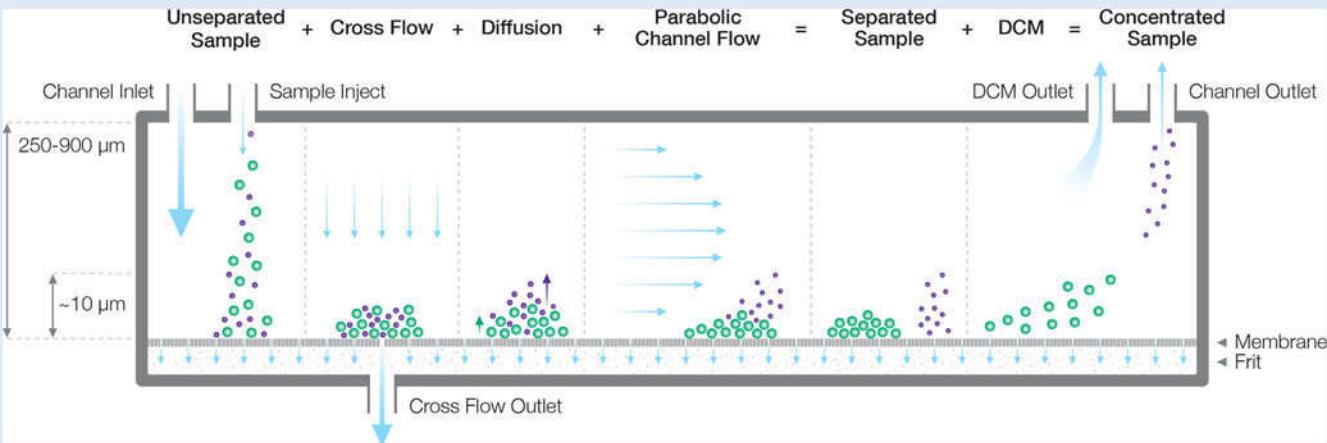
This elution order is reversed compared to the behavior in most size-exclusion chromatography (SEC) experiments. As a result, FFF-MALS is an orthogonal technique to SEC-MALS that has several advantages: (1) able to characterize larger aggregates that may be filtered or sheared by SEC columns; (2) applies less shear degradation stress on samples which can reveal true molar mass distributions; and (3) has wider dynamic range of separation due to variable cross flow and channel height, whereas SEC method development may require many different columns or variations of columns to achieve the same dynamic range.

Quick Reference Sheet: FFF Theory Overview

1  AF4 separation happens in a channel where flows are controlled by the Eclipse.

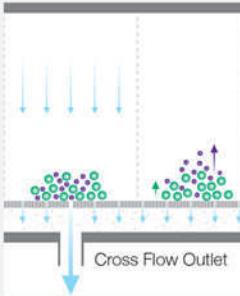
Variations and combinations of fluid flows enable separation by exploiting sample diffusion.

2 We'll look at a simplified breakdown of the separation process in the panels below. The figure below illustrates the key forces in play—which come together simultaneously to achieve separation.

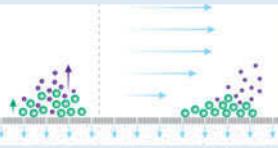


3 Sample is injected into the channel, where it may be concentrated into a narrow band in a step called "focusing." However, the main separation occurs during "elution." In the tunable elution step, cross flow pushes the sample to the channel's bottom, or "accumulation wall."

Although cross flow is pushing the sample down, the sample inherently moves against the force due to Brownian motion. Smaller species, which diffuse quickly, are statistically more likely to reach higher regions in the channel.



4 To exploit this equilibrium between cross flow and diffusion, channel flow is simultaneously applied. This laminar flow is parabolic in nature—flow near the edges is slower than flow near the middle. Smaller macromolecules, which diffuse more quickly, are statistically more likely to enter the faster fluid lanes, and so elute more quickly.



5 In the fractogram, typically small macromolecules will elute first, and larger macromolecules elute later. This order is reversed compared to size-exclusion chromatography (SEC).

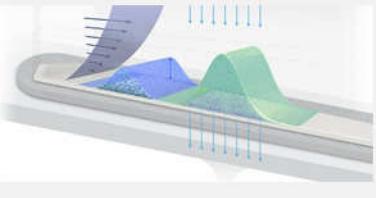


Figure 2. FFF Theory Overview Graphic

Hardware Installation Quick Guide

Your Eclipse should initially be installed by trained Wyatt scientists; however, it may be necessary to reconnect tubing lines, troubleshoot, or re-install an Eclipse that has returned from service or routine maintenance, as well as re-connect to an Agilent HPLC that has similarly been disconnected. As needed, this section provides an overview of the procedure for installing the Eclipse, connecting the HPLC system, and re-establishing communications with the software and computer.

Eclipse instruments that are returned from preventative maintenance (PM) or repair service, loaner instruments during service, and newly purchased Eclipse instruments come shipped with a solution of 50% alcohol in water, typically either an ethanol or isopropyl alcohol solution. If the HPLC and Eclipse are in co-miscible solvents, it is recommended to connect the fluid lines, establish communication with the VISION software, and then perform a solvent switch into the desired mobile phase. Steps for executing these procedures will be provided in subsequent sections, [Using Manual Control to Perform a Solvent Exchange with VISION](#).

An overview of the fluid connections and electrical connections is provided in Figure 3 and further explored in subsequent sections. Please see the [Eclipse Mobility Module Guide](#) section for information about fluidics for Mobility.

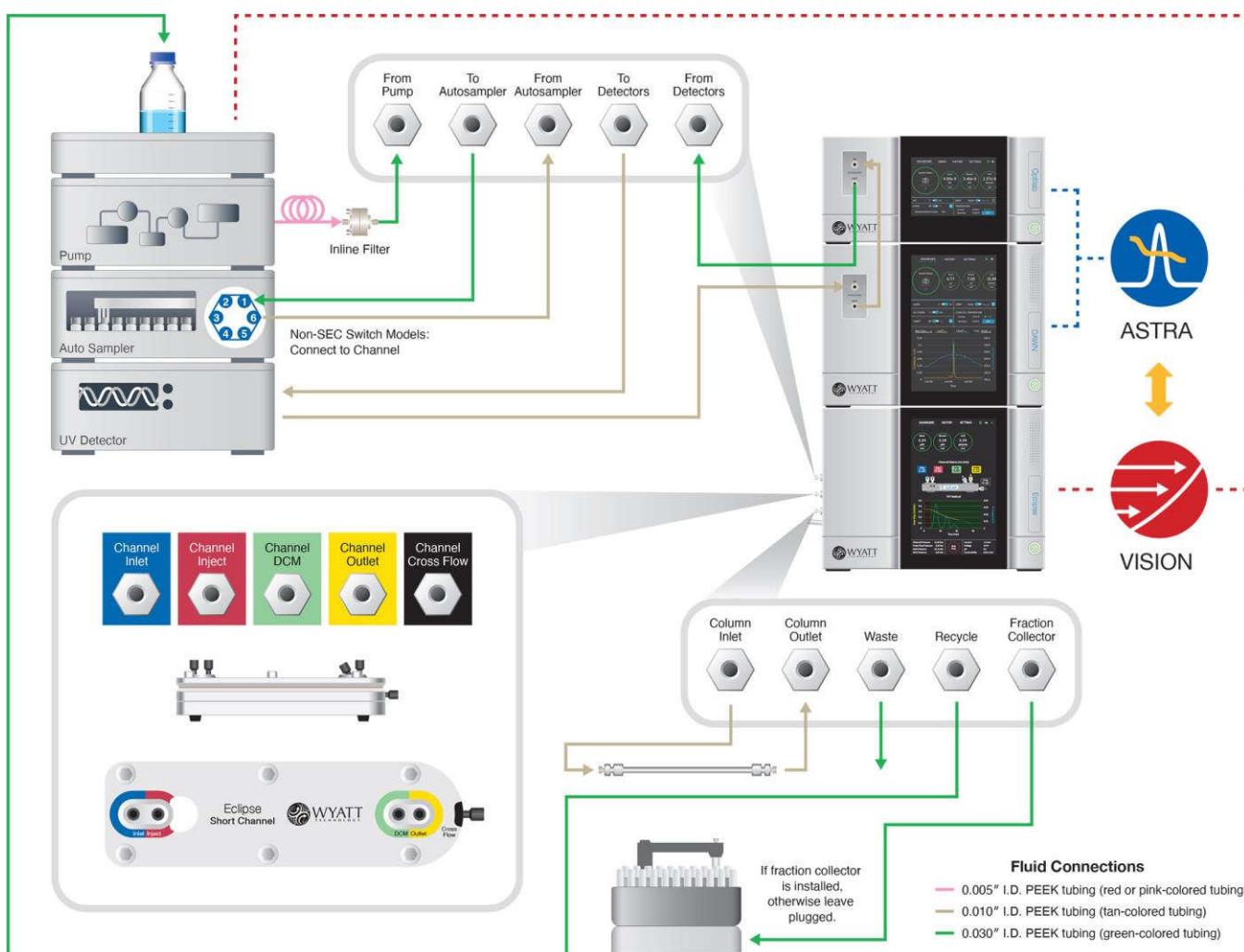


Figure 3. Overview of the Eclipse fluid and electronic connections.

Installing the Instrument and Network Connections

Once unboxed, the Eclipse is typically installed at the bottom of a Wyatt analytical detector suite. To minimize inter-detector tubing to reduce peak band broadening, the Eclipse should be placed to the right of the HPLC, as the fluid connections panel is situated on the left side of the Eclipse when viewing the front panel. You may consider placing the DAWN directly on the Eclipse and the Optilab on top to minimize tubing volume depending on the location of the UV detector. This is especially true if the Eclipse Mobility module is placed on top of the Optilab.

Analog detector signals, such as those from a UV, FLD, or third-party RI detector, are typically collected in the Wyatt DAWN for processing in ASTRA. These require a detector with analog output capabilities. Please refer to Appendix F – Agilent HPLC Connection Instructions for additional information on both the physical connections between Agilent and Wyatt hardware as well as instructions for configuring ASTRA to accept auxiliary voltages. Auto-inject signals from the Agilent HPLC can also be collected in the DAWN.

The Eclipse requires a power cable, an alarm out cable, and the Ethernet cable for communication with the computer. An analog signal, including a light scattering signal, can be configured into the Eclipse for collection in VISION for method development. The rear panel is shown in Figure 4.

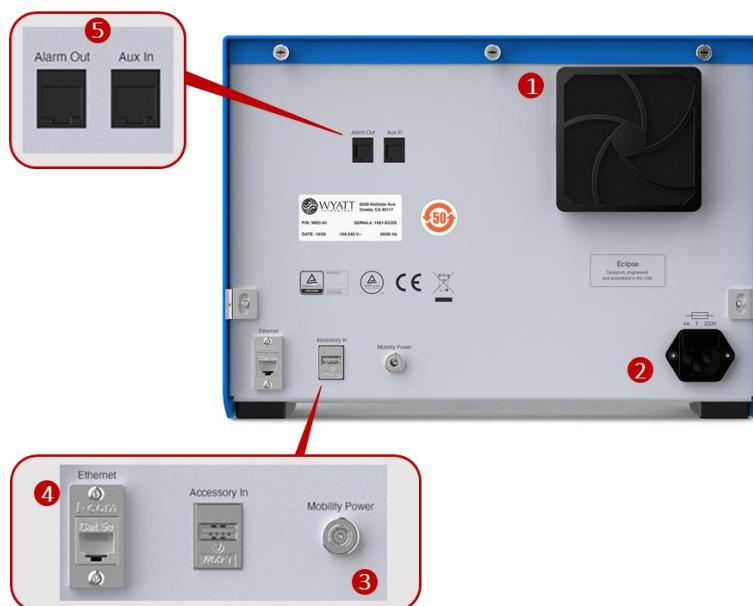


Figure 4. The back panel of the Eclipse: (1) The fan filter, which should be checked for dust and either cleaned or replaced once or twice a year; (2) the power cable—the Eclipse has a universal power input, so it is compatible with any 100-240 V (AC), single-phase, 50-60 Hz power source. A standard 125 V, 15 A power cable with a Type B plug is typically shipped; (3) the Mobility power connection—for connecting the Mobility module; (4) the ethernet port for communication to the computer via direct connection or through a switch box; (5) the RJ-12 inputs available on the Eclipse—the Alarm Out port can be used to export a signal when the Eclipse enters an alarm state and the Aux In can be used to collect an analog signal in VISION Design.

Alarms in the FFF-MALS system are controlled by the VISION software. Therefore, it is recommended to have the software launched whenever flows are applied to the system. In the event of loss of communication, critical alarms on the Eclipse—including overpressure or leak alarms—can stop the pump when a cable from the “Alarm Out” port is connected to the Agilent pump. For this additional safety, a special cable from Wyatt Technology (p/n 164704-2 for Agilent 1260 Infinity II) should be installed between the Eclipse and Agilent front end. With this cable, the Eclipse should be in an Active High state on the front panel settings tab in order to stop the pump when a critical alarm is observed in the Eclipse. If syncing a DAWN, Eclipse, and Agilent pump, the DAWN should be set to “Active Low” on

the front panel settings tab, the Eclipse should be set to “Active High” on the front panel settings tab, and the Agilent Pump is by default “Active Low.”

Analog signals from other detector instruments may be collected in VISION via the Aux In port with an RJ-12 cable. For cables supplied by Wyatt Technology, there are colored-coded pins: 1 (White); 2 (Black); 3 (Red); 4 (Green); 5 (Yellow); and 6 (Blue). Analog In 1 is collected on Red (Positive) and Green (Negative), whereas Analog In 2 is collected on White (Positive) and Black (Negative) pins.

Eclipse Network Connections

Typically, a 5-port or 8-port Ethernet (RJ-45) network switch box is used to connect Wyatt analytical detectors and the Agilent HPLC to a local computer. This connection was likely configured by trained Wyatt Technology personnel during instrument installation. A loaner Eclipse or Eclipse returning from service can be re-connected to this Ethernet network switch and a suitable IP address and subnet mask can be input on the settings tab on the front panel of the Eclipse. Using the same IP address will facilitate re-configuring with ASTRA and the computer. A typical IP address would be in the format 192.168.254.xxx where the first three sets of numbers (“192.168.254.”) match the computer adapter settings, the Agilent HPLC, and the other Wyatt analytical detectors, while the 3 digits of the last set of numbers should be unique. Although your static IP address may vary, an example set might be:

- Computer Adapter: 192.168.254.100
- Agilent HPLC: 192.168.254.11
- Eclipse: 192.168.254.101
- DAWN: 192.168.254.102
- Optilab: 192.168.254.103
- Mobility: 192.168.254.50

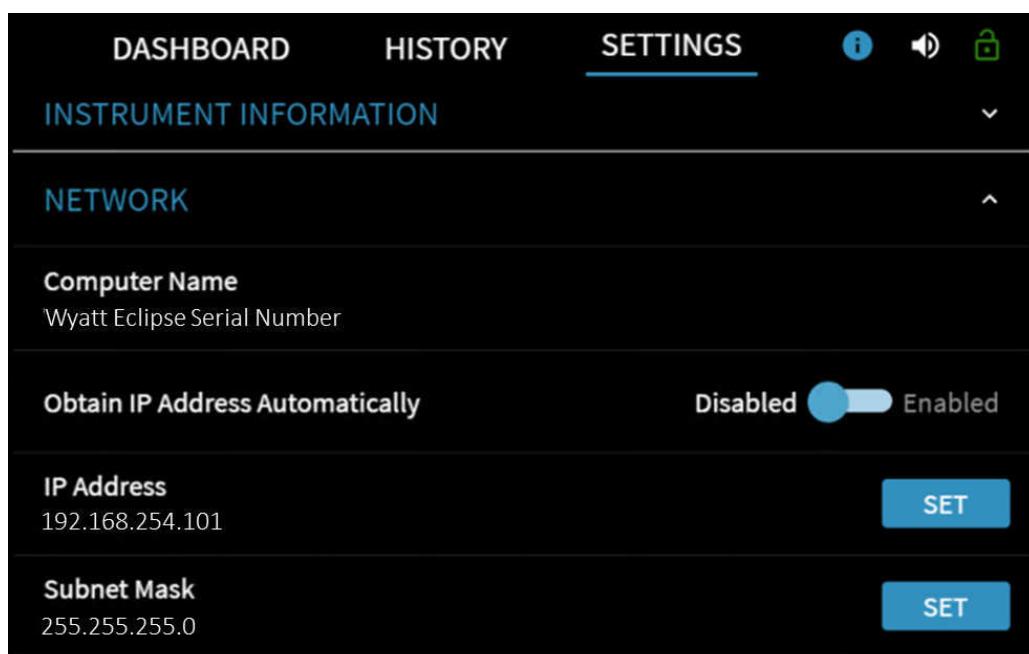


Figure 5. Eclipse Settings tab – the IP address and subnet mask can be set under **Network** in the Settings tab to re-establish communication with the Eclipse via the software. The communication can be checked by using the Windows Command Prompt to “Ping” the IP address. In the above example, “Ping 192.168.254.101” can be typed in the Windows Command Prompt to test connectivity with the Eclipse.

An example of the Eclipse settings page is shown in Figure 5. Please refer to the section, [Navigating the Eclipse Front Panel](#), for information on navigating the front panel display. The subnet mask should also match the computer adapter settings. Please refer to [TN1018 – Instrument Connection Guide](#) on the Wyatt Support Center for more information on instrument connectivity and troubleshooting instrument connections. Although written for ASTRA, the technical note covers PC-level troubleshooting that may help restore communications for the Eclipse with VISION. It will also guide you through re-connecting your other Wyatt analytical detectors that may be installed downstream of the Eclipse.

Please refer to your Agilent HPLC technical resources for determining the IP address of your Agilent hardware. The default Agilent IP address is usually 192.168.254.11. Agilent HPLC modules communicate to each other via CAN cables and to the computer via a LAN. Agilent LAN cards may have dip switches that can be used to determine the boot IP address or they may have a configuration switch with the FUSION electronics.

Since the default address is a so-called “local address,” the PC and module should reside in the same subnet for communication. An Agilent Instant Pilot (Gameboy) can be used to change the Agilent IP address. Additionally, the default values can be configured by accessing the rear of the Agilent module that contains the LAN connection. A LAN connection, not a CAN connection, is needed to establish communications with the computer and VISION 3 software. The two most common type of LAN interfaces are a dedicated LAN card and a built-in LAN configuration switch. These two options are summarized next.

Agilent LAN Card

Older Agilent models may have a LAN card with eight dip switches internally or on the card. With dip switches #5 & #6 up (and others down), the default address is 192.168.254.11 with a subnet mask of 255.255.255.0. This is illustrated in Figure 6.

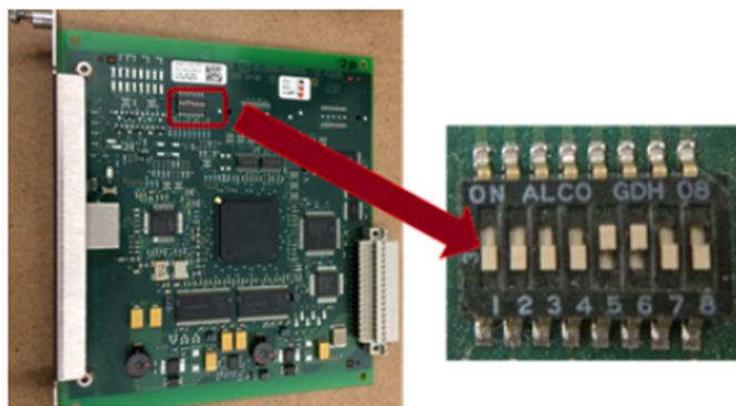


Figure 6. Agilent LAN card dip switches. With #5 and #6 switches up (and others down), the default IP address is 192.168.254.11 with a subnet mask of 255.255.255.0.

Agilent Configuration LAN Switch

Newer Agilent models may have six switches on the rear panel of the instrument. In this case, with all dip switches down (none up), the default address is 192.168.254.11 with a subnet mask of 255.255.255.0. This is illustrated in Figure 7.

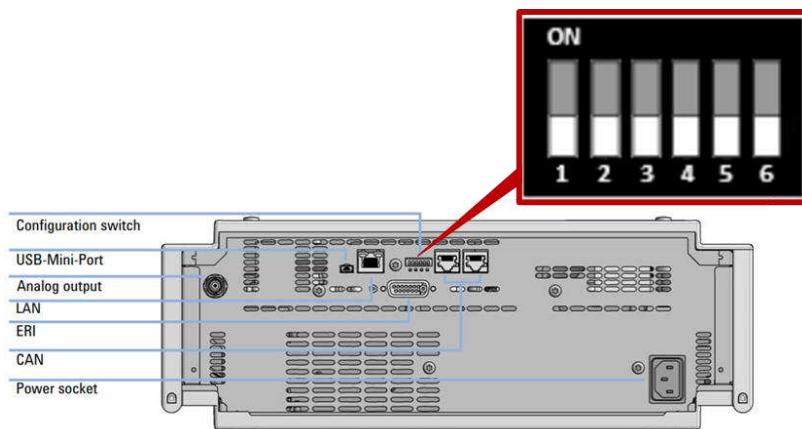


Figure 7. Agilent configuration switch. With all switches down (and none up), the default IP address is 192.168.254.11 with a subnet mask of 255.255.255.0.

For the most up-to-date information, please refer to Agilent hardware technical resources.

Eclipse Drain Tubing

Wyatt instruments feature a liquid drain system in the event of an internal or external leak or spill. Fluid on top of the instrument or inside the instrument is directed to a liquid leak port to protect the internal electronics. Typically, the bottom detector in an analytical stack is equipped with a drain line that can be placed in a waste bottle or reservoir in the event of a leak. An adapter and Versilon® tubing are provided in the hardware kit to direct any fluid to a waste bottle. Please see Figure 8 below.

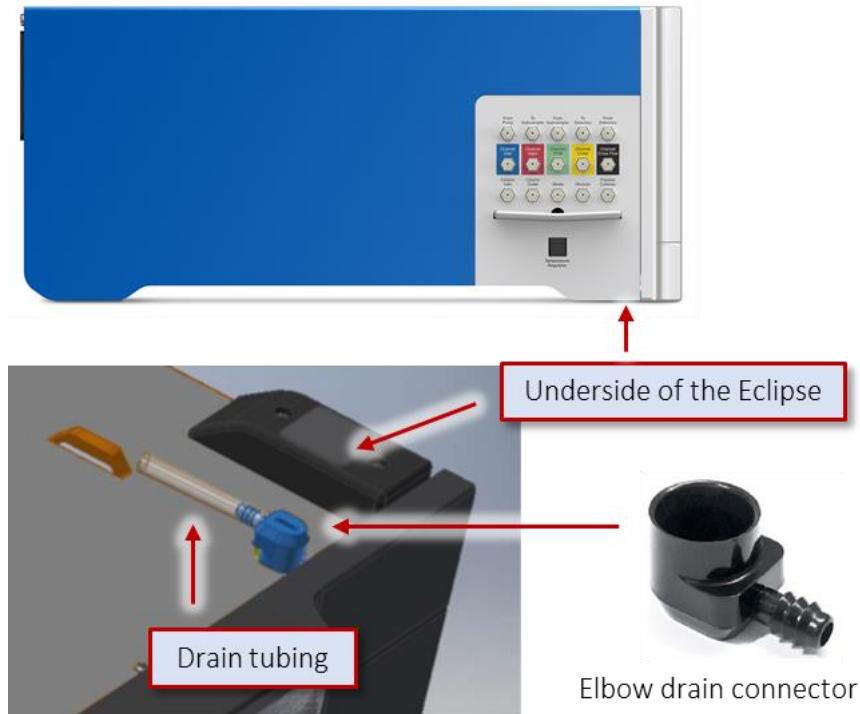


Figure 8. Connect the adapter (press firmly) on the underside of the Eclipse for the drain tubing.

You may connect the tubing to the elbow drain connector first. Press the elbow drain connector firmly into place on the underside of the instrument to secure it. To avoid the instrument feet from crushing the tubing, the tubing

can be zip-tied to a guide under the instrument or the tubing can be directed around the feet once the instrument is flat on the benchtop.

Eclipse Side-Panel Connections

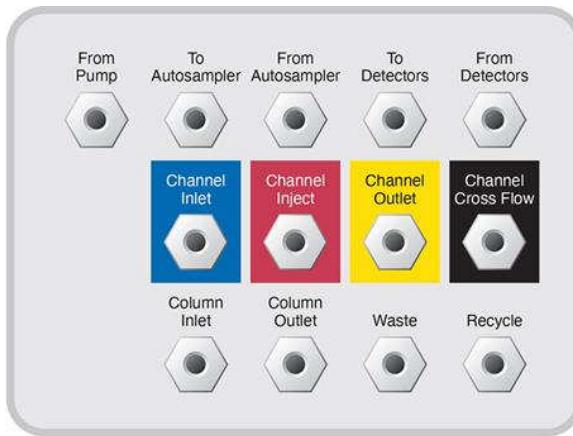
Depending on the Eclipse model, the configuration of the side-panel will vary. An overview of the side-panel connections for the various Eclipse models are shown in Figure 9. The Eclipse side-panel bulkhead unions are organized by row. The top row contains bulkhead unions connecting to the HPLC and downstream analytical detectors. If the SEC switching option is present, connections to and from the autosampler are needed.

The second row from the top is for the channel fluid connections. The Wyatt (NEON) generation channel has five ports available—designed for channel inlet, channel inject, dilution control module (DCM), channel outlet, and channel cross flow. If your Eclipse has the DCM option, the appropriate connection from the channel will be made. If your Eclipse does not have a DCM option, the corresponding port on your channel should be plugged. Channel cross flow ports are located on the side of the channel. If your Eclipse has an SEC switching option, the channel inject will connect to the Eclipse side panel. If it does not, the channel inject will connect directly from the autosampler.

The bottom row is for the column inlet and outlet (if you have an Eclipse with SEC switching option), waste, recycle, and fraction collector connections. If a fraction collector will not be used, the fraction collector bulkhead union should be plugged. A fraction collector port is not included in models without a DCM option.



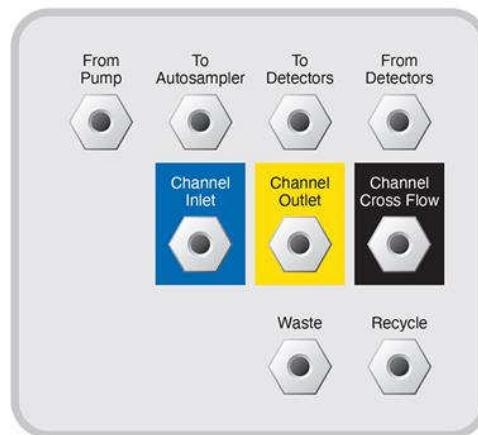
Eclipse with DCM + SEC (WECDS)



Eclipse with SEC (WECS)



Eclipse with DCM (WECD)



Eclipse Base Model (WEC)

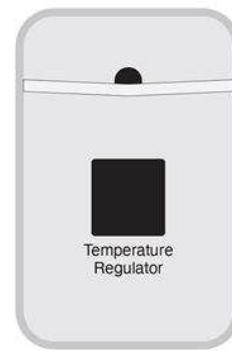
Liquid Leak Tray (top)
Channel Temperature
Regulator Connection
(bottom)

Figure 9. The side-panel connections for the various Eclipse models. The labels describe the connections to the HPLC, channel, column, downstream detectors, and waste/recycle.

In addition to the side-panel fluid connections, the side panel will include an opening for the fluid leak tray and the connection for the channel temperature regulator. In the event of a side panel liquid leak, fluid will drain into the side panel leak tray and activate one of the liquid leak alarms if the liquid is conducting, as with salt-containing buffers. Once the leak is identified and resolved, a cotton swab can be inserted to dry and clean the leak sensor. Please refer to the section, [Eclipse Liquid and Vapor Leak Detection](#), for additional instructions for resolving a liquid leak. The channel temperature regulator connection is required to establish communication and temperature control with the channel temperature regulator, which is discussed in the channel assembly section. Newer models also feature a channel leak sensor.

Bulkhead Frit Filter and Inline Filter Assemblies

In order to reduce the risk of clogs to critical components of the Eclipse, particularly to prevent particulates from entering the controller needle orifice of the flow controllers, bulkhead frit filters or inline filters are installed at key fluid connections on the Eclipse side panel. It is strongly recommended to install and regularly replace the frits and filter membranes on the bulkhead frit filter assemblies. Please see the section, [Maintenance Procedures](#), for recommended frequency and, [Replacing Bulkhead Frit Filter](#), for the procedure. Depending on the Eclipse model, you will have two or three filter assemblies. These are shown in Figure 10. For all Eclipse models, it is recommended

to maintain the frit filter on the **Pump** and an inline filter on the **Channel Cross Flow** ports. For Eclipse with DCM models, it is additionally recommended to also have an inline filter on the **Channel DCM** port.

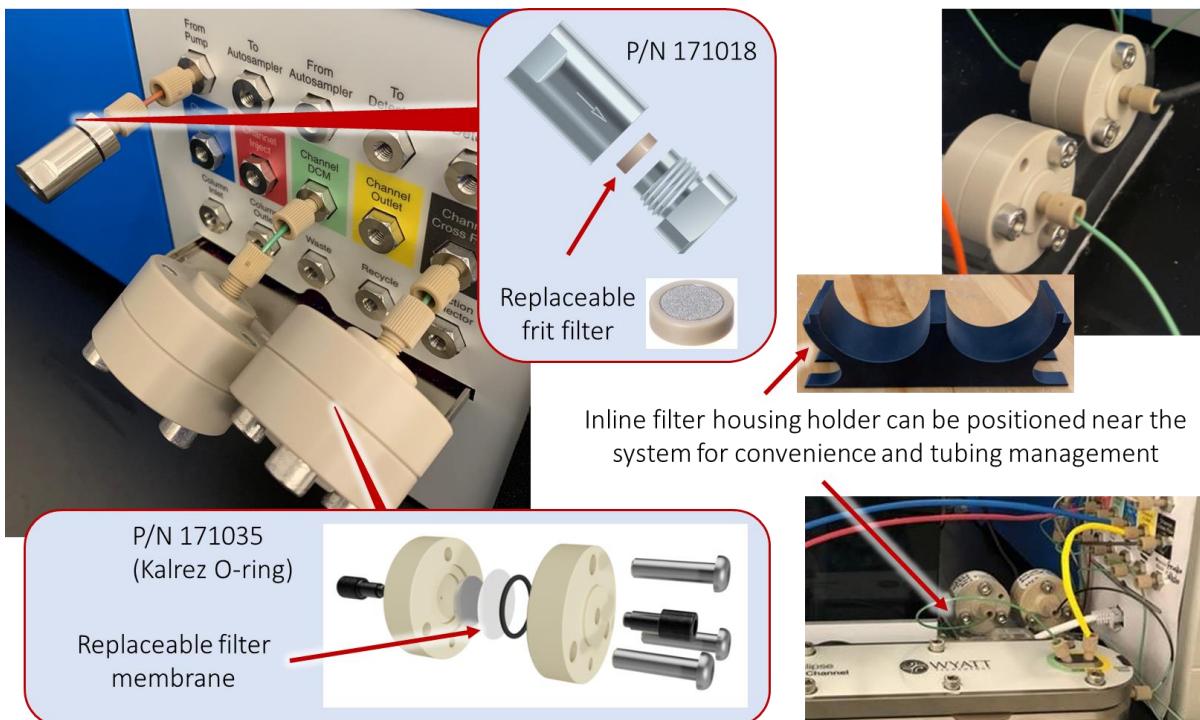


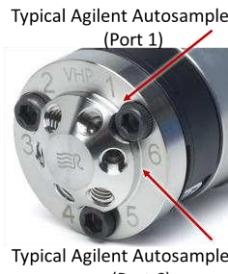
Figure 10. Bulkhead union frit filter assemblies should be installed on the “From Pump,” “Channel Cross Flow,” and “Channel DCM” fluid connection ports. Contact Wyatt Technology for more information regarding the filter holder.

These will help prevent potential clogs from entering sensitive Eclipse fluid pathways. Because they are not installed on sample fluid pathways, they will not remove or alter samples prior to analysis. Clogged frits can, however, prevent cross flow or DCM flow from reaching the set point. These 20 µm frits or 0.22 µm filter membranes should be replaced when observing or troubleshooting poor DCM or cross flow regulation. Please see the [Maintenance Procedures](#) section for instructions for replacing these filters.

Fluid Connections

The Eclipse and HPLC will be interconnected with fluid connections that may seem intimidating but each serves a core function, for example connecting the pump to the Eclipse or the Eclipse to the autosampler. These connections are illustrated in Figure 3 between the HPLC modules and the Eclipse. Please refer to [Appendix D – Eclipse Flow Diagrams](#) for figures illustrating the fluid pathways internally and externally for each Eclipse model. These diagrams also describe the appropriate tubing between fluid connections. At installation, your Wyatt representative will install the correct external tubing to and from your HPLC modules and to downstream detectors. A summary of the tubing is provided in Table 1.

Table 1. Summary of Fluid Connections

	Eclipse Side Panel	Module	Tubing (1/16" O.D.)	Notes
HPLC & Detector (10/32 Coned Fittings Both Ends)	From Pump	Inline Filter Outlet (or from Pump)	Orange (0.020" I.D.) or Green (0.030" I.D.) PEEK	Use coil of narrow tubing between pump and inline filter to put pressure on the pump to minimize pump pulsations and improve performance.
	To Autosampler	Port 1 for standard Agilent Autosampler Rheodyne	Green (0.03" I.D.) PEEK	
	From Autosampler (SEC switch models)	Port 6 for standard Agilent Autosampler Rheodyne	Tan (0.010" I.D.) or Red/Pink (0.005" I.D.) PEEK (possible that red PEEK restricts flow)	In non-SEC Eclipse models, connect directly from Port 6 (Agilent) to channel inject (port is color-coded red).
	To Detectors	First analytical detector (typically UV or MALS detector)	Tan (0.010" I.D.), Red/Pink (0.005" I.D.), or Yellow (0.007" I.D.) PEEK	Need to tune channel pressure (>10 bar) based on detector flow rates. Narrower PEEK (0.005" I.D) can be used to increase channel pressure for flow regulation, especially with DCM.
	From Detectors	Last analytical detector (typically RI detector)	<ul style="list-style-type: none"> If RI: Green (0.030" I.D.) PEEK If no RI and using fraction collector: Tan (0.010" I.D.) PEEK 	Please see TN6504 for Fraction Collector configurations.
Channel (Super flangeless)	Channel Inlet	Super flangeless ferrule into Channel Inlet	Green (0.030" I.D.) PEEK	Color-coded blue sleeve
	Channel Inject (SEC switch models)	Super flangeless ferrule into Channel Inject	Tan (0.010" I.D.) or Gray (0.015" I.D.) PEEK	Color-coded red sleeve (from Autosampler if not an SEC switching model)
	Channel DCM (DCM Models)	Super flangeless ferrule into Channel DCM	Green (0.030" I.D.) PEEK	Color-coded green sleeve
	Channel Outlet	Super flangeless ferrule into Channel Outlet	Tan (0.010" I.D.) PEEK	Color-coded yellow sleeve
	Channel Cross Flow	Super flangeless ferrule into Channel Cross Flow	Green (0.030" I.D.) PEEK	Color-coded black sleeve
Eclipse	Column Inlet (SEC switch models)	Inlet of guard column	Tan (0.010" I.D.) PEEK	
	Column Outlet (SEC switch models)	Outlet of the last analytical column	Tan (0.010" I.D.) PEEK	Max column pressure with the Eclipse is 75 bar
	Waste	Waste reservoir	Green (0.030" I.D.) PEEK	
	Recycle	Solvent reservoir	Green (0.030" I.D.) PEEK	
	Fraction Collector	If applicable	Green (0.030" I.D.) PEEK	Remain plugged if FC not plumbed.

Improving HPLC Performance: Degasser

Wyatt Technology strongly recommends an inline degasser for removing air bubbles or dissolved gases in the mobile phase, which can negatively impact analytical detector signals. Agilent HPLCs may have an integrated degasser which can function effectively up to 5 mL/min. Please check your Agilent hardware manual for more information. Your Eclipse ship kit includes a splitting connection for convenience or when using third-party degasser with less efficiency. The flow can be split across multiple channels entering the degasser and then re-combined exiting the degasser if increased efficiency is needed. With this configuration, although total flow may be higher than the rated value, the flow is split across multiple channels to improve degassing efficiency.

Improving HPLC Performance: Pump

Wyatt Technology's analytical detectors and Eclipse hardware are often sensitive enough to detect the pump pulsations even in HPLC pumps. There are strategies you can take to minimize these effects on the system. The compressibility of the solvent can be input into the Agilent hardware to help smooth fluid delivery and reduce pump ripple and pulsations. Adjusting these settings in your VISION RUN method is discussed in [Considerations for some HPLC Instrument Methods](#). An improvement can also be made by increasing the back pressure on the pump, which can help keep seals in place and improve performance. Agilent HPLC pumps can operate at up to 400 or 600 bar. The pressure on the pump can be increased by adding a coil of tubing directly after the pump but before the inline filter—this ensures that all backpressure is applied just to the pump and not to any inline filter, column, channel, or detector downstream of the pump. Depending on typical flow rates, an appropriate pump pressure (measured at the pump inlet) with *just* the coil of tubing should be in the 150 bar range at 4 mL/min with an aqueous buffer. This will help improve pump performance and reduce noise in the inject flow controller. This setup is demonstrated in Figure 11.

Note: If you are observing inject flow fluctuations, you may need to increase your pump pressure.

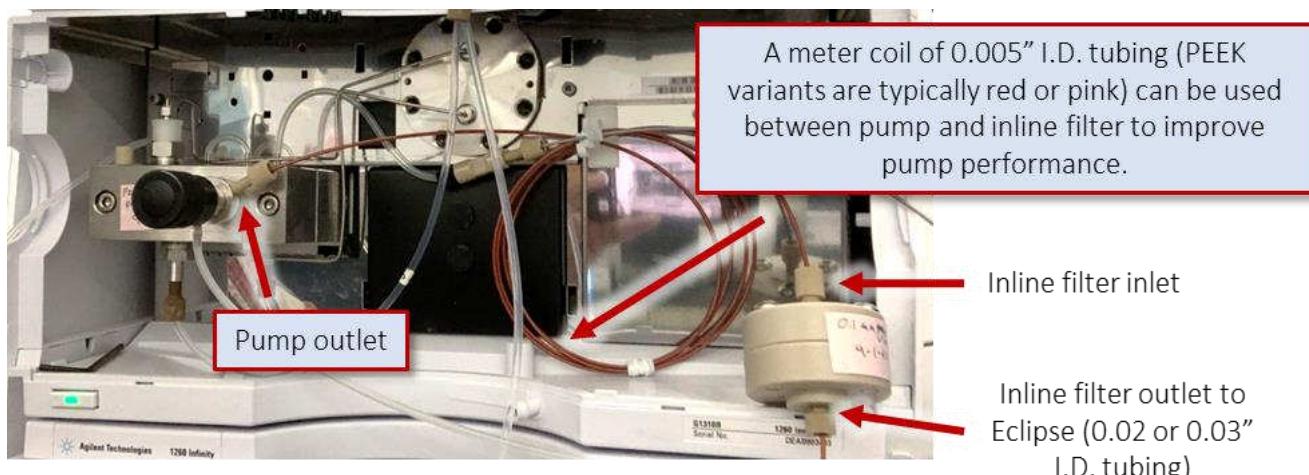


Figure 11. A coil of PEEK tubing (0.005" I.D., colored red or pink) between the pump outlet and the inline filter inlet can help improve pump performance. Do not add restrictive tubing after the inline filter, which will put excessive backpressure on the inline filter housing.

When operating at higher flow rates, Agilent isocratic or quaternary pumps may need reduced upper pressure limits (200 bar) to go above 5 mL/min total flow. As a result, when operating at higher total flow rates (> 5 mL/min) with an isocratic pump, it is recommended to remove the coil of tubing and set the pump pressure limit to 200 bar in the HPLC method. Add a shorter coil as needed to reduce pump or inlet flow ripple effects.

NOTE: Agilent may require a lower pump pressure limit (200 bar) to achieve high flow rates (> 5 mL/min) in many isocratic pumps. If high flow rates will be used, the pulse dampening coil should be removed or shortened to ensure the max pressure is not reached with method flow rates.

Connecting a Fraction Collector

If your Eclipse has the DCM option, there will be a fraction collector bulkhead union on the side panel. The last analytical detector can be connected to the **From Detectors** port on the Eclipse side panel. The **Fraction Collector** port on the side panel can then be connected to the fraction collector inlet. The fraction collector waste line should go to the waste reservoir. In this configuration, the system will recycle or direct fluid to waste when the fraction collector is not running but sends all detector flow to the fraction collector when the fraction collector is enabled in the Eclipse method editor:

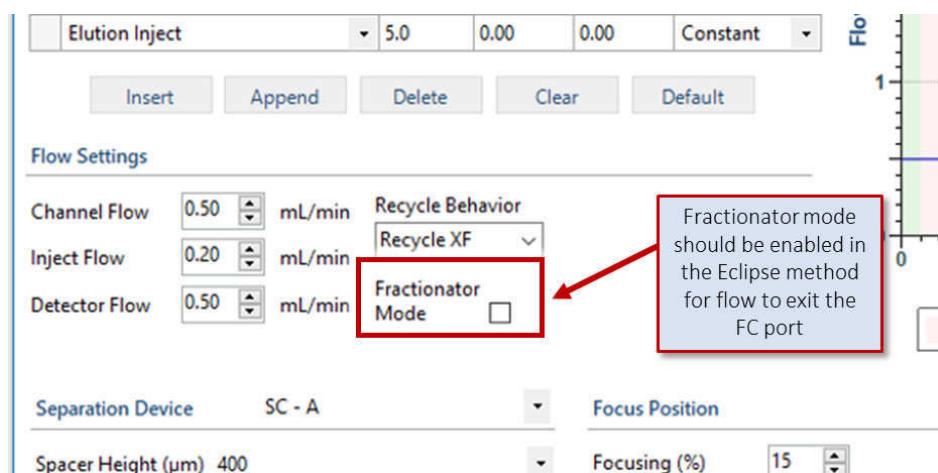


Figure 12. Flow via the fraction collector port in the Eclipse + DCM models requires enabling “Fractionator Mode” in the Eclipse method and subsequently enabling collection in the fraction collector method. For Eclipse models without the DCM option, the Fractionator Mode toggle is not used. Instead, collection is enabled or disabled in the fraction collector method.

If your Eclipse does not have the DCM option, you will connect your fraction collector to the last analytical detector that will be used. Because the Optilab is often bypassed due to band broadening and low backpressure tolerances, this might be the UV or MALS detector that is connected directly to the fraction collector. In this case, the fraction collector outlet line is connected to the **From Detectors** port on the Eclipse side panel. Please see [TN6504 Configuration Fraction Collectors with VISION](#) for additional guidelines when using a fraction collector.

Replacement Tubing and Chromatography Fittings

For replacement Eclipse or channel tubing, please contact Wyatt Technology. Tubing for the channel comes pre-swaged with the appropriate fittings and is color-coded for your convenience. In addition to the tubing color, which determines inner diameter, the channel tubing also has an insulating sleeve that is color-coded for connections to the channel. Fluid connections to the Eclipse, HPLC, analytical detectors, columns, and channels will typically be one of two styles—10-32 coned fittings and Super Flangeless™ ferrules with metal locking ring. The 10-32 coned fittings are used everywhere on the Eclipse side panel and HPLC. The Super Flangeless fittings may be used on the channel and often the inline filter. Although your tubing will come pre-swaged, Figure 13 illustrates how a super flangeless ferrule is swaged with locking metal ring and the 10-32 coned fitting for comparison.

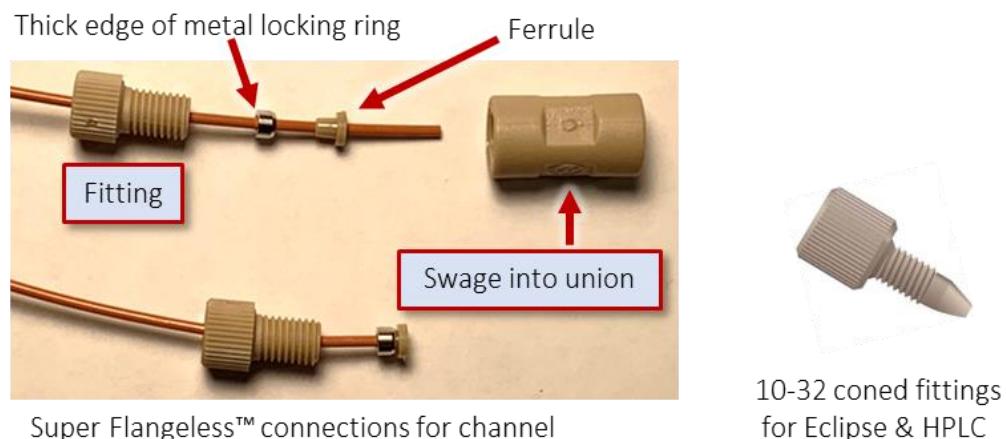


Figure 13. (Left) Super Flangeless™ ferrule with metal locking ring for channel and inline filter housing and (right) 10-32 coned fitting for Eclipse side panel and HPLC.

If it is necessary to swage your own tubing, the tubing can have the fitting inserted first, then the metal locking ring with the thickest edge nearest the fitting, and then the ferrule with the narrow cylindrical portion toward the locking ring. The fitting can be tightened into a union with an extra $\frac{1}{2}$ rotation when finger tight to lock the metal ring onto the ferrule. Remove the fitting, then inspect to ensure it is not loose. It can now be used to make fluid connections. 10-32 coned or stainless-steel fittings, which leave some tubing protruding from the fitting, should be swaged directly into the desired port. More information can be found in [TN3101 – Swaging Stainless Steel Tubing](#) or with regards to inline filter installation, [TN3504 – Wyatt Inline Filter Installation and Use](#).

Channel Fluid Connections

All Eclipse channels feature 5 fluid connections—the channel inlet, channel injection, channel DCM, channel outlet, and channel cross flow. For Eclipse models without the dilution control module (DCM), the channel DCM port will need to be plugged. Other connections are made between the channel and Eclipse bulkhead union as shown in Figure 14. A minor exception to this typical order is the Dispersion Inlet channel, where the inlet and injection ports are reversed, however, all Eclipse channels are color-coded for your convenience.

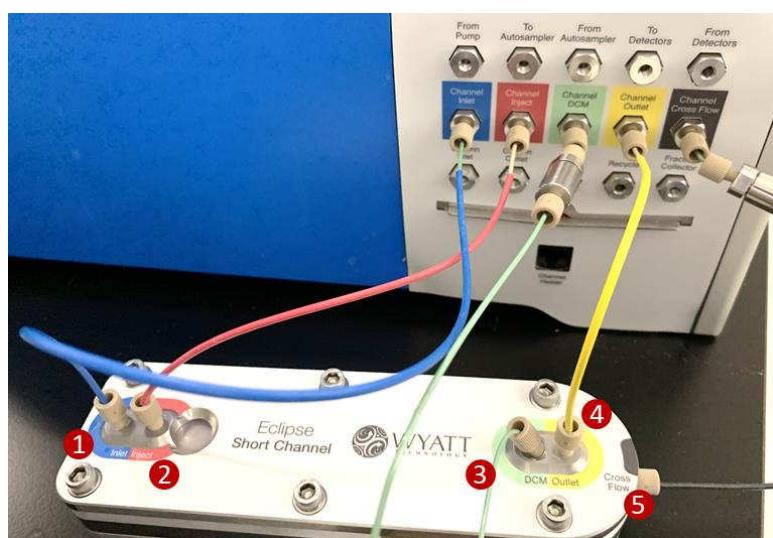


Figure 14. Fluid connections between the Eclipse and the channel, including (1) channel inlet, (2) channel inject, (3) channel DCM, (4) channel outlet, and (5) channel cross flow. For Dispersion Inlet channels, the channel inlet and channel inject are reversed. For Eclipse model without DCM, the channel DCM port should be plugged.

Channel Temperature Regulator Connection

The channel temperature regulator is included with all Eclipse channels, which include the Eclipse Short Channel (SC), Long Channel (LC), Dispersion Inlet Channel (DC) (which had been called the frit-inlet channel in legacy Eclipse instruments), Semi-Preparative Channel (SP), and Mobility Channel (MC). It is not waterproof and the channel temperature regulator, especially the micro-USB port, should not be exposed to liquids. Instructions for removing or installing the channel temperature regulator are described in the channel cleaning section. It is not necessary to remove the channel temperature regulator for routine membrane/spacer replacement unless you suspect the bottom block of the channel to be contaminated.

WARNING: The channel temperature regulator is not waterproof. Do not submerge channel temperature regulator in water or clean when connected with other channel components. Avoid salt precipitation at electrical connections by cleaning up liquid leaks and drying the exterior of the channel after membrane installation. Additional instructions are provided in the channel membrane installation and cleaning sections.

The channel temperature regulator is controlled via a micro-USB to RJ-45 connection cable as shown in Figure 15. It can regulate temperatures from ambient up to 50 °C.

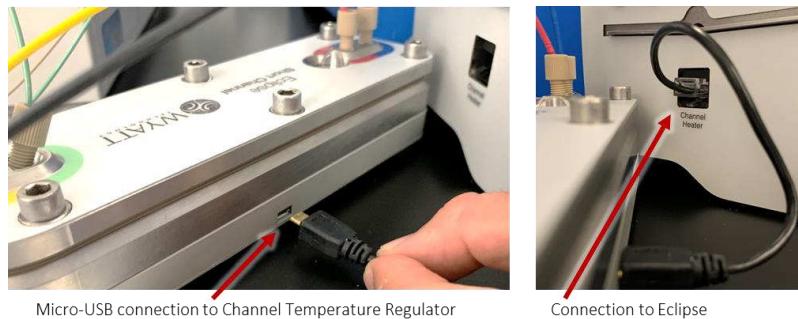


Figure 15. Connection between channel temperature regulator (underneath the channel) to the Eclipse chassis.

It is recommended to set the channel temperature to slightly above ambient (+ 2-5° C) for best regulation. If sub-ambient temperatures are needed, or temperatures above 50 °C, please contact Wyatt Technology for additional information.

NOTE: Since the original product launch, Wyatt Technology now offers channels with dedicated leak sensor and better leak protection against spills or fluid leaks. These feature a permanently affixed cable on the channel temperature regulator for connection to the Eclipse and require a new internal board. Please contact Wyatt Technology for more information.



Bypassing the Channel for Solvent Exchange or Membrane Installation

A 5-port union is included with the Eclipse hardware kit that can be used to bypass the channel. It is recommended to remove the channel from the fluid pathway when performing a system solvent exchange, when replacing the channel membrane, or when cleaning the system. In the following sections, the solvent exchange protocols and new membrane installations will be explained. Where applicable, the fluid connections can be removed from the channel and installed on the 5-port union in order to minimize exposure to dust or particulates as well as facilitate system cleaning or solvent exchanges. This is shown in Figure 16.

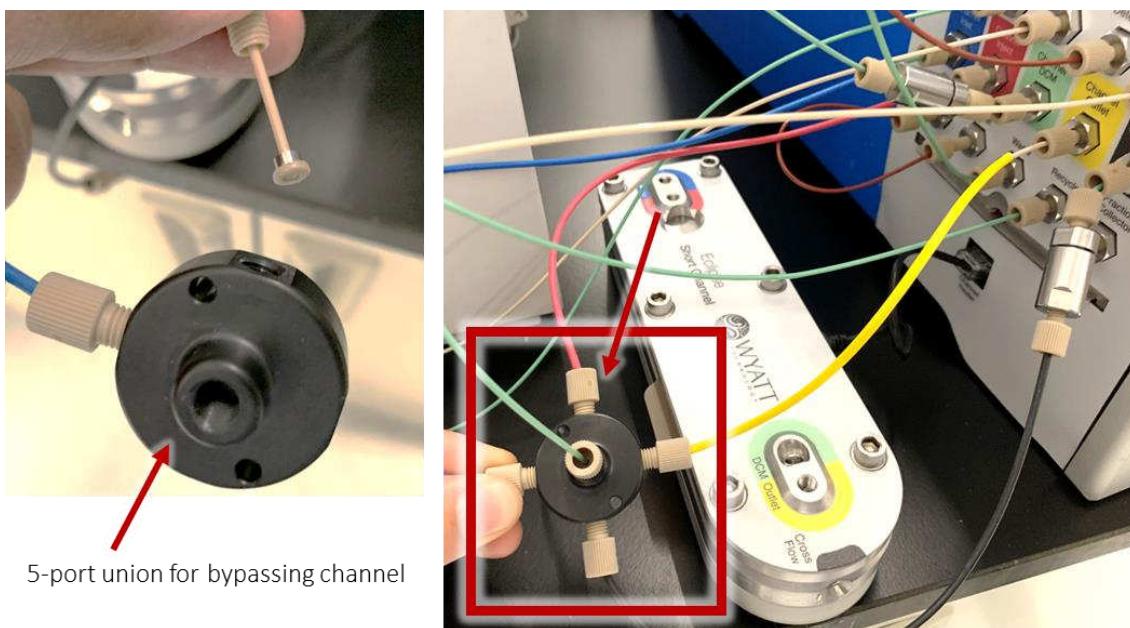


Figure 16. 5-port union for bypassing the channel. The order or orientation of the connections are not critical. If the Eclipse does not have a DCM option, one of the ports can be plugged or a 4-port union can be used.

Connecting the Column for SEC Mode or Column Installation

If your Eclipse has the SEC switching option, you can similarly bypass the column by using a short piece of tubing to directly connect the Column Inlet to Column Outlet port as shown in Figure 17. This can facilitate solvent switching the system without risking shock to the column. This is also helpful to avoid shocking the column when configuring a column or method for the first time. Even if no column is installed, it is recommended to regularly flush the SEC pathway to prevent contamination or clogs. The SEC mode pathway can be stored in a 20 - 50% alcohol mixture to prevent microbial growth.

CAUTION: If you are using a column for the first time, it is recommended to perform the initial method creation and all hardware configuration with the column bypassed (short loop of tubing between column inlet and outlet) in order to avoid inadvertent damage to the column. Once the method and hardware are configured, then the column can be safely installed and it will be less likely that the column will get shocked.

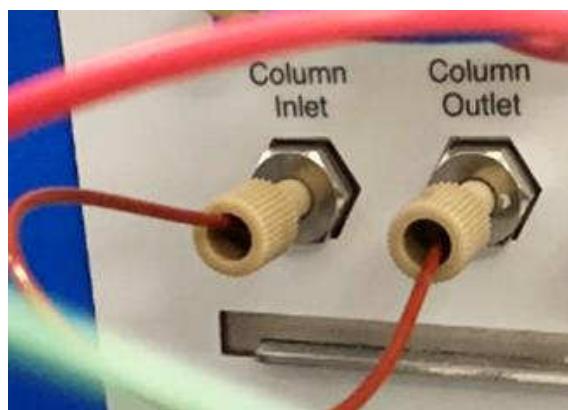


Figure 17. Bypassing SEC column with a short length of PEEK tubing between the column inlet and column outlet in the Eclipse with SEC switching option.

Navigating the Eclipse Front Panel

The Eclipse has a display interface with multi-touch controls which allow you to monitor, configure, and control the function and operation of the Eclipse. You can tap, swipe, pinch, and spread your fingers to perform useful actions on the screen as described in Table 2.

Table 2. Using the Multi-Touch Controls

Multi-touch Controls	Function	Actions
	Tap to click	Tap with one finger to select an interface option. You can tap to view drop-down lists and tap to select commands such as OK or Cancel.
	Slide to scroll	Press and drag with one finger on the screen to scroll through the interface. Press and slide up to scroll down through the Settings tab, or press and slide left to scroll right through the Status Indicators on the Dashboard tab, or vice-versa. You can also press and drag the blue scroll bar shown on the Dashboard and Settings tabs to scroll.

	Press and pinch or spread to zoom	<p>Two fingers are needed to zoom in or out on display graphs. Using your thumb and pointer finger, press the display and spread two fingers apart to zoom in on a graph. Move two fingers together to zoom out on a graph.</p>
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The front panel (Figure 18) has a top ribbon display, which provides the **Dashboard**, **History**, and **Settings** tab. There are additionally icons for status indicator, an alarm mute/unmute button, and a touchscreen disable button. For the status indicator: a green checkmark indicates there are no warnings or alarms, a yellow caution icon means an issue requires attention, a red warning icon means the system is not ready or an alarm is active, and a blue icon indicates a maintenance action is recommended. Tapping these icons provides a shortcut to the Alarms section on the History tab.

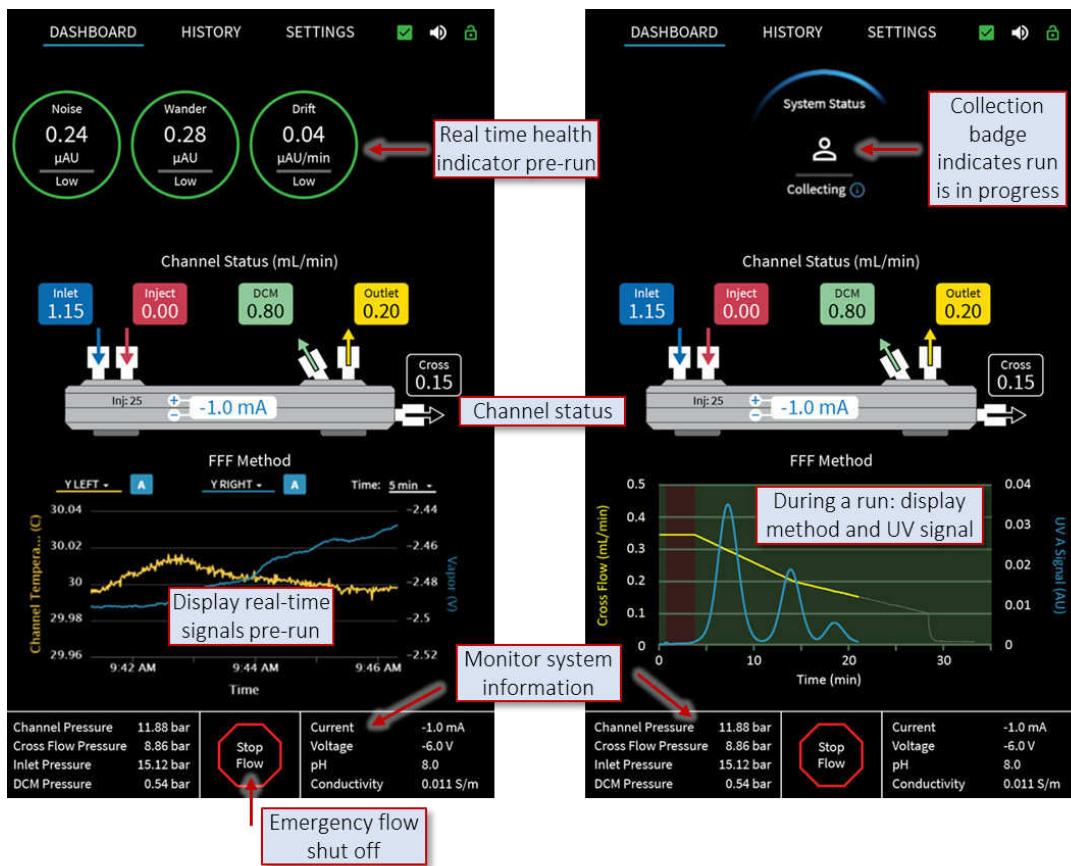


Figure 18. Eclipse front panel display during pre-run (left) and collection (right).

The display shows real-time data that assists in observing experiment progress and current system performance. You can select a data stream to display in the graph for each y-axis by tapping on the fields above the graph. Other tabs like History show status updates and the Settings enables modification of certain hardware parameters, like vapor sensitivity and network settings.

VISION Installation Overview

Once the Eclipse is connected to the computer via Ethernet cable from the rear panel of the instrument, it can be controlled via the VISION software. Please refer to the VISION ReadMe on the software installer or refer to the VISION User's Guide for instructions for installing the software. To start the VISION software after installation/upgrade, choose Windows® Start Menu → Program Files → Wyatt Technology VISION 3.x.x. or launch VISION via a desktop shortcut.

NOTE: This document does not cover procedures that are required for the installation or use of VISION Security Pack (SP). Please refer to the VISION Software User Guide for information related to VISION SP operation.

VISION RUN Configuration

If running the VISION software for the first time, it is necessary to activate the program. In the main window, select the **LICENSE MANAGER** (License Manager) tile, and click the **Enter Product Key** (Enter Product Key) button to enter the Wyatt Technology supplied activation key(s) for VISION DESIGN and VISION RUN. These steps are shown in Figure 19.

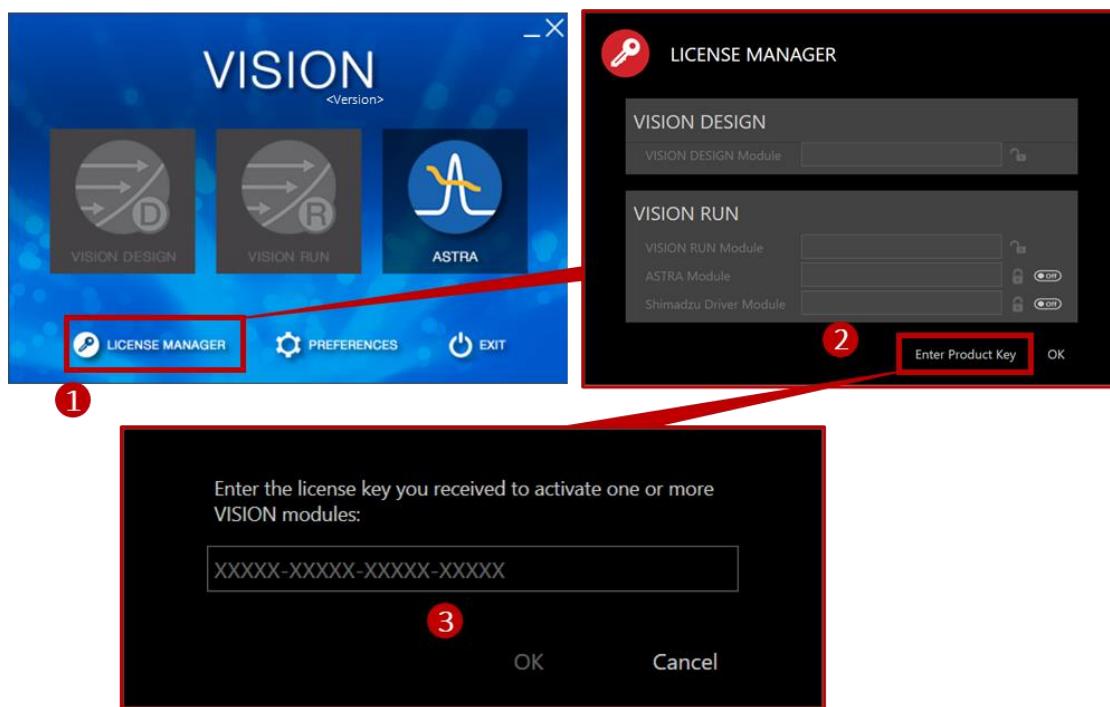


Figure 19. Activating VISION via License Manager.

After inputting the product key, VISION will indicate whether the key is valid or invalid. Once a valid key is entered, the License Manager window will display the key for the VISION DESIGN module and VISION RUN module. The ASTRA module key is automatically enabled with VISION RUN. Although no numeric key will be displayed for the ASTRA module, the module will work as long as a VISION RUN key is displayed. Hovering the mouse cursor over the unlock icon will show "Product Key" text.

NOTE: VISION 3 with an Eclipse is only compatible with Agilent HPLCs.

VISION is capable of controlling ASTRA via a software development kit (SDK). This enables VISION RUN sequences to generate both VISION DESIGN EMDFs and ASTRA data files. This SDK key is automatically applied to ASTRA when

using VISION 3.1 with ASTRA 8.0.1 or higher. If using older versions of VISION 3.0 to control ASTRA 8.0 during sequence runs, an ASTRA SDK license key must be entered in the ASTRA software. This can be done by launching ASTRA and navigating to System → Feature Activation. Enter the product key provided by Wyatt Technology to enable the SDK. Please note that in VISION 3.1 and ASTRA 8.1, this key is automatically applied and no longer visible in ASTRA 8.1. These instructions do not apply for the most recent versions of the software.

NOTE: If using ASTRA 8.0.1 or lower, an ASTRA SDK key needs to be manually entered in ASTRA. The SDK key is automatically applied with VISION 3.1.1.9 and ASTRA 8.1. The SDK feature is not visible in ASTRA 8.1.

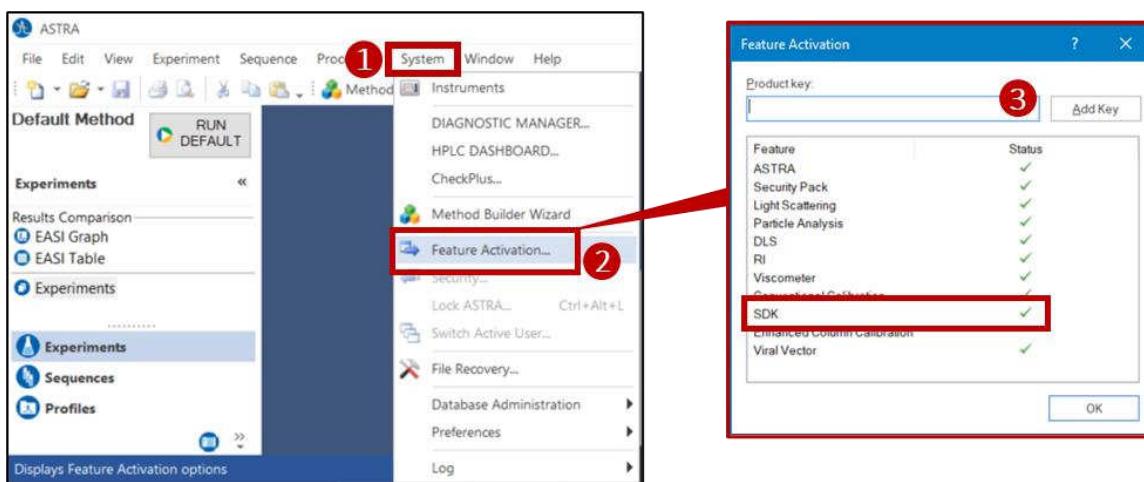


Figure 20. Enabling VISION control over ASTRA via SDK feature activation in ASTRA 8.0.1 or earlier.

Other settings may be required for proper operation of the software. Please consult the VISION ReadMe for a list of software improvements and complete software installation instructions.

Configuring ASTRA for Eclipse Operations

With the ASTRA module enabled in the VISION license manager (Figure 19) and the ASTRA SDK feature activated in ASTRA (Figure 20), the VISION RUN software will automatically generate ASTRA data files as part of the VISION RUN sequence. However, as part of this functionality, a suitable ASTRA method will need to be created. Although VISION will adjust the ASTRA method collection time to match the sequence row run time (based on the HPLC and Eclipse method), it is necessary to create an ASTRA method with the appropriate analytical detector physical instruments established. Please refer to [TN1007 ASTRA Quick Guide](#) for more information on creating a new method or editing an existing method.

An initial FFF-MALS method can be used to run a standard such as BSA or PSL bead to determine system constants like normalization, alignment, and band broadening. Typically, your initial method with system parameters is created by Wyatt Technology personnel during installation.

Configuring VISION for Eclipse Operations

In this section, recommendations for configuring VISION and an overview of the functions will be outlined. In subsequent sections, procedures for flushing, cleaning, and other software procedures will be discussed in more detail.

VISION Overview

The VISION software assists with experiment design, method development, HPLC control, data acquisition, and data processing. VISION comprises of two modules, VISION DESIGN and VISION RUN. VISION DESIGN can be used to develop your FFF method and VISION RUN can be used to execute experiments in a sequence. A summary of the capabilities for the different software modules are described below in Table 3.

Table 3. Functions and their applicability in VISION DESIGN, VISION RUN, and ASTRA.

Function	VISION DESIGN	VISION RUN	ASTRA
Simulate FFF-MALS separations based on hydrodynamic radius and FFF theory	✓		
Control the Agilent front-end and Eclipse; create HPLC & Eclipse methods		✓	
Create methods for other Wyatt analytical detectors and collect analog signals			✓
Run FFF-MALS experiments from sequence tables		✓	
Generate Eclipse Method Data Files (EMDFs) and ASTRA data files (.afe8)		✓	
Perform light scattering (LS) analyses based on MALS, DLS, and concentration			✓
View HPLC and Eclipse flow rates and pressures	✓	✓	
View spectral data in 3D from an HPLC UV or FLD module	✓		
Analyze Eclipse Mobility Module data (electrophoretic mobility and zeta potential)	✓		
Optimize Eclipse methods based on experimental data, including LS signals	✓		

Once the data has been collected, the light scattering data can be analyzed in ASTRA and electrophoretic mobility data (with the optional Eclipse Mobility Module) can be analyzed in VISION DESIGN along with information about the flows and pressures. Additionally, the method can be optimized further in VISION DESIGN even with light scattering signals. Figure 21 outlines the workflow for designing, executing, analyzing, and improving experiments with VISION and ASTRA.

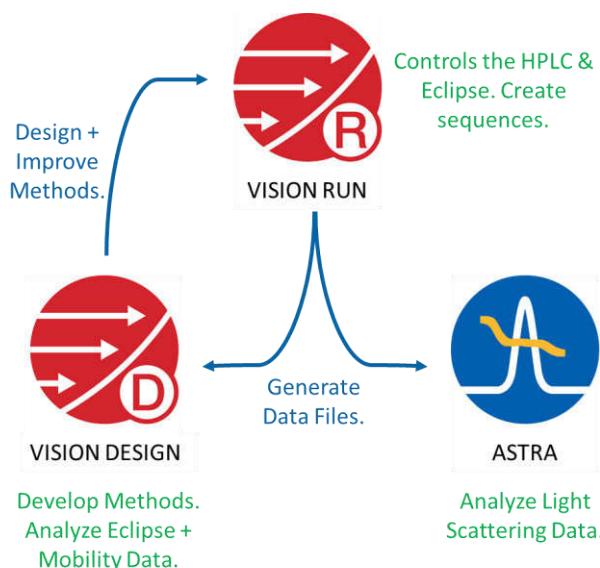


Figure 21. An overview of the workflow when using VISION DESIGN, VISION RUN, and ASTRA. VISION DESIGN can be used to develop methods from scratch using hydrodynamic radius, and then subsequently improve the method using experimental data. Additionally, it can analyze data from the Eclipse Mobility Module. VISION RUN is the control center for the HPLC and Eclipse and is used to execute methods via sequence tables. ASTRA is Wyatt Technology's software for analyzing light scattering data.

In Figure 22, the software integration of the HPLC stack, the Eclipse, the separation device (for example, an FFF channel), a DAWN multi-angle light scattering detector, and an Optilab differential refractive index (dRI) detector with the VISION and ASTRA software are shown. ASTRA collects data from the DAWN and Optilab. VISION controls the Eclipse and HPLC, and via sequences can generate both EMDF and ASTRA data files.



Figure 22. A simplified overview of the Eclipse FFF-MALS solution. The HPLC and Eclipse are controlled by the VISION software, which can generate ASTRA data files from sequences with data collected from Wyatt analytical detectors. Fundamentally, the Eclipse is a flow separator that takes a single pump flow and diverts that flow to achieve FFF-MALS separations in a channel. After separation, the sample can be analyzed via downstream analytical instruments like UV, MALS, and RI detectors.

VISION RUN Instrument Configuration

If you already have an HPLC and Eclipse configured, VISION RUN can be launched by selecting the appropriate tile in the VISION hub. Then the appropriate instrument configuration can be selected and the software launched **Launch** (Figure 23). If your instrument does not need to be added or configured, skip to the next section entitled **VISION RUN**.

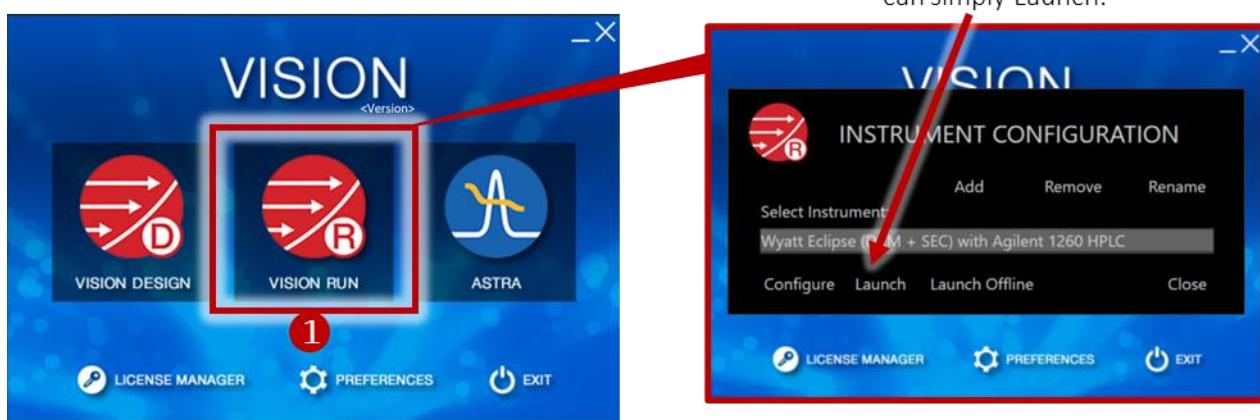


Figure 23. VISION RUN launch window.

If your HPLC stack has not been configured, a new entry will need to be added by clicking **Add** (Add) and then entering a name for the system. Once the name is entered and the system is added to the list, it can be configured to communicate with your HPLC and Eclipse. Select **Configure** (Configure) to open the Instrument Configuration Window (Figure 24).

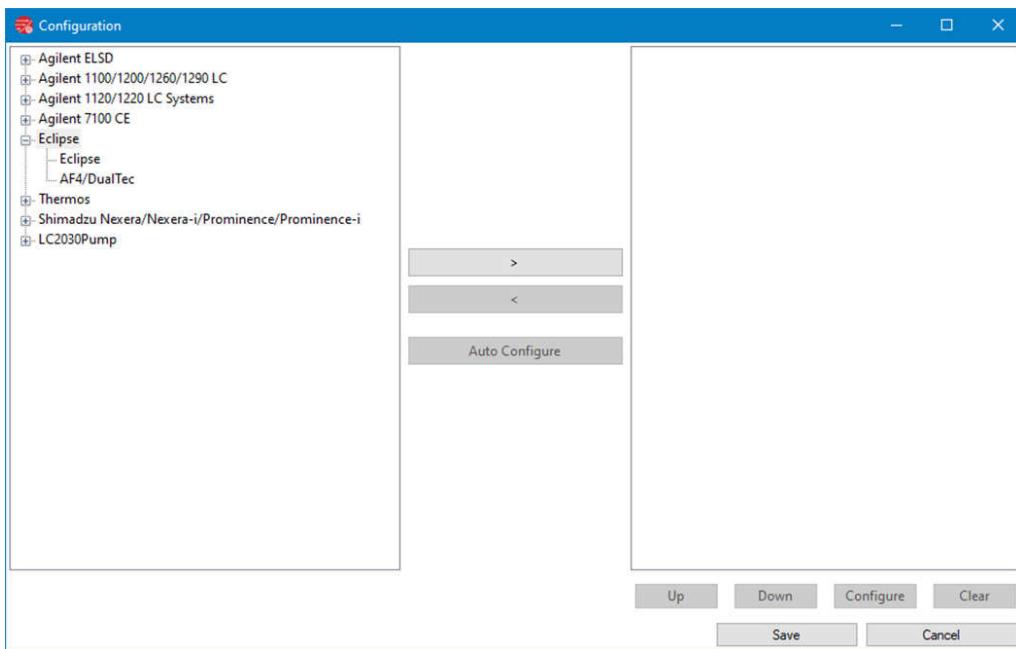
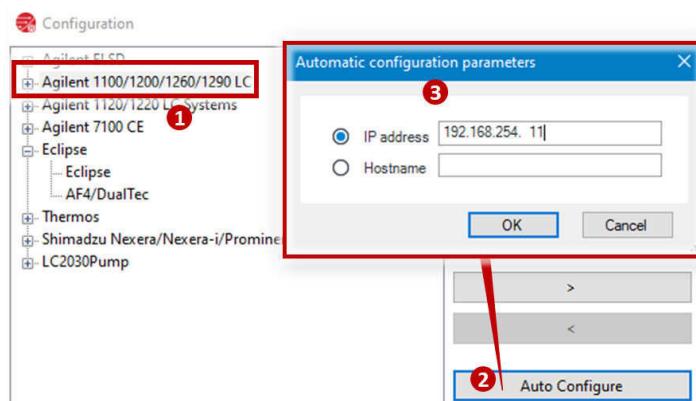


Figure 24. Agilent and Eclipse configuration window.

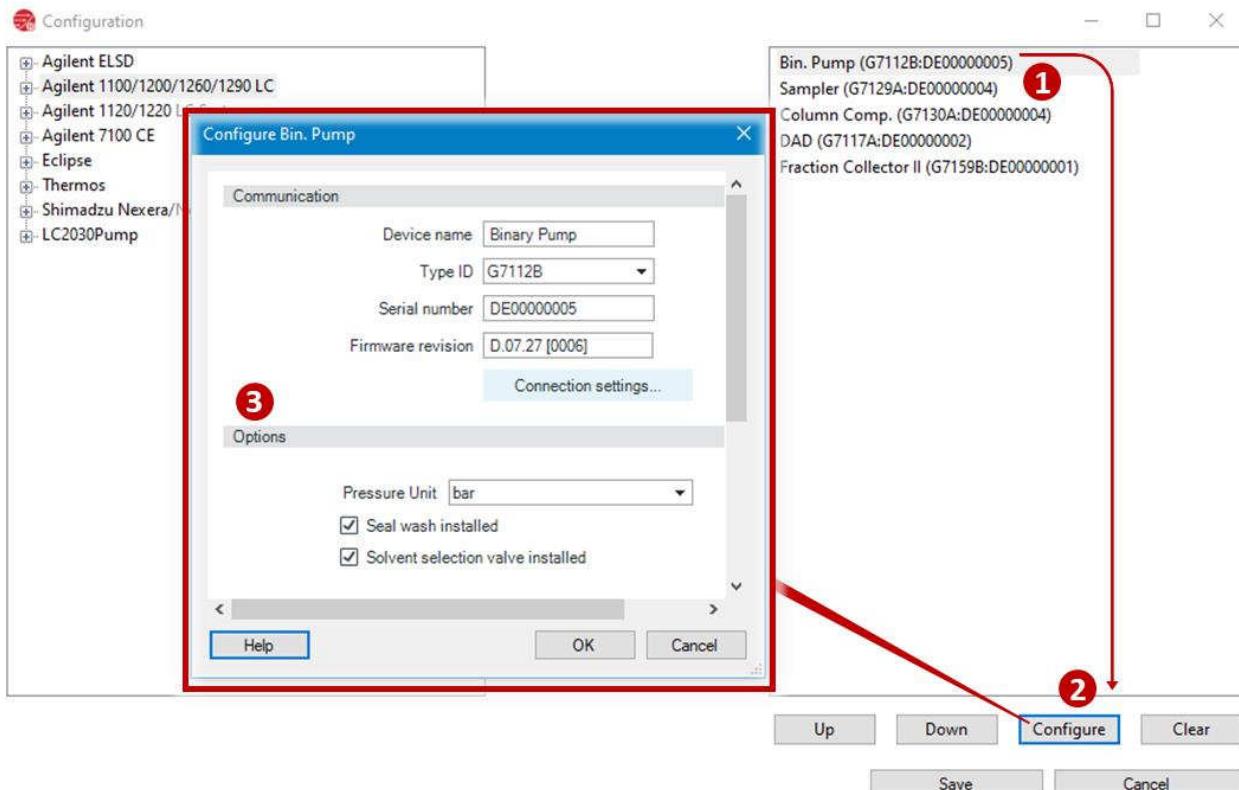
With reference to Figure 24, the Agilent HPLC and Eclipse can be configured by following the instructions below:

1. Select the top-level Agilent listing on the left-side of the screen (i.e., Agilent 1100/1200/1260/1290 LC). You do not need to expand the selection as long as all of your Agilent modules are interconnected via CAN cables with the LAN cable communicating with the PC. You can then select **Auto Configure** (Auto Configure). The IP Address for the Agilent HPLC can be entered into the “automatic configuration parameters” dialog window. The default IP address for Agilent HPLCs is 192.168.254.11.

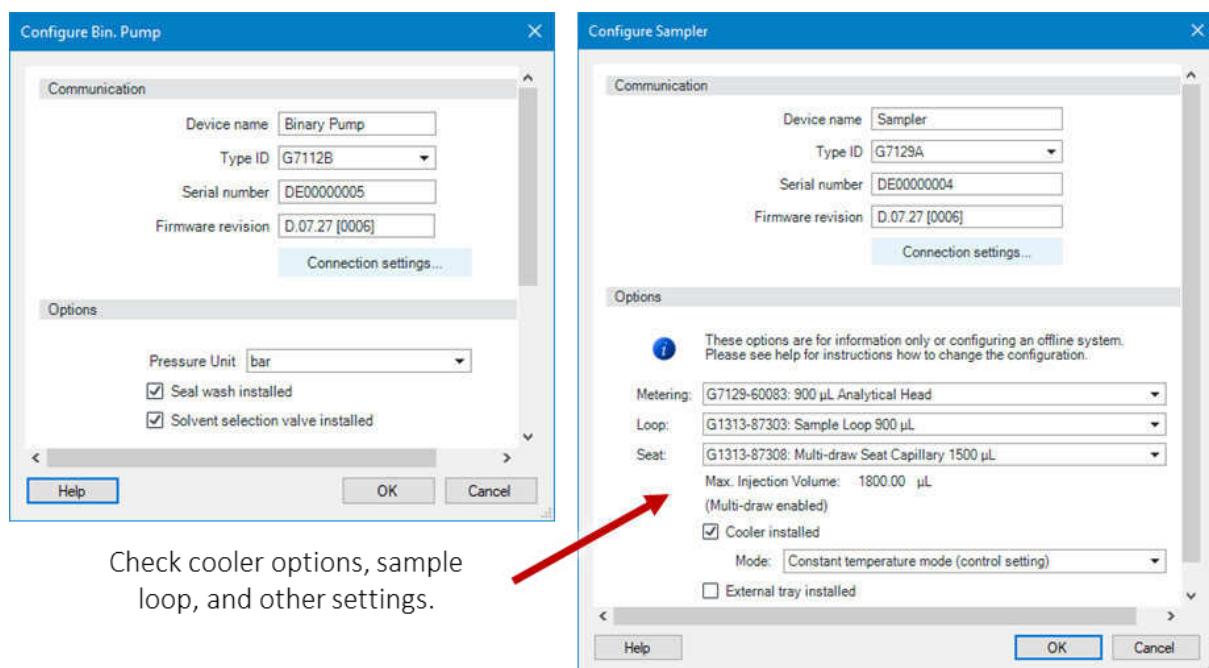


If you do not know the Agilent HPLC IP address, please contact Agilent for the most up-to-date information. In many older Agilent HPLC systems, the default address is set by having the 5 & 6 dip switches up and the other switches down. For many newer Agilent HPLC systems, the default address is set by having all dip switches down (6 total) located on the rear panel of the module.

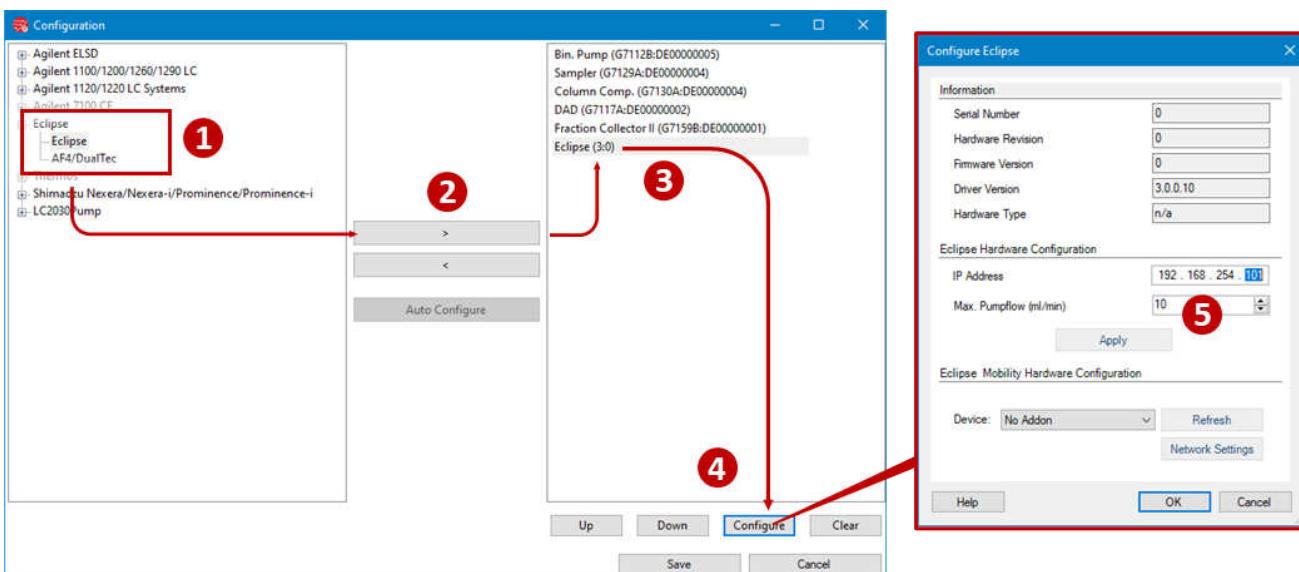
2. With the appropriate Agilent HPLC IP address entered, the instrument modules should automatically be detected and added to the right-side of the screen. Additional settings for each module can be accessed by selecting **Configure** (Configure).



3. Although most settings may be pulled from the Agilent Instrument Control Framework (ICF), and would not need adjustment, it can be helpful to check that certain options and configurations are correct. These include whether a seal wash is installed for the pump and the correct sample loop or multi-draw kit settings for an autosampler.



4. Once the HPLC is configured, you should also add the Eclipse to the instrument list. To do this, expand the Eclipse node on the left and select your Eclipse model—either current generation Eclipse or legacy Eclipse AF4 or DualTec. If you are not sure what kind of Eclipse you have, you can look at the front panel of your instrument—anything with a 10" touch-screen will be the current generation and legacy Eclipse will have a small blue display. Move the Eclipse over to the right-side (**>**) and select **Configure** (Configure). In this window, you can type in the Eclipse IP address (found on the front panel under Settings → Network as shown in Figure 5) and select **Apply** (Apply).



5. After selecting Apply, the Eclipse serial number and firmware version should populate the fields. At this time, the Eclipse Mobility module—if installed, can also be configured by selecting from the dropdown. The default IP address for Eclipse Mobility is 192.168.254.50.
6. The instrument configuration window can be saved, which will close the window and return you to the VISION RUN Instrument Configuration window.
7. You can launch the software with the correct configuration selected.

VISION RUN Settings

Many of the configuration settings will be retained between sessions when using VISION RUN; however, the next few sections will cover recommended settings and procedures that should be performed when using VISION for the first time and are helpful to check and confirm when running VISION after the initial installation.

A detailed walkthrough of the software, including creating methods and running sequences will be described in the section, [VISION RUN Operation Workflow](#). If your software has already been configured and these settings have been established, you can proceed to subsequent sections.

An overview of the VISION RUN interface is provided in Figure 25. Influenced by the Agilent ICF, the layout and structure will be familiar to those who have used Agilent HPLC software in the past. At a glance, the methods and sequences are located on the left-panel (collapsible) and can be loaded by double-clicking on the desired file, which will initiate the first line of the method. The instrument modules can be individually viewed and modified in the main instrument status window and will form the basis for creating methods and controlling the instruments. Real-time data can be displayed at the bottom of the window, including ASTRA basic collection data.

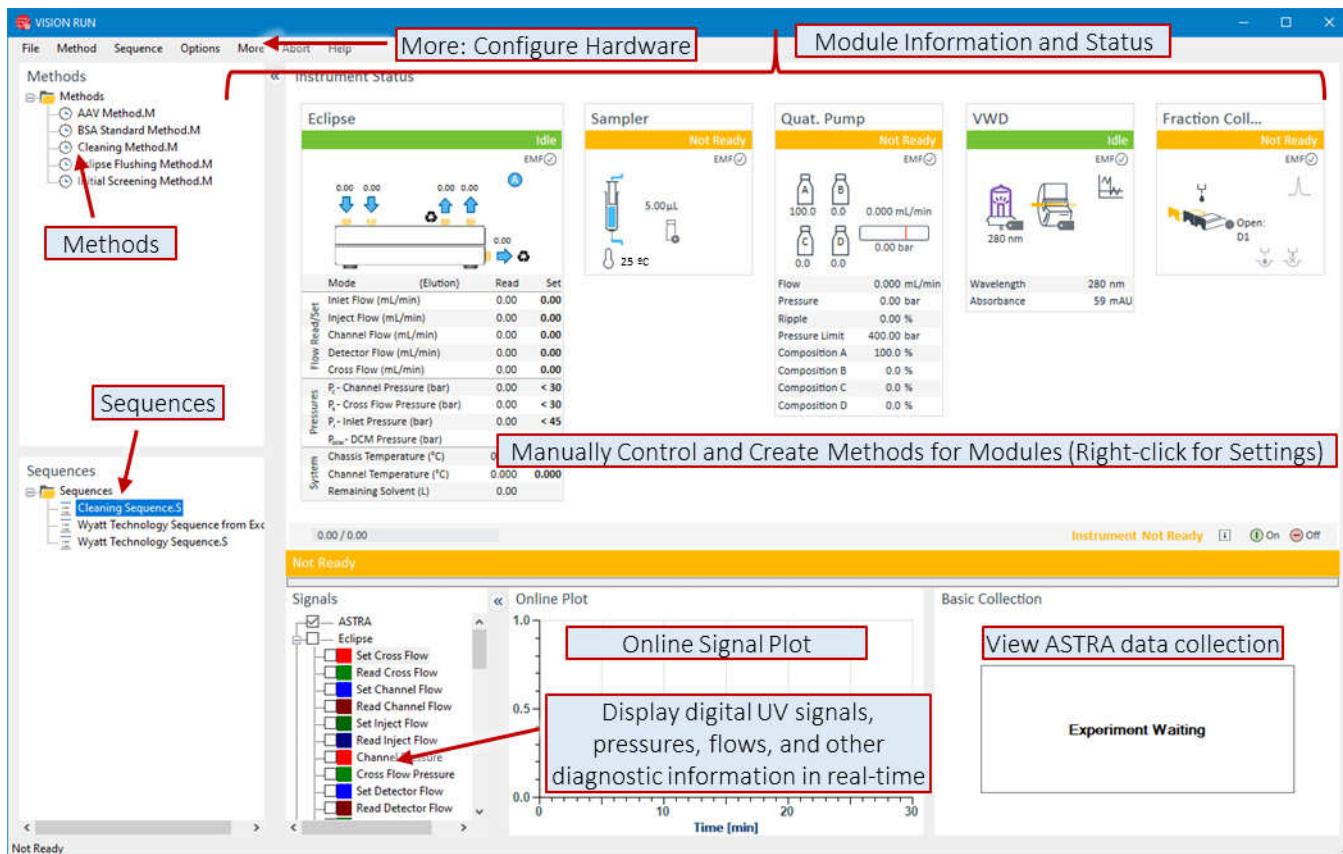


Figure 25. VISION RUN user interface overview

In the next sections, recommended software settings will be outlined.

Separation Device and Hardware Configuration

The Eclipse separation device and hardware configuration can be accessed by navigating to **More** → **Eclipse** → **Separation Device Configuration / Hardware Configuration** as shown in Figure 26.

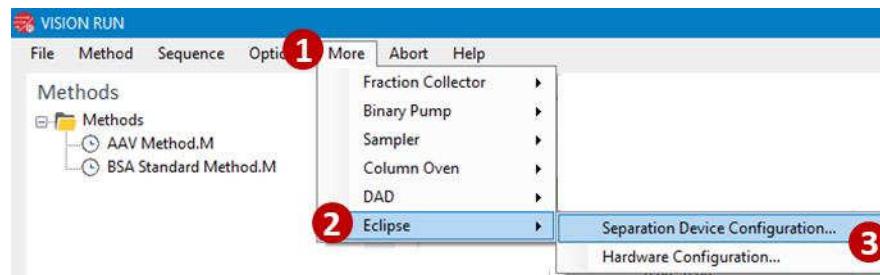


Figure 26. Accessing either the Eclipse separation device or hardware configuration window.

The windows are shown side-by-side in Figure 27, with the separation device configuration window on the left and the hardware configuration window on the right. Under the separation device configuration window, the currently installed device(s) can be configured. An Eclipse with SEC switching option can be configured with a column in Port B. Non-SEC switching option models can only be configured with a single separation device. This will enable that port to be selected and manually controlled as well as assist with record-keeping on the properties of the currently installed device. Under the hardware configuration, the chassis target temperature can be set to a temperature above the ambient temperature of the lab. The other settings can be left at the default value. One invaluable option

is the Rinse After Sequence toggle. When enabled, the system will go to a low flow rinse mode after the sequence is complete. This can also be enabled under the manual control window described later.

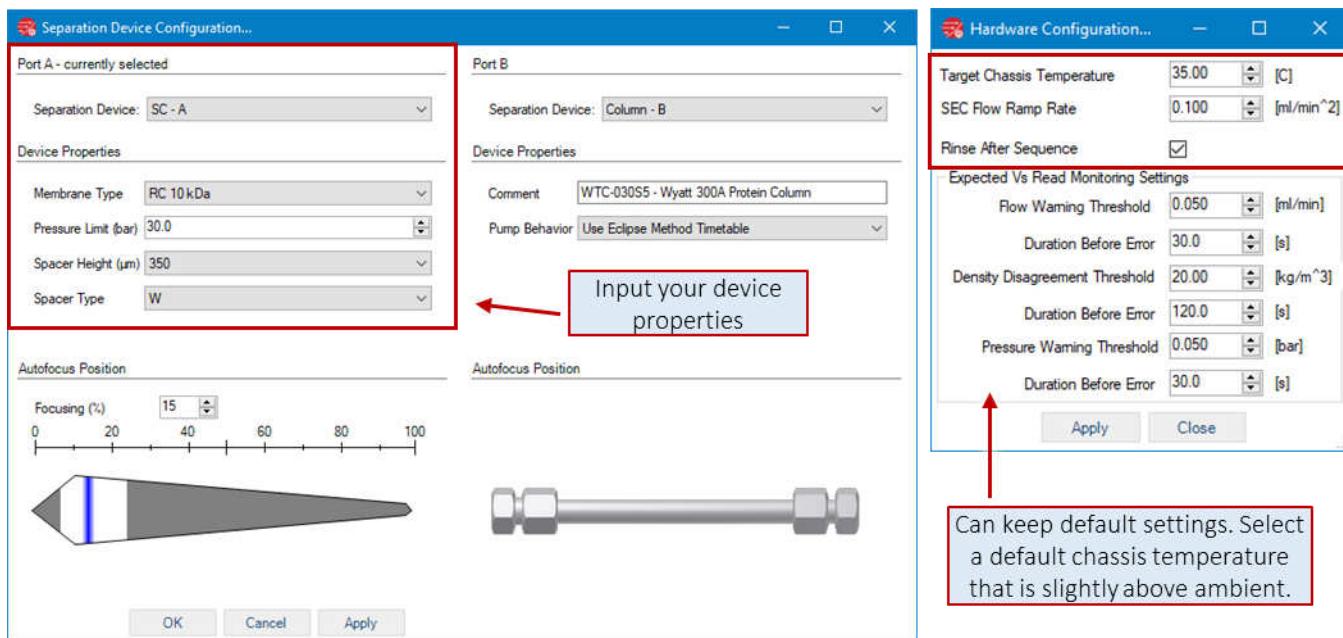


Figure 27. Separation device configuration (left) and hardware configuration (right) windows.

With the initial configuration complete, VISION RUN is ready for basic operation. In the next section, the manual control window will be discussed. Afterwards, the solvent exchange protocol for exchanging solvents in your system will be outlined using the manual control option.

Eclipse and HPLC Control Settings

Although most instrument parameters are set at the method level, there are instrument settings that Wyatt Technology recommends at the instrument (or control) level. This section will discuss instrument settings that are *not* located in the method editor windows. Method settings, including recommended UV settings and HPLC pump compressibility settings, are described in the [Considerations for some HPLC Instrument Methods](#) section.

Eclipse Performance and Tuning

The Eclipse is a sophisticated instrument that is capable of taking a single pump flow and directing that flow in different ways to achieve FFF-MALS separations. A number of sensitive performance and tuning functions are available that are recommended to be adjusted for optimal performance and system responsiveness—although quality data can still be achieved even with default settings. Other settings may need to be updated by users when changing the method. Which settings need regular updating and which should not be adjusted are described in the following sections. These options are accessed by right-clicking on the Eclipse widget and selecting “Performance and Tuning.”

WARNING: Adjusting the settings in the Performance and Tuning window can cause damage to the system. Contact Wyatt Technology Support for assistance before altering any of the settings.

Valve Preset Values (*Rarely need adjusting with VISION 3.1.1 or earlier*)

The top of the Performance & Tuning window contains valve preset values that will be determined at installation by Wyatt Technology scientists and only rarely, if ever, need to be adjusted. These valve presets determine the value at which the controller valves associated with the flow regulators (CoriFlow) open, which improves response

time for flow application. It is equivalent to the “Learn Open” function that was previously utilized in legacy Eclipse. After installation, it is only necessary to run this function if recommended by Wyatt Technology Support upon inspection of the EDMF. At the very least, no valve preset should be at a value of 0% and are typically in the 20 – 35% range.



Figure 28. Autofind functions under Performance & Tuning

Note: With VISION 3.1.2 and later, there is a toggle next to “Autofind All” that enables automatic preset determination. When this is enabled, it is no longer required to update or check these valve presets. Contact Wyatt Technology Support for assistance before altering any of the settings.



Automatically determines preset values, and so no longer requires any adjustment to the preset values (recommended).

There are four valve presets—crossflow, inlet, inject, and pressure. Although these values should not change significantly over time, the “Autofind” function can be run when instructed to by Wyatt Technology. After selecting the “Autofind” button, the Eclipse will enter a tuning state (the Eclipse may go from a green idle state to a yellow not-ready state). The Eclipse will automatically determine appropriate values over several minutes and then populate the fields with new values.

Focus Flow Offset (Method-dependent, may need adjusting regularly)

As opposed to the valve presets, the focus flow offset (FFO) may need to be regularly updated. This is because the FFO is based on the flow rates used in the elution and focus steps. The FFO is designed to help improve the detector flow regulation between focus and elution modes, which improves signal stability. The software will increase the detector flow only during the focus steps so that the channel pressure in focus is equal to the channel pressure in elution, or in DCM Eclipse instruments, the DCM pressure. A detector flow increase is referred to as the focus flow offset.

The “Autofind” button applies the flows of the currently loaded method’s first elution and first focus step. There will be an interval of time at those flow rates in focus mode, and then in elution mode. It averages the pressure from the last few seconds of each mode and calculates the FFO. You can fine-tune this value by running the autofind multiple times.

It is important to note that the FFO (typically some small amount of flow in the 0.05 – 0.2 mL/min range) is added to the total pump flow during focus mode, thus ensuring more consistent pressure when switching between focus and elution and better regulation. Any discrepancy between the set flows in the method and the read flows may be due to the FFO during focus mode.

The FFO will need to be updated whenever changing the method focus flow significantly for best performance.

Controller Settings (Do Not Need Adjusting)

While the focus flow offset does need regular adjustment for the best performance and the valve presets may require adjustments rarely, the loop setting values in the Performance & Tuning window do not typically need adjustment. These settings are the DCM Pressure Control, Chassis Temperature, and Channel Temperature Loop Settings. These values do not need to be adjusted; however, the recommended kp, ki, kd, nave, and Max P-Diff settings are provided in Figure 29. Changes to these values may cause oscillations in the DCM pressure and flow control or temperature. These settings should not be altered. Contact Wyatt Technology Support for assistance before altering any of the settings from those specified below.

Dcm Pressure Control Loop Settings

Integral:	0.888	Error:	-0.001	Output:	1.646%	Setpoint:	0.0000	[ml/min]	Disable	Enable		
kp	2.0000	ki	0.0200	kd	0.0000	nave	1	Max P-Diff	0.020	Max %	0.920	Set

Chassis Temperature Loop Settings

Integral:	18.342	Error:	-0.088	Output:	87.737%	Setpoint:	30.0000	[C]	Disable	Enable		
kp	0.5000	ki	0.0500	kd	0.0000	nave	5	Max P-Diff	0.050	Max %	1.000	Set

Channel Temperature Loop Settings

Integral:	-5.011	Error:	-1.958	Output:	0.000%	Setpoint:	28.0000	[C]	Disable	Enable		
kp	0.5000	ki	0.0500	kd	0.0000	nave	5	Max P-Diff	0.050	Max %	1.000	Set

Figure 29. Settings such as the DCM Pressure Control, Chassis Temperature, and Channel Temperature Loop Settings are provided here for reference but do not need adjusting.

Eclipse CoriFlow Cross Flow Capacity Adjustment

If you are working with very low cross flows (< 0.04 mL/min), enhanced control over flow can be improved by adjusting the cross flow capacity. Please note that if you have experience with the Eclipse AF4 or DualTec cross flow capacity, the regulation in the Eclipse (NEON) is much more improved. A default cross flow capacity of 6.67 mL/min can regulate down to 0.03 – 0.04 mL/min cross flow with sufficient back pressure. If cross flow control below that threshold is needed, the cross flow capacity can be adjusted in the Performance & Tuning window.

Only the cross flow capacity needs to be adjusted, and can be reduced to 2 – 3 mL/min. The default value of 6.67 mL/min and the window is shown in Figure 30. With Eclipse (NEON) and VISION 3, there is no need to adjust a conversion factor anywhere—the capacity here is automatically applied to the software and hardware.

Bronkhorst Controller Parameters

Cross Flow Controller

Kp	15.0000	Ti	0.1500	Td	0.0000	<input checked="" type="checkbox"/> Capacity	6.6670	Apply	Zero
----	---------	----	--------	----	--------	--	--------	-------	------

Inject Flow Controller

Kp	10.0000	Ti	2.0000	Td	0.0200	<input checked="" type="checkbox"/> Capacity	1.0000	Apply	Zero
----	---------	----	--------	----	--------	--	--------	-------	------

Inlet Flow Controller

Kp	8.0000	Ti	0.2000	Td	0.0000	<input checked="" type="checkbox"/> Capacity	10.0000	Apply	Zero
----	--------	----	--------	----	--------	--	---------	-------	------

Pressure Controller

Kp	2000.0000	Ti	0.2500	Td	0.0000		Apply	Zero
----	-----------	----	--------	----	--------	--	-------	------

Detector Flow Meter

<input type="checkbox"/> Capacity	3.0000	Apply	Zero
-----------------------------------	--------	-------	------

Figure 30. Bronkhorst Controller Parameters section of the Performance and Tuning window for Eclipse. The only parameter that should be adjusted here is the Capacity factor.

NOTE: In order to adjust the capacity, VISION 3.0.1.12 or higher is required.

To make a change to the capacity, enable to toggle. This determines whether the field can be edited or not. Once enabled, the field can be edited to the value you desire. Only the capacity of the cross flow controller is ever typically adjusted. It is important to note that controller stability decreases with reduced capacity, as a result the valve exhibits more noise when the capacity is reduced. *As a result, it is recommended to use the highest capacity possible up to the default value of 6.67 mL/min, which enables excellent cross flow control down to 0.03 – 0.04 mL/min.* Your specific cross flow regulation can be viewed in the Online Plot or in VISION DESIGN. Once you have input the desire capacity, hit “Apply” to change the software and hardware capacity settings. You can then toggle off the checkbox to prevent editing. The toggle only determines whether the field can be edited, and the value must be applied to take effect.

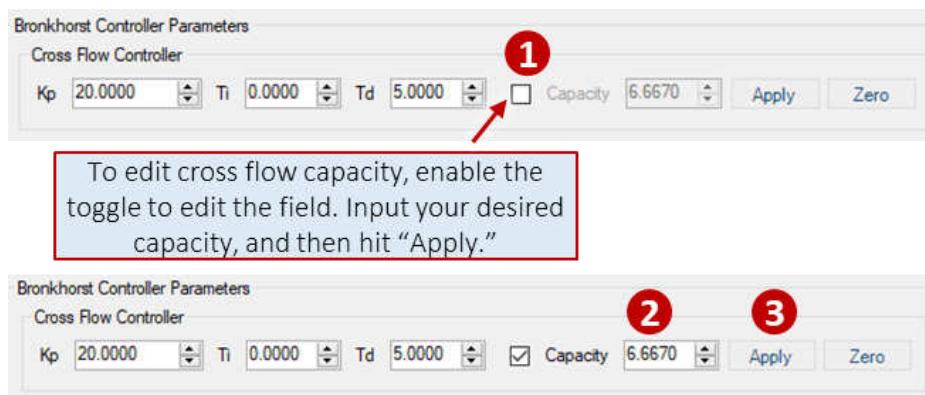


Figure 31. If cross flow regulation below 0.04 mL/min is needed, you may adjust the cross flow capacity in the Performance & Tuning window. Only the cross flow capacity is every typically adjusted. You will need to (1) enable the field to be edited by checking the toggle, then (2) edit the field, and (3) Apply the changes. Once applied the toggle can be checked off.

HPLC Pump Settings

The Eclipse features the Intelligent Solvent Management (ISM) system as a convenient function that is capable of recycling both detector and cross flow or either one individually while tracking total recycled flow rates. As a result, the Eclipse module controls the solvent and bottle filling parameters because it is tracking the recycled solvent flow rates, whereas the HPLC pump is unaware that solvent recycling is happening. *As a result, it is important to disable the HPLC pump bottle fillings and ensure all solvent management is done through the Eclipse.*

CAUTION: Enabling the Agilent HPLC pump bottle fillings may result in premature pump stoppage as the pump bottle fillings does not track recycled solvent via the Eclipse ISM system. Please only use the Eclipse solvent manager for solvent tracking. Additionally, as of June 2022, the Eclipse ISM is only compatible with a single solvent reservoir. Update the values whenever switching solvent reservoirs for binary or quaternary pumps. Contact Wyatt Technology Support for assistance before altering any of the settings.

To disabled the HPLC pump bottle fillings, right-click the pump module and navigate to “Bottle Fillings.” In the bottle fillings window, you should disable the actions.

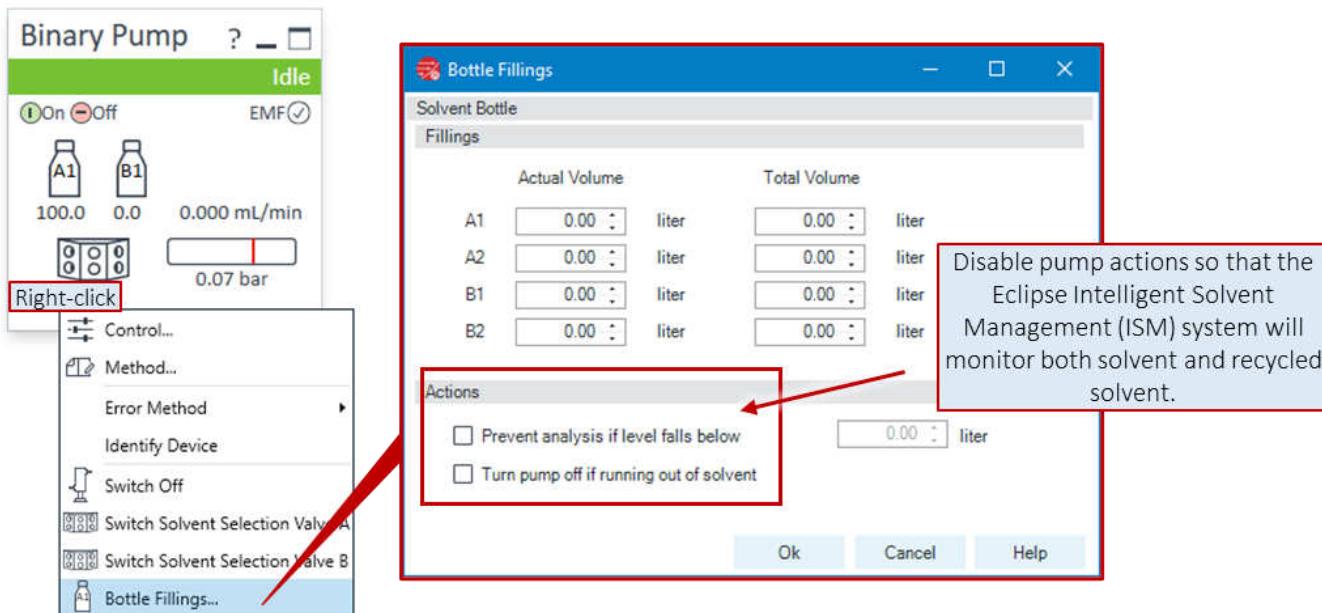


Figure 32. Disable HPLC pump bottle fillings when using the Eclipse with Intelligent Solvent Management (ISM) system to avoid conflicts. The Eclipse will track recycled solvent that the HPLC pump is unaware of. This can result in the HPLC pump preventing analysis or turning off even though there is still solvent available.

Eclipse Intelligent Solvent Management System

The Eclipse Intelligent Solvent Management (ISM) system can be accessed by right-clicking the Eclipse widget and selecting “Manage Solvent.” In this window, the current solvent capacity of the main solvent reservoir (that which contains the solvent reservoir specified in the pump method and the recycle line from the Eclipse). This is so the Eclipse can track the current solvent volume and factor in the amount of recycled solvent. This window is shown in Figure 33.

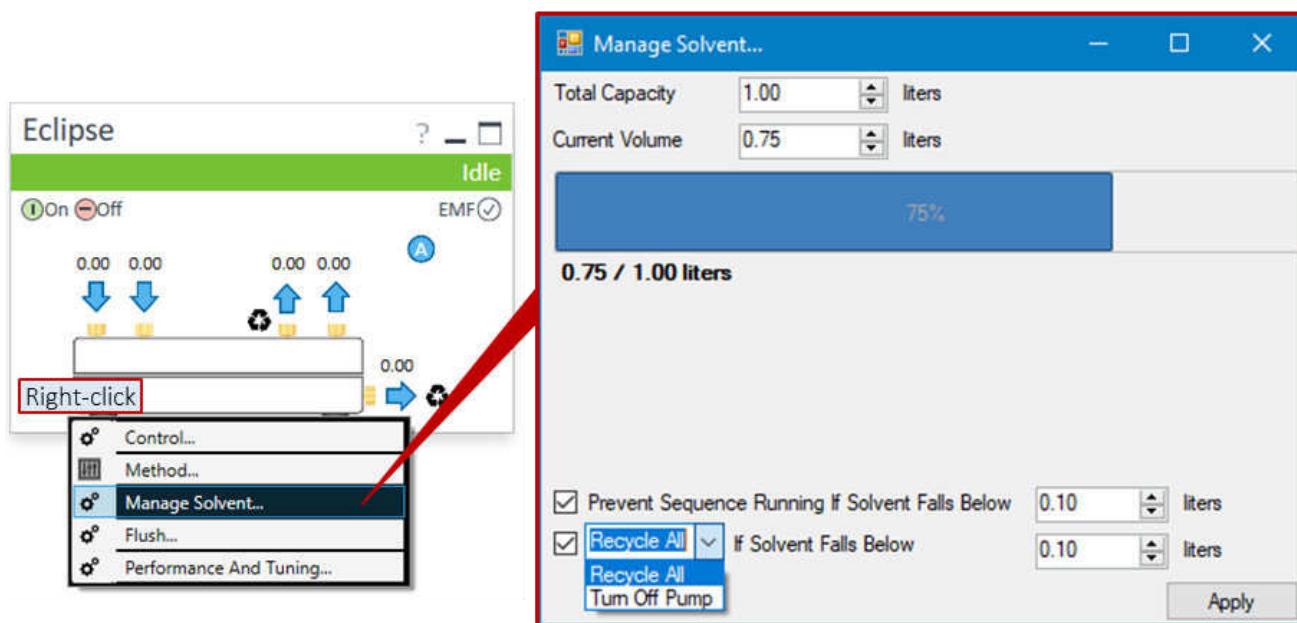


Figure 33. Eclipse Intelligent Solvent Management (ISM) system window.

The first toggle, “Prevent Sequence Running if Solvent Falls Below” will prevent the sequence from starting if the Eclipse predicts the solvent will fall below a certain volume. This will ensure you have an appropriate amount of solvent before starting the run. The second toggle will apply a protocol if the solvent level falls below a value, either turning off the pump or recycling all solvent to avoid running dry.

NOTE: The Eclipse Intelligent Solvent Management (ISM) system should be updated whenever the solvent reservoir is replaced with fresh solvent or if you observe a mismatch between the actual solvent in the bottle and the reading on the Eclipse widget. The Eclipse will display the current assumed solvent volume at the bottom of the widget.

The current solvent level estimated by the Eclipse can be found at the bottom of the Eclipse widget. The VISION interface and instrument information will be discussed in [VISION RUN Operation Workflow](#).

Autosampler Settings

Most autosampler settings are defined at the method level; however, if a cooler is installed for your autosampler, you can define whether the control-level or the method-level determines the temperature. The control-level is recommended if you want to keep the autosampler at a certain temperature at all times. These settings are only available if a cooler is installed and by right-clicking on the autosampler widget.

In VISION 3.1.2 or later, VISION RUN can execute injector programs defined by the sampler. The injector programs can be enabled or disabled in the VISION RUN sequence. Only one injector program can be applied at a time, and injector programs cannot be saved.

UV or FLD Detector Settings

Most UV or FLD settings are defined at the method level; however, the analog output range may be defined under the control options. For Agilent UV detectors, it's recommended to export UV on the 1 V output range. This can be accessed by right-clicking on the UV widget and navigating to “Control.” Other settings defined at the method level, such as offset, are described in the section, [Considerations for some HPLC Instrument Methods](#).

Fraction Collector Settings

Please refer to [TN6504 - Configuring a Fraction Collector with VISION](#) for more information about appropriate settings and considerations. It is recommended to decouple or de-link the pump from the fraction collector and specify the detector flow manually. This is because while the pump may deliver 3 mL/min of flow during an FFF-MALS experiment, only a fraction of that flow enters the analytical detectors. As a result, a linked pump may underfill vials in the fraction collector. Additionally, delay volumes between Agilent detectors and the fraction collector may be set at the control-level. Other options are available at the method level.

Eclipse Manual Control Overview

The Eclipse Manual Control window enables flow control over the system and Eclipse without the need to load a method. This can be used to equilibrate a system before a run, to perform a solvent exchange (described in the section [Using Manual Control to Perform a Solvent Exchange with VISION](#)), to flush the system after a new channel has been installed (described in [Flushing Membrane without Eclipse + DCM Model \(WEC, WECS\)](#)), and to troubleshoot pressures by enabling direct control over the Eclipse and its associated modes (Elution, Focus) with or without injection.

The combination of the Eclipse module and the manual control window are important for fully understanding the capabilities and diagnostic protocols for operating the Eclipse system. The manual control window can be accessed by right-clicking on the Eclipse module widget. This is shown in Figure 34.

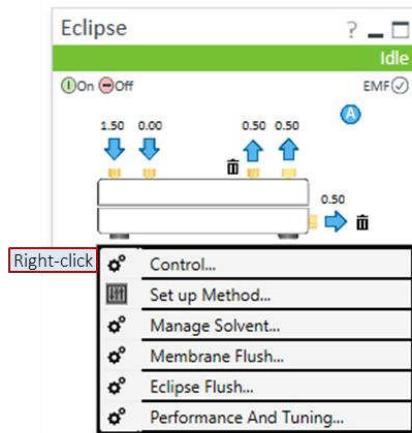


Figure 34. Accessing the Eclipse manual control window by right-clicking on the Eclipse widget.

The Manual Control Window is shown in Figure 35 for a short channel installed on an Eclipse with the dilution control module (DCM).

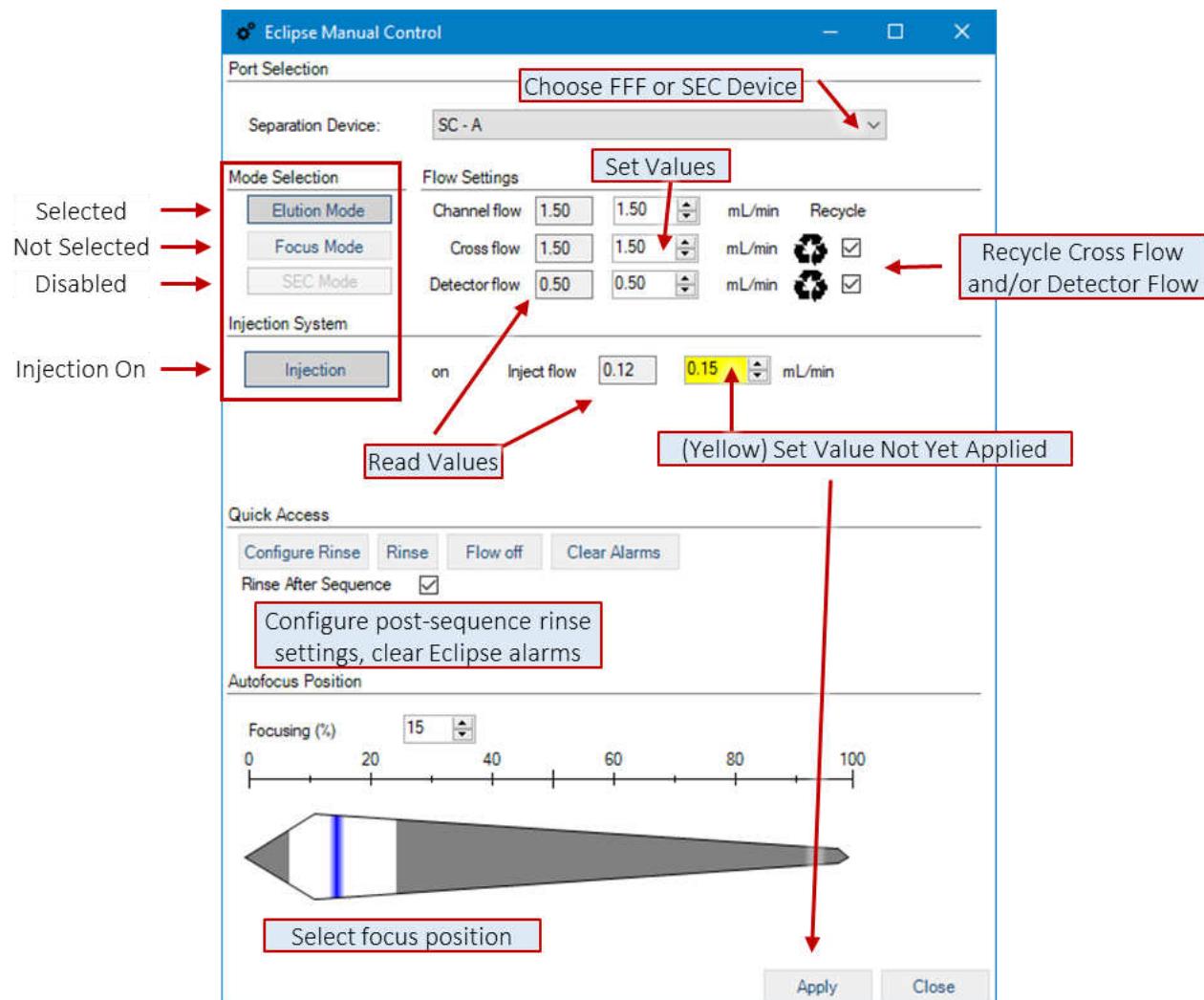


Figure 35. The Eclipse manual control window with a short channel. With the Eclipse + DCM models, the channel and detector flow can be specified. Without DCM, only the channel flow can be specified, as all channel flow is detector flow.

Under Manual Control, you can (independently of the method):

- Select the configured separation device and switch between FFF and SEC modes (which will enable or disable different flows).
- Switch between elution and focus modes, including with or without injection flow.
- Specify channel flow, cross flow, detector flow (with DCM), and injection flow rates and compare set values to actual values.
- Recycle the cross flow and/or detector flow.
- Control the focusing position.

CAUTION: Flow settings applied under manual control will draw solvent from the reservoir specified by the pump method. Ensure the desired solvent bottle is configured in the pump method prior to adjusting flows in manual control.

Changes in the flow rates will be indicated by a yellow-highlighted field **0.15** until Apply is selected, which confirms the flow rates. The currently selected mode is indicated by a dark gray box with a dark blue border as shown in Figure 36.



Figure 36. Manual control over Elution or Focus mode can be selected. In this case, the selected mode is Elution, which has a darker gray box and blue border.

The quick access buttons can be used for additional commands, as outlined below:

- **Clear Alarms:** Clears an Eclipse error, for example, an overpressure alarm, leak alarm, vapor alarm, or valve error if the alarm state has been addressed. Persistent alarms cannot be cleared; however, the audible alarm sound can be muted on the Eclipse front panel:



- **Flow Off:** Immediately switches off pump flow. Can also be applied from the Eclipse instrument front panel.



- **Configure Rinse:** Enables customization of the rinse flow settings when selecting the Rinse button or after a sequence when “Rinse after Sequence” is toggled on. This option can be enabled in either the manual control window or the [Eclipse Separation Device](#) and Hardware Configuration.
- **Rinse:** Applies the currently configured flow settings under the rinse configuration.

The **Configure Rinse** option allows for custom flow settings which can even be used to rinse the system in elution mode with or without injection flow at a specified cross flow and detector flow. For the Eclipse with SEC switching option, both ports can be rinsed and the time interval between port switching can be specified. The rinse settings window is shown in Figure 37. It is always recommended to maintain a low flow after a sequence to reduce the risk of salt precipitation or solvent evaporation.

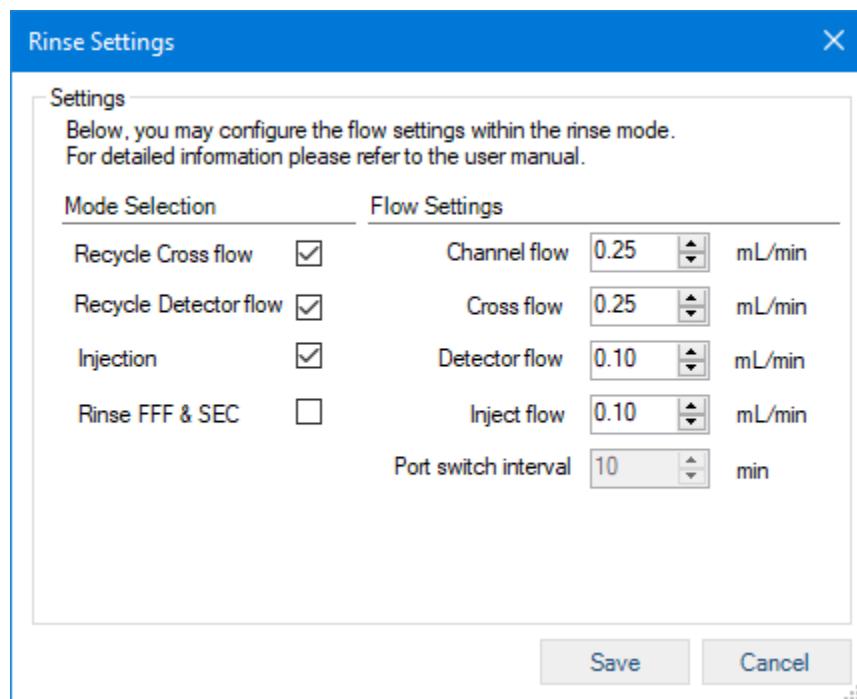


Figure 37. Configure Rinse can be used to specify flow rates for rinse mode.

For long-term storage, it is recommended to switch the system into an appropriate storage solvent (i.e., 20-50% alcohol solution). In the following section, [Using Manual Control to Perform a Solvent Exchange with VISION](#), the manual control window will be used to perform a system solvent exchange. If you understand the procedures for creating methods and running sequences, you can alternatively create a solvent flushing method as described in the section, [Method for Automating Solvent Exchanges](#). If your system has already been solvent exchanged, you can proceed to the [Eclipse Channel Overview and Membrane Installation](#) section. If your channel has already been prepared, you can proceed to the [VISION RUN Operation Workflow](#) section or if you already have a method, the [Creating and Running a Sequence](#) section.

Using Manual Control to Perform a Solvent Exchange with VISION

In this section, the solvent exchange procedures will be outlined for converting both the HPLC and Eclipse to the appropriate mobile phase for your experiments using the manual control window. Always use co-miscible solvents when flushing your system. Some co-miscibility recommendations are provided in Figure 38. If your Eclipse has arrived or returned from service at Wyatt, it will be stored in 50% alcohol solution. Going to a buffer like PBS will require an intermediate pure water flush, and going to organic solvent will require an intermediate alcohol flush and possibly an intermediate organic solvent flush.

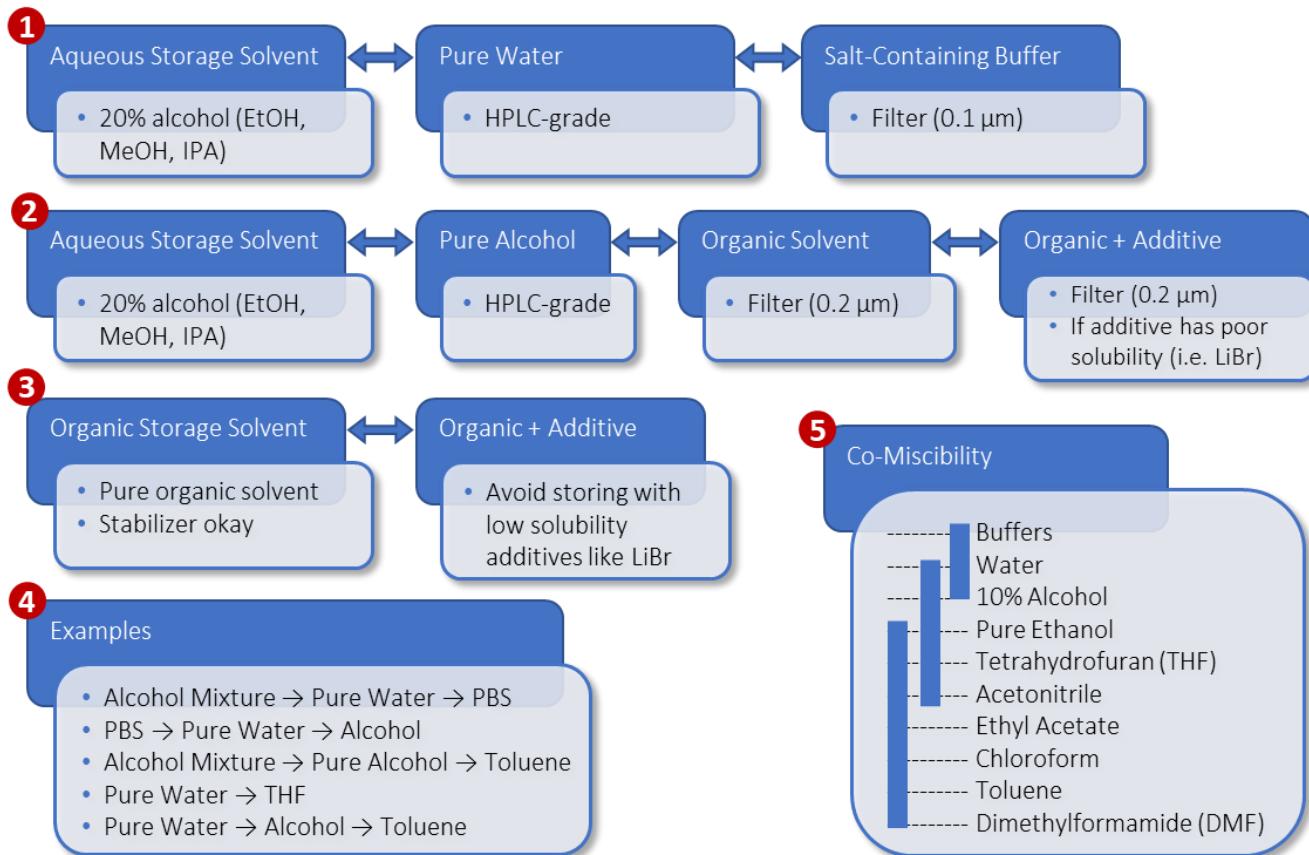


Figure 38. Overview of solvent exchange steps. (1) Going to or from salt-containing buffers like PBS from an aqueous storage solvent; (2) Going to or from additive-containing organic solvents from an aqueous storage solvent; (3) Going to or from additive-containing organic solvents from a pure organic storage solvent; (4) Examples of common solvent exchanges; (5) An overview of solvent co-miscibility. **NOTE:** For organic solvent applications, FFF is primarily limited to THF and toluene as compatible solvents due to the regenerated cellulose membrane compatibility. The co-miscibility chart shows other solvents that may be used in SEC mode.

For aqueous buffers, you may consider bypassing the channel with a 5-port union for the system solvent exchange, before installing the channel as described in the membrane installation section, [Replacing Channel Membrane and Spacer](#). However, for organic solvents, it is typically recommended to install the channel and flush with water because rinsing the membrane with organic solvents can easily deform the membrane and make membrane installation challenging. Always use co-miscible solvents when flushing your system.

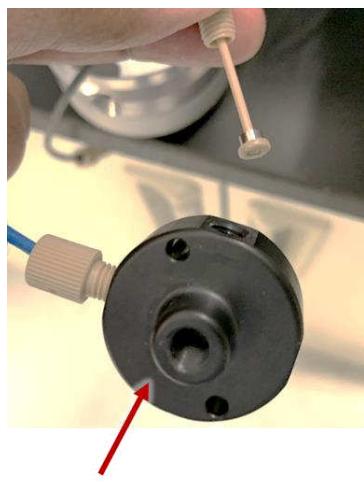
NOTE: The following procedure will walk you through performing a solvent exchange with the manual control window that was introduced above. The process can be partially automated by running a solvent exchange method, which is described in the [Method for Automating Solvent Exchanges](#) section. Although the manual flushing can be quicker, using a method can be more convenient.

The following procedure will walk you through a solvent flush with the Eclipse:

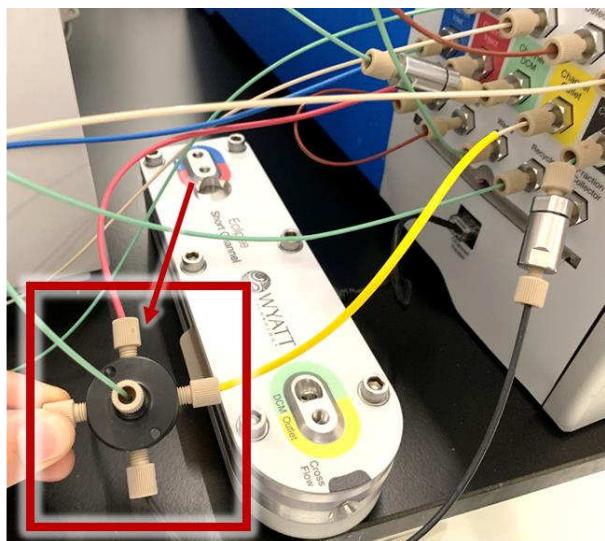
NOTE: The Eclipse has an internal function that can detect significant solvent density differences in the flow meters. This will display a caution badge on the front panel of the Eclipse, referred to as the “Solvent Density Disagreement” alarm. While performing a solvent exchange between solvents of different densities (i.e., from alcohol mixtures to water), the Eclipse front panel may be beneficial for monitoring the progress of the solvent exchange.



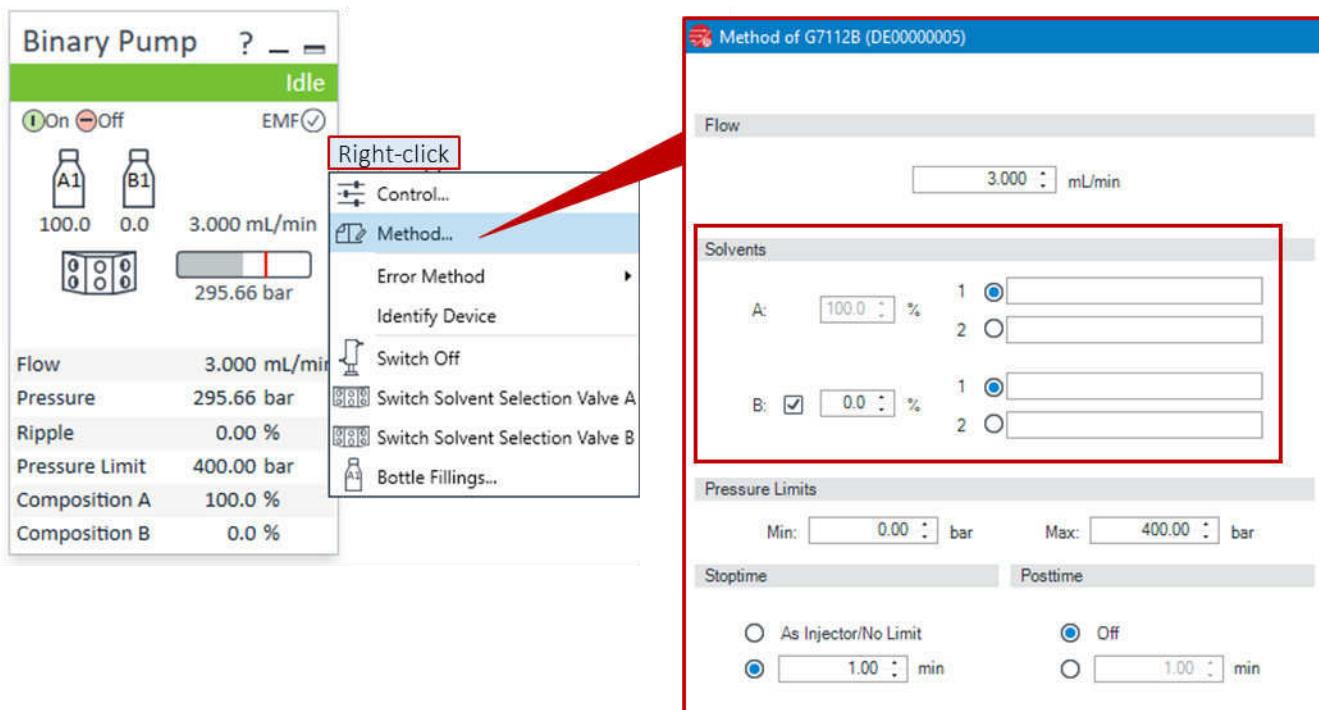
1. Make sure the storage solvent of your Eclipse and analytical detectors is compatible with the mobile phase in your HPLC before connecting. Remove columns from the system (after reducing flow rate slowly) and bypass the channel with a 5-port union unless exchanging the solvent in the channel in the case of organic channels assembled with water. The order does not matter for connecting to the 5-port union.



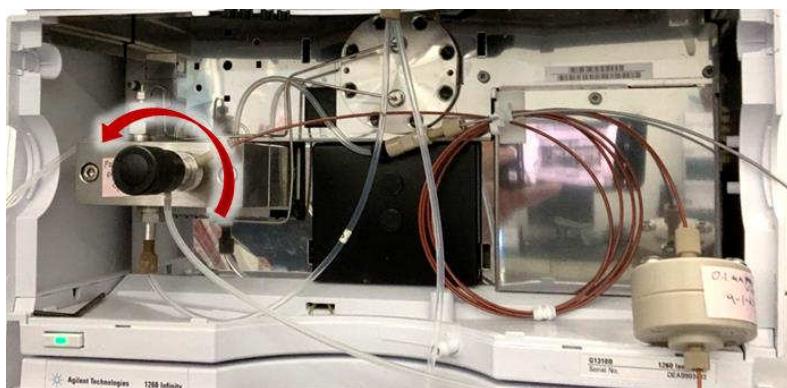
5-port union for bypassing channel



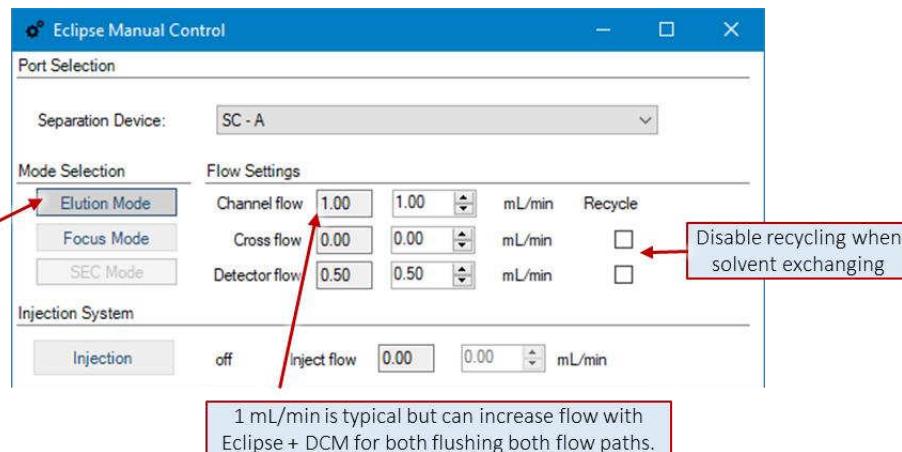
2. Identify the correct solvent reservoir (if using a binary or quaternary pump) with the solvent that will be flushed through the system. This solvent reservoir should be selected in the pump method by right-clicking and modifying the method to ensure the correct solvent will be pumped through the system.



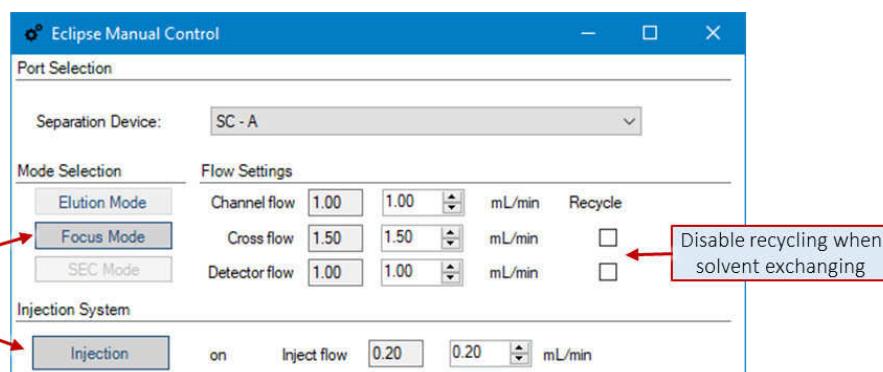
3. Once the solvent is selected in the VISION RUN method, when flows are applied via the manual control window, the pump will deliver the specified flow from the solvent reservoir selected. The degasser and pump will be flushed next. First, determine the volume of the degasser from the manufacturer specifications. With a discrete degasser module and associated tubing, the total fluid volume may be around 20 mL. The pump itself should be purged for at least 15 mL of volume.
4. **(Optional)** The degasser and pump can be flushed separately at high flow rates by first opening the black purge valve on the Agilent HPLC pump and then setting the flow in the Eclipse manual control to 5 mL/min. Flow for at least 5 - 7 minutes depending on the degasser volume. With the purge open, it does not strictly matter which flows are applied in manual control, as no flows will enter the system with the purge valve open.



5. Reduce the flow rate from 5 mL/min to 0 mL/min and close the purge valve completely.
6. Next, place the system in elution mode without injection and apply a channel flow of 1.5 mL/min with 0 mL/min cross flow. If you have an Eclipse with DCM model, you can apply 2 mL/min channel flow and 1 mL/min detector flow. Flow until the pressures stabilize or at least 10 - 15 minutes. If you did not purge the degasser or pump as described above, you can opt to flush the system with the purge closed for longer at these lower flow rates or increase the flow rate as needed to keep the system under reasonable pressures.

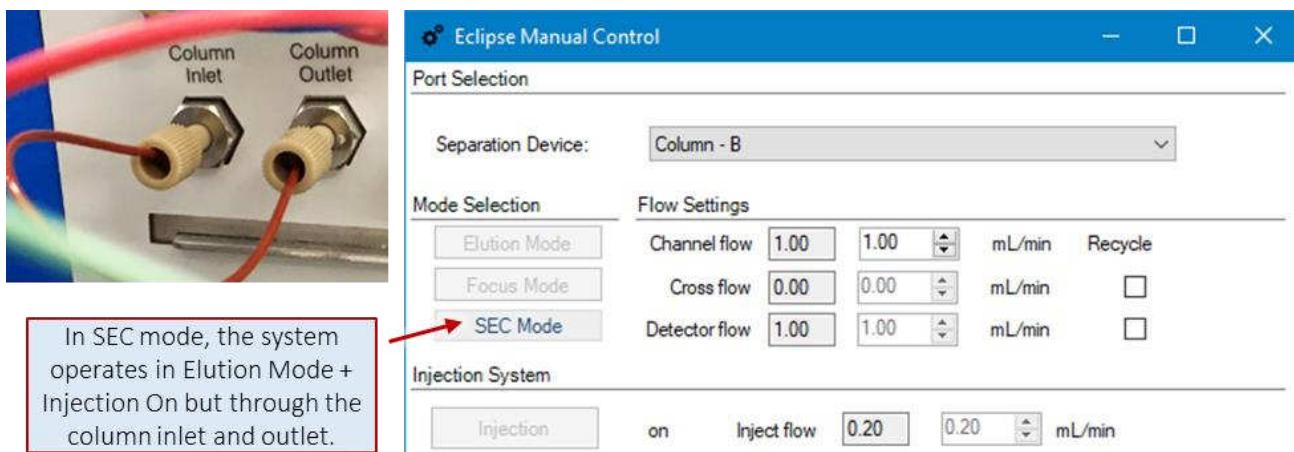


7. Turn on injection (**Elution Mode + Injection On**) and flush for 10 minutes with 1 mL/min channel flow and 0.1 – 0.2 mL/min inject flow.
8. With the elution pathway flushed, the focus pathway can be flushed next. Switch to **Focus Mode + Injection On** with a channel flow of 1 mL/min and a cross flow of 1.5 mL/min. Purge the system under these conditions for 15 - 20 minutes.

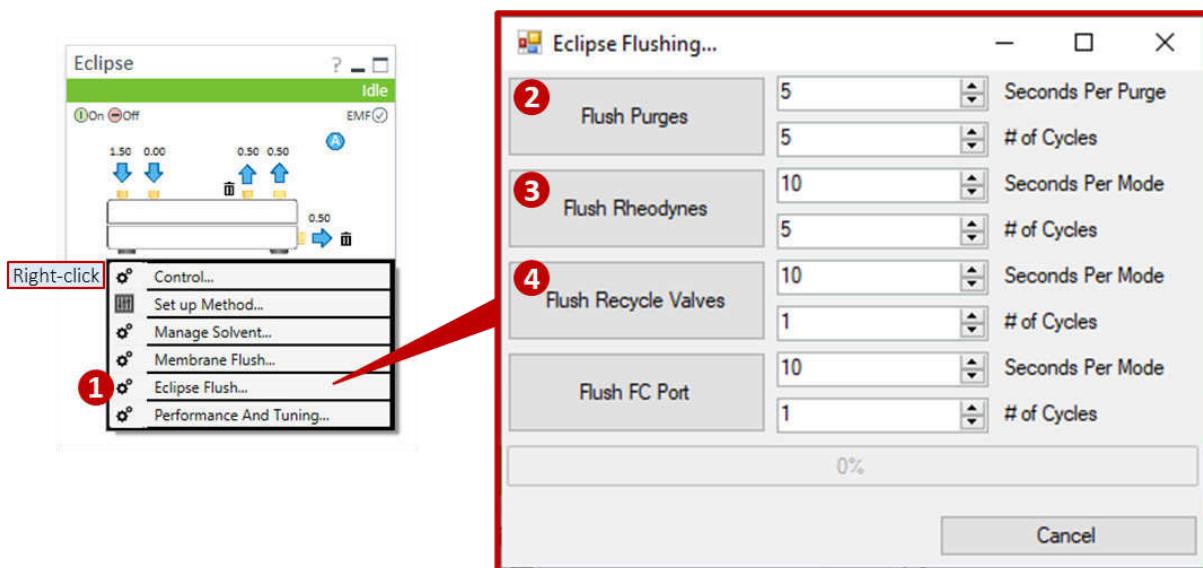


9. You may now turn off injection and flow in just **Focus Mode** for 5 minutes with the same flow rates as above, 1 mL/min channel flow and a cross flow of 1.5 mL/min.
10. After the four pathways (Elution Mode, Elution Mode + Injection On, Focus Mode, and Focus Mode + Injection On) have been flushed (~ 40 - 60 minutes total flushing), the next step will depend on the Eclipse model you have. An Eclipse with SEC switching option will need to have the SEC port (Port B) flushed to remove residual solvent. If the SEC port is stored in a storage solvent like 20 – 50% alcohol and will not be used, flushing that port may be omitted. If you will need to flush the SEC port as well, proceed to Step 11. If you do not have an SEC switching option, proceed to Step 14.
11. To flush the SEC port, first ensure that there is a PEEK tubing bridge connection between the column inlet and the column outlet. If your column requires flushing, it is recommended to flush to waste for at least 2 – 3 column volumes before re-connecting the column outlet as to avoid clogs. Column flushing can also be done later.
12. To flush the column fluid pathways with or without a column installed, switch the separation device from the channel to the column, which will automatically apply the flow rate gradient specified under **More > Eclipse > Hardware Configuration** described in Figure 27 as part of the section, **Separation Device and Hardware Configuration**. The default value is a flow gradient of 0.1 mL/min² to prevent shock to the column. When switching from the channel to the column pathway, the system will shut down the flow (as channels are insensitive to pressure changes) and then ramp the flow rate from 0 mL/min to the specified channel

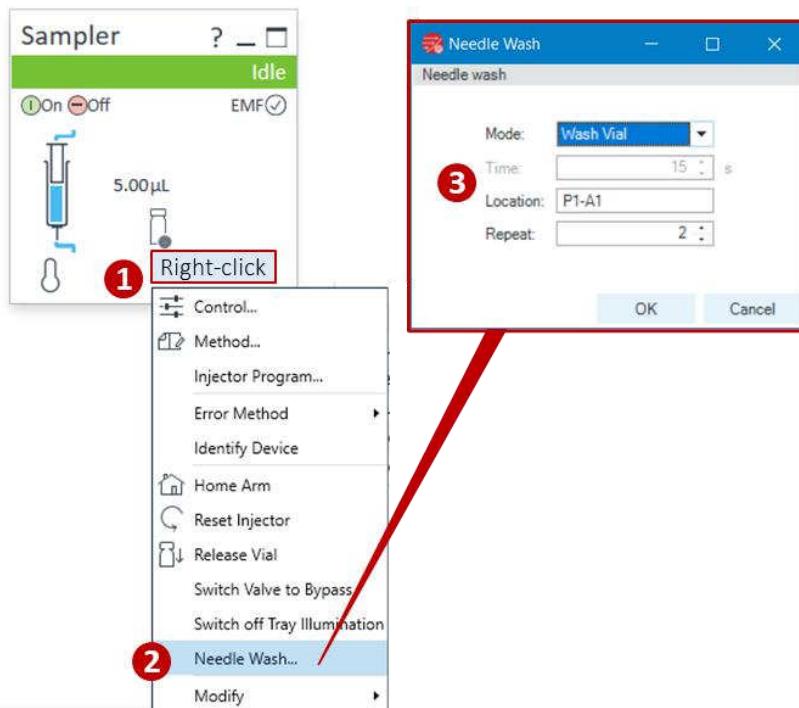
flow (column flow rate). For example, it will take 10 minutes to ramp from 0 mL/min to 1 mL/min through the column after switching the separation device under manual control.



13. Flush the SEC pathway for 10 minutes if no column is installed or at least 3 - 5 column volumes if the column is also being flushed. Consider disconnecting the column outlet and flush to waste to avoid column particulates from entering the system. The maximum operating pressure with a column installed is 75 bar when connected to the outlet. If that pressure limit is reached, the flow rate will need to be reduced until it is within an acceptable range. Do not make or break column connections with flow rate (> 0.1 mL/min) applied, as this may shock the column.
14. Now that the Eclipse is flushed, the internal valves can be purged to complete the solvent exchange. These next steps will remove residual solvent in the instrument purges and Rheodynes. To access the final purges, right-click on the Eclipse widget in VISION RUN and select “Eclipse Flush...” which will open the Eclipse flushing window. Depending on your Eclipse model, there will be different flushing options available to you:
 - a. The “Flush Purges” should be performed to complete the solvent exchange by flushing the various purge valves in the system.
 - b. The “Flush Rheodynes” option will flush the Focus/Elution Rheodyne and the SEC/FFF Rheodyne to fully exchange solvents in those mode-switching valves.
 - c. The “Flush Recycle Valves” option will flush the valves responsible for switching between waste and recycle for both the detector flow and the cross flow fluid outlet lines.
 - d. The “Flush FC Port” will flush the fraction collector pathway and is recommended to flush the fraction collector pathway and fraction collector. Even if a fraction collector is not installed, this flushing option can be used to ensure an appropriate storage solvent is stored in the internal fluid lines.
 - e. The “Membrane Flush” option will be utilized later, as part of the discussion for flushing a new membrane and resetting the injection counter in the section, [Flushing Membrane with Eclipse + DCM models \(WECD, WECDS\)](#) when the DCM option is present.



15. After following these instructions, the FFF-MALS system should now be completely flushed to a new solvent. As a last step, the autosampler needle and loop can be rinsed to exchange the solvent in the autosampler. Instructions will vary based on the autosampler but in some cases, you can right-click on the autosampler and select “Needle Wash” to select a wash vial for rinsing.



16. A similar procedure can be performed for a fraction collector by right-clicking and selecting “Rinse.”
17. If this is an intermediate solvent, perform this procedure again for the next solvent. If this is a storage solvent, the system is ready for long-term storage. If the system is flushed into the appropriate mobile phase for experiments, you can proceed to the channel membrane installation.

Once the solvent exchange is complete, the “Solvent Density Disagreement” caution badge should no longer be displayed on the front panel. If it is still present, proceed to flush the system further and repeat the software flush automations.

Although the strategy for using manual control for solvent exchange is typically faster (as you can physically open the pump purge valve to flush the pump and degasser more quickly), one minor limitation of this strategy is that it requires attending to the system throughout the process. Another strategy can be employed by running a method to automate the solvent exchange, including injections of the intermediate solvent to help flush out the autosampler as well. This can be beneficial for performing an overnight solvent exchange. Creating a solvent flushing method for solvent flushing or cleaning will be discussed in the section, [Method for Automating Solvent Exchanges](#).

In the next section, installing the membrane and preparing the channel for experiments will be discussed. If your channel has already been prepared, you can proceed to the [VISION RUN Operation Workflow](#) or if you already have a method, the section [Creating and Running a Sequence](#).

Eclipse Channel Overview and Membrane Installation

The channel separation device (Figure 39) in combination with the Eclipse is crucial for successful FFF-MALS separations. After installation of the Eclipse hardware and VISION software, routine maintenance of the Eclipse and especially the channel is required to maximize instrument uptime and acquire good data. The separation channel consists of consumable materials, including an FFF membrane, that will need to be replaced regularly. In the following sections, the procedures for installing a new membrane will be discussed.



Figure 39. (from left to right) Eclipse short channel, long channel, dispersion inlet channel, semi-preparative channel, and mobility channel.

Channel Design

The channel consists of an upper block and a lower (bottom) block made of stainless steel. Beneath the lower block, the channel temperature regulator is secured via bolts that go through four rubber feet. In between these two blocks are a supporting frit material (Inconel 625 or ceramic), two O-rings (perfluoroelastomer), an ultrafiltration membrane (typically regenerated cellulose (RC) or polyethersulfone (PES) on a polypropylene support layer), and a spacer or specialized top block that defines the height of the channel. Channel length is based on the channel type (short or long) whereas channel height is defined by the spacer or top block.

There are two channel designs—a variable height channel that uses a spacer to define the channel height, and the fixed height channel that has a defined spacer height built into the top block. The advantage of the variable height is the flexibility for method development and exploring different channel heights; whereas the fixed height channel is more convenient to assemble but has limited flexibility for adjusting the channel height. Because cross flow can still be dynamically tuned, a broad separation range is nonetheless possible with the fixed height channels even with one height.

The variable height channels include either Mylar (organic applications) or a polycarbonate coated with PTFE affixed by glue (aqueous applications) spacers. The fixed height channels have a defined thickness incorporated into the design. They cannot be adjusted, instead each top block has a different specified height.

An expanded view of a disassembled variable height channel is provided in Figure 40. The channel only needs to be completely disassembled when performing a thorough cleaning, for example if microbial growth, salt deposits, or clogs have been isolated to the channel. For normal operation, as described later in this guide, the consumable membrane and spacer (if polycarbonate with PTFE coating) can be replaced and the channel does not need to be completely disassembled.

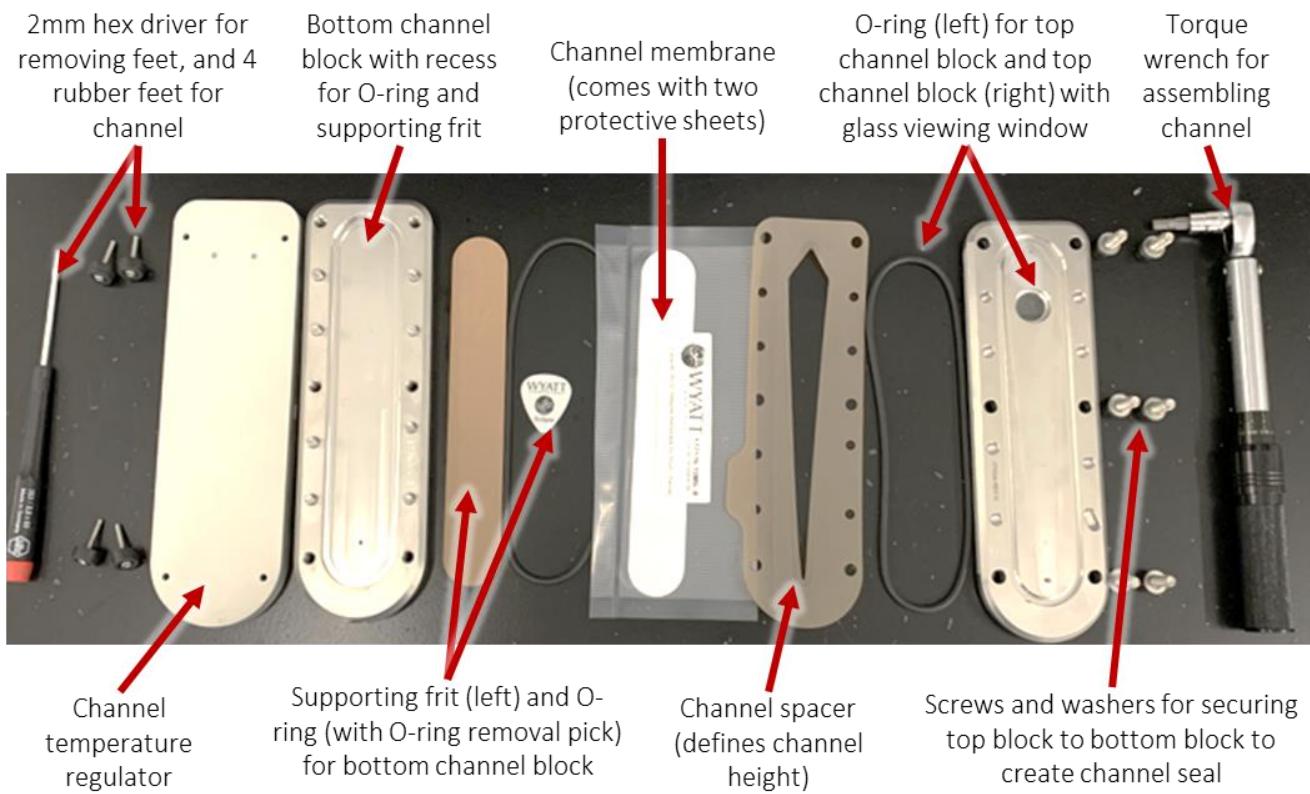


Figure 40. Disassembled channel and tools for disassembling and re-assembling the channel.

These components come together to create a sealed channel, which has a separation zone and enables FFF-MALS separations. For method development, the height of the channel determines the parabolic flow profile, thus affecting separation by altering the flow velocities at certain heights. The support frit, the membrane, and the height of the channel and their impact on the sample separation in FFF-MALS are illustrated in Figure 41.

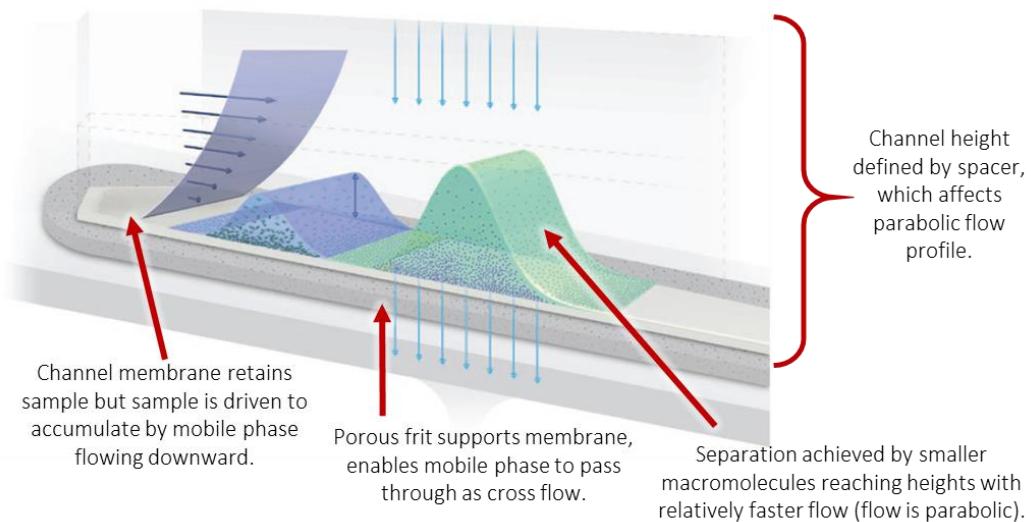


Figure 41. An illustration of the separation zone in an FFF-MALS channel where the height is defined by the spacer, and the membrane and supporting frit enable cross flow to pass through and exit whereas sample is retained.

In Table 4, the options for Wyatt Technology Eclipse channels are summarized. Please refer to the Eclipse Accessories Options Brochure or the Channel User's Guide for Eclipse for the latest channel offerings.

Table 4. Available Eclipse Channels (December 2021) and Spacer Height Options

Eclipse Channel Options	Channel Temp. Regulator	Spacer Height Options (Typical Effective Height) ^a
Variable Height Short Channel (SC)	✓	Aqueous Laminated Spacers (Polycarbonate with PTFE Coating): <ul style="list-style-type: none"> • 275 µm (195 µm effective height) • 400 µm (320 µm effective height) • 525 µm (445 µm effective height) Organic Spacers (Mylar) <ul style="list-style-type: none"> • 250 µm (190 µm effective height) • 350 µm (290 µm effective height) • 490 µm (430 µm effective height)
Fixed Height Short Channel (SC)	✓	Available Top Block Options: <ul style="list-style-type: none"> • 250 µm (195 µm effective height) • 350 µm (320 µm effective height) • 500 µm (445 µm effective height)
Variable Height Long Channel (SC)	✓	Aqueous Laminated Spacers (Polycarbonate with PTFE Coating): <ul style="list-style-type: none"> • 275 µm (195 µm effective height) • 400 µm (320 µm effective height) • 525 µm (445 µm effective height) Organic Spacers (Mylar) <ul style="list-style-type: none"> • 250 µm (190 µm effective height) • 350 µm (290 µm effective height) • 490 µm (430 µm effective height)
Fixed Height Long Channel (SC)	✓	Available Top Block Options: <ul style="list-style-type: none"> • 250 µm (195 µm effective height) • 350 µm (320 µm effective height) • 500 µm (445 µm effective height)
Variable Height Semi-Preparative Channel (SP)	✓	Aqueous Laminated Spacers (Polycarbonate with PTFE Coating): <ul style="list-style-type: none"> • 525 µm (445 µm effective height) • 650 µm (570 µm effective height) • 900 µm (820 µm effective height)
Variable Height Dispersion Inlet Channel (DC)	✓	Aqueous Laminated Spacers (Polycarbonate with PTFE Coating): <ul style="list-style-type: none"> • 275 µm (195 µm effective height) • 400 µm (320 µm effective height) • 525 µm (445 µm effective height)
Variable Height Mobility Channel	✓	Aqueous Laminated Spacers (Polycarbonate with PTFE Coating): <ul style="list-style-type: none"> • 275 µm (195 µm effective height) • 400 µm (320 µm effective height) • 525 µm (445 µm effective height)

^a After channel compression with recommend torque settings, the effective height will be smaller than the uncompressed channel height. **NOTE: These typical effective heights are estimated values and do not represent true effective height values which are determined in the software with a size standard.**

Mylar spacers are required for organic solvents like THF or toluene, as the glue and polycarbonate are damaged by these solvents. Although Mylar can be used for both aqueous and organic, it is recommended to use the laminated spacers for aqueous applications because of the superior seal and reproducibility of these spacers.

Selecting a Membrane Material

The two most common types of membrane material are regenerated cellulose (RC) and polyethersulfone (PES) as shown in Figure 42. Selecting the membrane is an important aspect for method development and plays a critical role in the performance of FFF-MALS separations. Unlike other chromatography methods where the stationary phase and its interaction with the sample are key to separations, FFF-MALS requires minimal channel interaction so that separation is purely based on hydrodynamic size and diffusion coefficients.

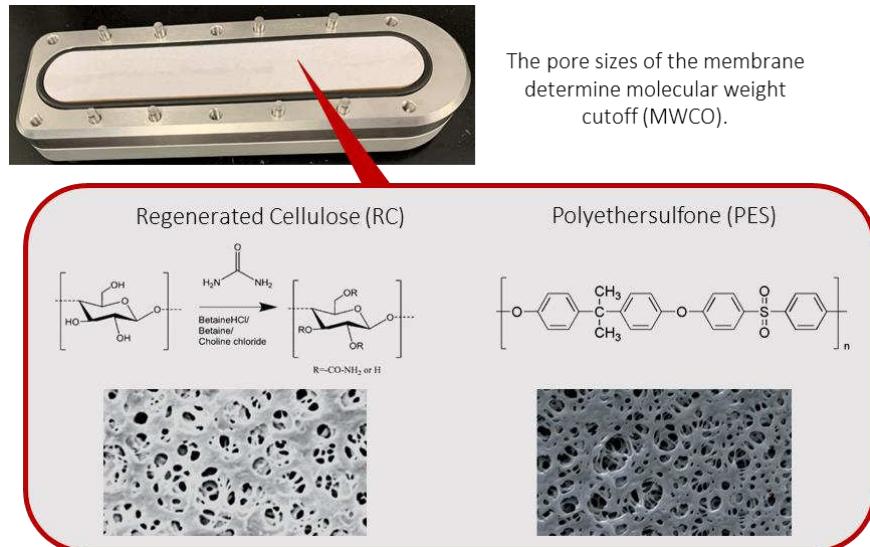


Figure 42. Microscopic view of regenerated cellulose and polyethersulfone membrane materials.

Membrane Material Comparison

The difference is in the chemistry of the membrane layer. RC is more hydrophilic compared to PES and is generally better suited for proteins and hydrophilic polymers. Both RC and PES are negatively charged at pH values above 5, but PES carries a stronger surface zeta potential compared to RC. Therefore, PES is suitable for particles which are also negatively charged so that the electrostatic repulsion minimizes adsorption on the membrane. A comparison of the membranes is provided in Table 5.

Table 5. Membrane Material Attributes

Property*	Polyethersulfone (PES)	Regenerated cellulose (RC)
Protein Binding	Low	Very Low
DNA Binding	Very Low	Low
Chemical Resistance	Low	Very High
Organic Solvent Compatibility	No (Aqueous and alcohol only)	THF, Toluene, DMF (short-term; DMF will damage membrane)
pH Resistance	1 - 14	3 – 12
Maximum Temperature	80 °C	80 °C
Hydrophobic Interactions	Higher	Lower
Cationic Interactions	Higher	Lower
Anionic Interactions	Lower	Higher

*Lower interactions are preferred.

For organic solvents, the RC membrane is the default choice. For proteins, cationic samples, or samples with hydrophobic groups, the RC membrane will provide the most electrostatic repulsion for minimizing adsorption to the membrane. For DNA and anionic samples, the PES may be better suited.

Other Membrane Materials

Other membrane materials from vendors such as Sterlitech (<https://www.sterlitech.com/>) can be used; however, successfully cutting and incorporating custom-cut membranes can be challenging and great care must be taken to ensure suitable performance.

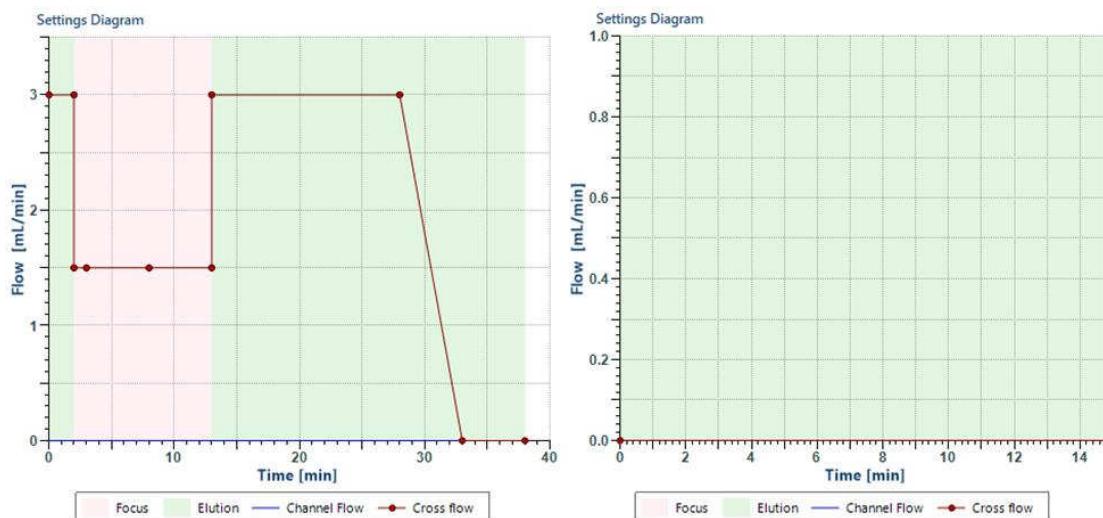
Test membranes can be created by using the channel frit as a template with an X-acto knife on a cutting board. However, for the most robust results, membranes can also be cut using a craft cutting machine equipped with a fine point blade. Please contact Wyatt Technology for additional instructions for cutting custom membranes and guidelines for producing custom membranes for your channel.

Membrane Adsorption Test

If you are not sure if your sample may adsorb to the membrane, there are a few strategies you can employ to assess the situation.

Zero Cross Flow Test

During a typical FFF-MALS experiment, focus flow concentrates the sample and cross flow drives the sample to the accumulation wall—the membrane surface. If the sample may interact with the membrane, you can compare two injections—one with cross flow and one without cross flow (i.e., Elution + Injection with no cross flow). By comparing the UV or the LS with and without cross flow, you can gauge the mass recovery and determine whether your sample is sticking to the membrane.



Filter Test

Another option is to purchase a syringe-tip filter with a very large pore size (0.45 – 1 μm or greater) and perform the following:

1. Determine a method for measuring sample concentration (e.g., UV-Vis, IR)
2. Determine the appropriate mobile phase for your sample for FFF-MALS or SEC-MALS analysis
3. Use this mobile phase to dissolve or suspend your sample. A typical concentration for many proteins would be 0.5 – 1.0 mg/mL.
4. Pass the sample through the large pore-size membrane (the goal is to not remove any sample by steric filtration but rather adsorption only). Go slowly (at least 2 minutes) to ensure residence time for potential adsorption to occur.
5. (Alternatively) You can incubate the sample with a small amount of the FFF membrane for 10 – 30 minutes.

6. Compare the concentration before and after exposure to the membrane material.

Even if your sample adsorbs to the membrane, you can perform a few injections (if not sample limited or use a chemically similar sample) to saturate the active sites on the membrane. Or you can soak the membrane in the sample solution prior to installing the membrane.

Membrane Molecular Weight Cutoffs (MWCO)

The molecular weight cutoff (MWCO) of the membrane determines the lower limit of a linear coil polymer that would pass through the ultrafiltration membrane. For example, a 10 kDa RC membrane would be compatible for FFF-MALS separations of linear coil polymers greater than 10 kDa, whereas smaller polymers might pass through the cross-flow waste. Because proteins are far more compact than linear polymers, a MWCO that appears compatible might still lead to a loss of protein. Using the Optimization Calculator in Wyatt's DYNAMICS® software, the estimated hydrodynamic radius for different sizes can be estimated. Some estimates are provided in Table 6; however, they should be considered purely an estimate and should not be taken as an absolute value or limit. For example, although the 66.5 kDa protein standard BSA (with a 3.5 – 4 nm hydrodynamic radius) may theoretically be able to pass through a 30 kDa RC membrane (rated for linear polymers with an estimated 4.4 nm hydrodynamic radius), it may only affect mass recovery and may still lead to monomer detector peaks. Some other common sizes in addition to BSA monomer (3.6 nm) are 40 kDa dextran (~ 4.7 nm), 30 kDa polystyrene (~ 4.4 nm), 200 kDa polystyrene (~ 12.6 nm). Sizes for polystyrene latex beads are typically specified as sphere radius or diameter.

CAUTION: Using a MWCO (rated for linear polymer) that is higher than your sample may result in a loss of sample through the cross flow waste. For example, a 30 kDa RC membrane can result in significant loss of BSA monomer (66.5 kDa), even though dimer or other aggregates will be retained.

Table 6. Estimated hydrodynamic sizes for different models using DYNAMICS software.

Membrane Molecular Weight Cutoffs and Molecular Standards	Estimated Hydrodynamic Size from DYNAMICS®	Minimum Theoretical Linear Polymer	Minimum Theoretical Branched Polymer	Minimum Theoretical Globular Protein
1 kDa Linear Polymer Model (MWCO)	0.7 nm	1 kDa	~ 1.5 kDa	~ 2 kDa
5 kDa Linear Polymer Model (MWCO)	1.6 nm	5 kDa	~ 7 kDa	10 kDa
10 kDa Linear Polymer Model (MWCO)	2.4 nm	10 kDa	14 kDa	25 kDa
30 kDa Linear Polymer Model (MWCO)	4.4 nm	30 kDa	52 kDa	105 kDa

From the table above, the different membrane cutoffs can be estimated to retain samples with a hydrodynamic radius larger than the corresponding linear polymer model. For example, a 10 kDa membrane can be expected to be compatible with samples with a hydrodynamic size greater than 2.4 nm. The reason the minimum MWCO is not always used is because the small pores can restrict flow, so ideally the largest MWCO that is compatible with your samples is recommended for the best performance.

When to Replace Channel Membrane and Spacer

The channel membrane and PTFE-coated laminated spacers are consumable items for your channel. They will need to be regularly replaced for optimal performance. Your channel membrane should be replaced every 2 – 4 weeks or when elevated system noise or system peaks, which may be indicative of a fouled membrane, are observed. Membranes which are conditioned with a sample should be replaced when moving to a different application or type of macromolecule—for example when switching from proteins to nanoparticles. A membrane surface

conditioned with a type of macromolecule can often lead to poor mass recovery with dissimilar samples. It is recommended to also replace the PTFE-coated laminated spacer when replacing the membrane for highest data quality. Mylar spacers and the Kalrez O-rings can be used for a long time with care.

A comparison of the light scattering chromatogram between a new membrane after cleaning and a membrane that has deteriorated is provided in Figure 43. Replacing the membrane is often the first recommendation when unexpected results are observed.

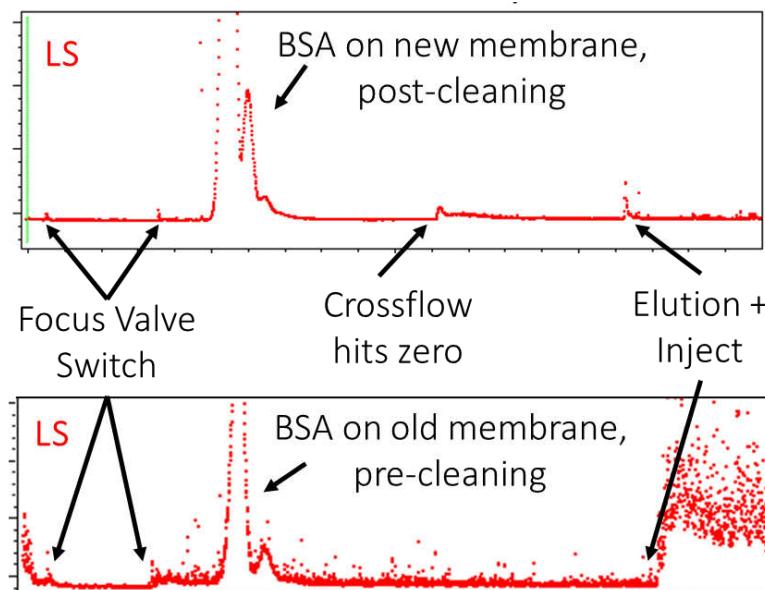


Figure 43. Similarly-scaled comparison of bovine albumin serum (BSA), a protein standard, on a new membrane, post-cleaning compared to BSA on an old membrane, prior to cleaning. The trimer peak is not obscured on the new membrane, post-cleaning. Lower noise during “Focus” compared to during “Elution,” large system peaks during valve switches, reduced peak resolution, or large contamination peaks from the membrane when cross flow reaches 0 mL/min may indicate a dirty membrane that should be replaced.

A step-by-step procedure for replacing the membrane and spacer will be discussed in the next section, followed by a one-sheet reference page overview.

Replacing Channel Membrane and Spacer for Variable Height Spacers

This section is written for variable height channels, which feature a spacer for tunable channel heights. For installing the membrane for a fixed height channel, please refer to the section [Replacing the Channel Membrane with Fixed Height Channels](#). The first step for replacing the channel membrane is to bypass the channel in the HPLC + Eclipse setup. Disconnect the channel temperature regulator micro-USB connection cable and then remove the fluid fittings from the channel and connect them to a 5-port union as shown in Figure 16 in the section [Bypassing the Channel for Solvent Exchange or Membrane Installation](#). Installation will be facilitated by the following:

- A clean benchtop as a working surface
- Disposable gloves for handling the membrane and new spacer
- Access to a sink or squirt bottle with DI water for rinsing the membrane
- Torque wrench (included in the Eclipse hardware kit)
- A new membrane
- A new spacer if using a laminated spacer (PTFE-coated polycarbonate)

- (Optional) 1% detergent solution and alcohol for additional channel cleaning
- (Optional) Wyatt-supplied flexible O-ring removal pick if cleaning the ceramic frit

If the channel requires more cleaning due to contaminated frit, please refer the [Variable Height Channel Cleaning Guide](#) section. Some differences for the Mobility channel are described in [Eclipse Mobility Channel Installation](#). Please follow the instructions below for replacing the channel membrane and spacer in a variable height channel:

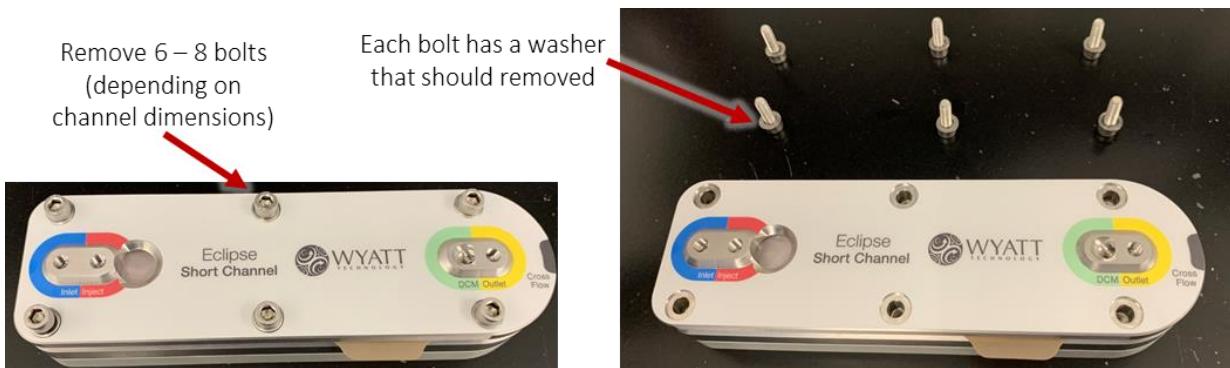
1. Place the channel on a flat, clean surface. Either a T-handle driver or a torque wrench with the correct-sized bit can be installed so that the bolts securing the top block of the channel to the bottom block can be removed. The bit can be installed by pressing down the button on the back of the torque wrench and sliding the bit into place.



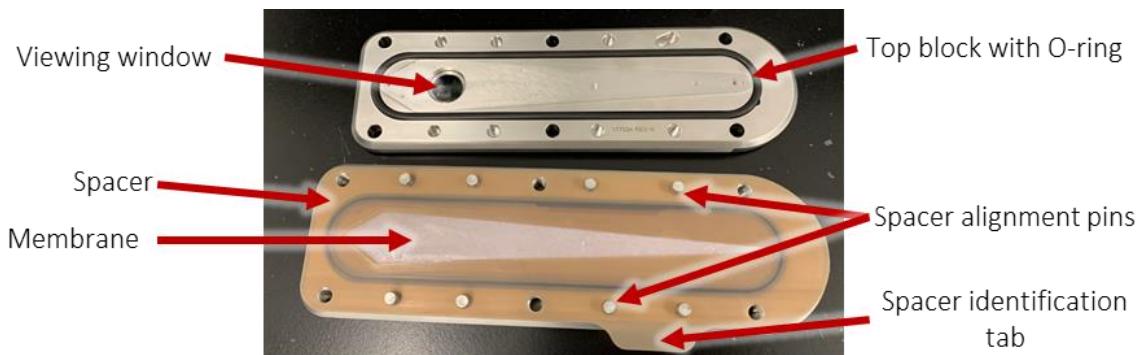
2. Although the torque wrench is required for re-assembling the channel, specific torque settings are not required when removing the bolts and is used here to reduce the number of tools needed. To avoid damage to the torque wrench, you may also use a standard T-handle driver. The torque wrench will have a directional switch, so that it can either tighten or loosen with automatic return functions so you don't need to reposition the wrench—that is, there should be resistance in the counter-clockwise (loosening) direction but no resistance in the clockwise (tightening) direction depending on the switch direction on the torque wrench head. If the switch is to the right (when looking at it with the torque wrench pointed up while holding the handle), the torque wrench can be used to loosen the bolts by rotating counter-clockwise from a comfortable position and then returning to that position.



3. The bolts will just need to be loosened until they can be unscrewed and removed by hand. Each bolt has a washer. Take care not to lose the washers and remove the 6 bolts (short channel), 8 bolts (long, semi-mobility, or dispersion channel), or 10 bolts (semi-preparative channel) from the top block of the channel and set them safely aside.



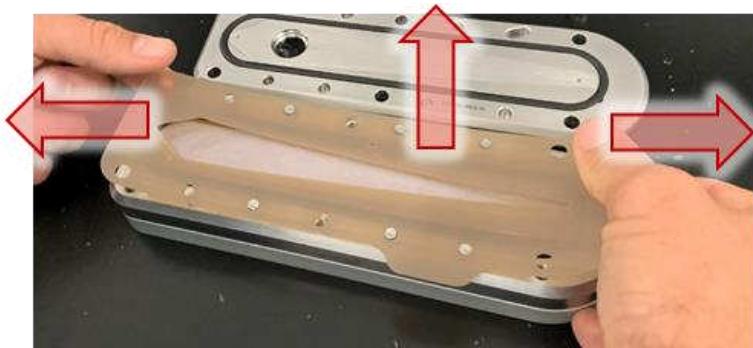
4. Remove the top block, which will may or may not contain a viewing window and the O-ring on the underside. The spacer will remain with the bottom block, held by positioning pins. The O-ring typically does not need to be removed and cleaned unless you observe salt deposits or anything that might prevent a good seal. Instructions for cleaning the entire channel will be described in the section, [Variable Height Channel Cleaning Guide](#). If the O-ring is damaged, it should be replaced.



5. A squirt bottle of distilled water or alcohol can be used to rinse the fluid ports on the top block of the channel. The surface of the top block can also be scrubbed with cleaning solution. Additionally, the holes for the bolts should be dried to minimize solvent getting into the threads. This can be done with compressed air or with Kim wipe.



6. If the spacer will be re-used (e.g., Mylar spacer), the spacer should be carefully removed to minimize damage or tearing. A damaged spacer should always be replaced. Laminated spacers (PTFE-coated polycarbonate) have a superior seal but may deform under compression. These should be replaced whenever the channel is disassembled. The spacer can be “pulled outwards” to stretch and make the removal upward from the alignment pins easier.

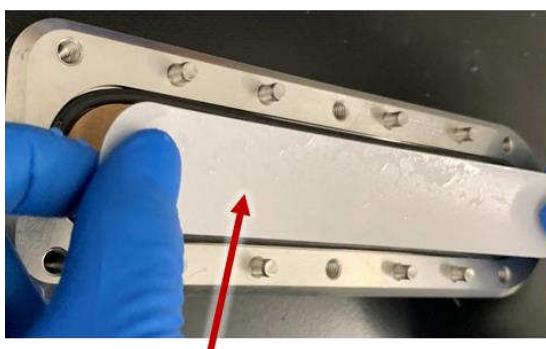


7. The spacer should be cleaned with water and optionally 1% detergent solution, taking care to scrub the interior edge of the spacer with a gloved finger to fully clean. New laminated spacers should be rinsed with water to remove any residue or dust from the packaging or manufacturing process.



Clean inner
edges of the
spacer

8. Once the spacer is removed, the membrane can be removed and discarded. Membranes should never be re-used as the spacer is imprinted on the membrane and it does not reliably seal or perform well when re-used.



Remove the old
membrane



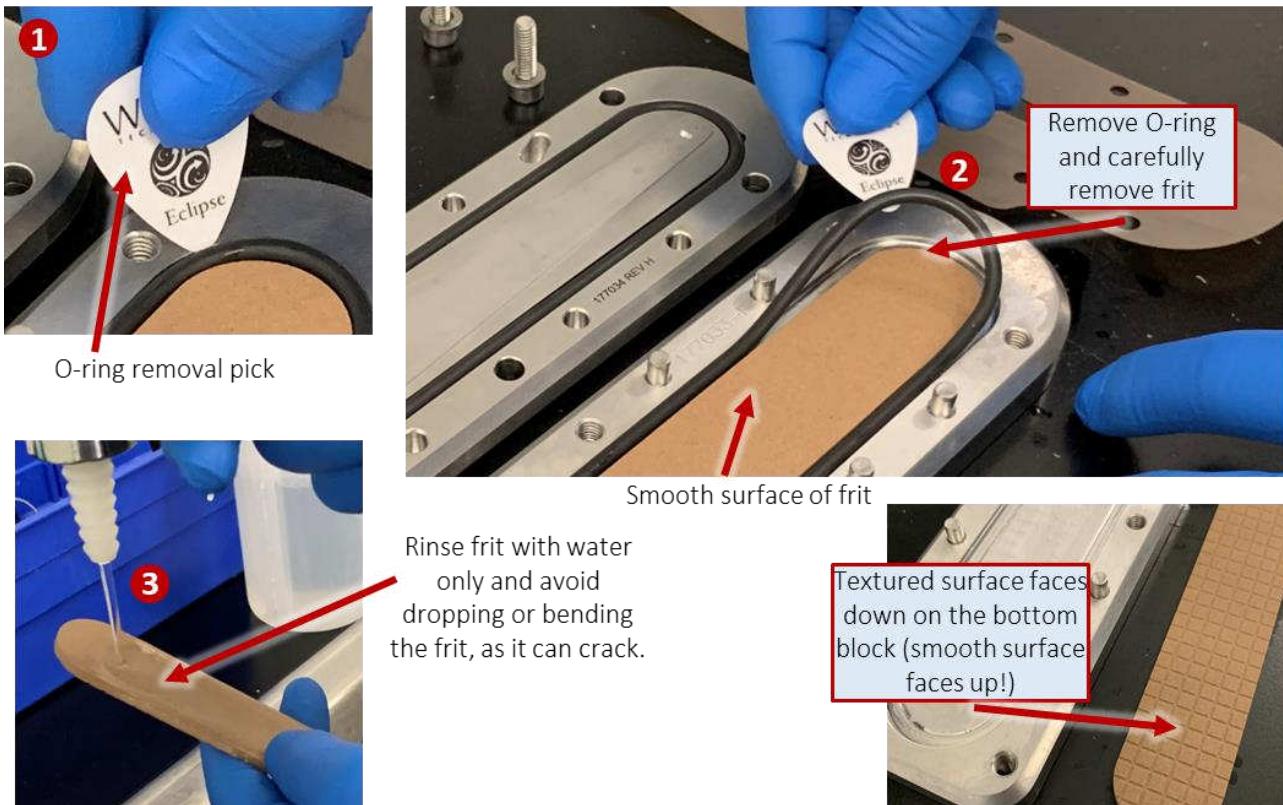
Always install a
new membrane

- If the membrane was not centered (i.e., sample has gone around the membrane and through the frit) or if you suspect the porous frit to be contaminated, you may consider cleaning the frit and bottom block of the channel. Instructions for cleaning the bottom block will be discussed in the [Variable Height Channel Cleaning Guide](#) and is typically not needed for standard membrane replacement, especially if the membrane was positioned correctly. You may remove the ceramic frit and rinse with water if desired (avoid soap which can be difficult to remove from the pores) but handle the frit gently to avoid damage.

WARNING: The ceramic frit can easily crack if bent or dropped. Be careful when removing or cleaning. Contact Wyatt Technology for ceramic frit replacements if you observe any cracks or damage to the frit.

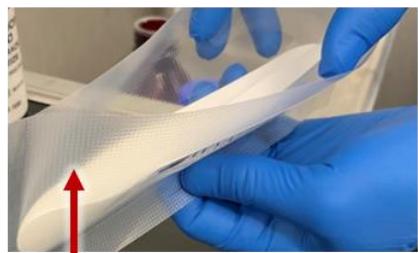
- (Optional) If you choose to remove the ceramic frit to rinse with water, it is recommended to use the plastic O-ring removal pick to remove the O-ring so that the ceramic frit can be more easily removed. The ceramic frit can be removed from the bottom block and rinsed with water. It can then be re-installed with the textured surface facing down (not visible) and the smooth surface facing up.

CAUTION: The Kalrez O-ring is an expensive component. Only use the Wyatt O-ring removal pick included in the hardware kit to remove the O-rings inside the channel. Sharp edges or dental picks may damage the O-ring or the channel. Contact Wyatt Technology to purchase replacement O-rings if damaged.

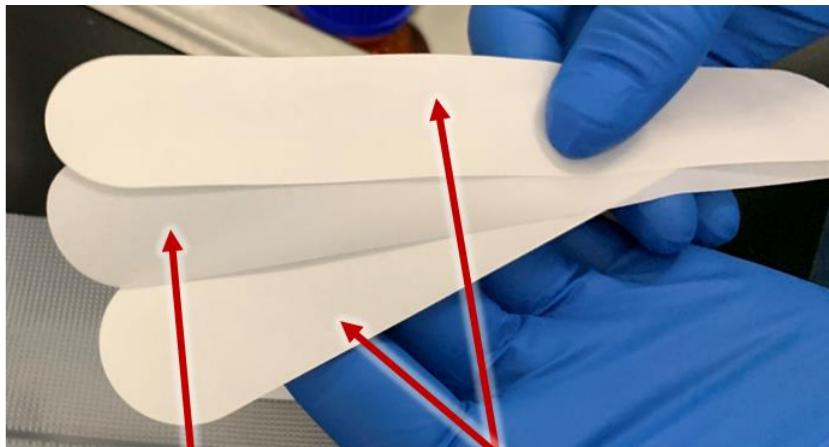


- Always wear gloves when handling a new membrane. The new membrane should be removed from the packaging. The membrane consists of a porous material such as regenerated cellulose or polyethersulfone on a polypropylene backing. As part of the membrane cutting process and to protect the membrane, it is sandwiched by two paper spacers.

CAUTION: The paper spacers sandwiching the membrane are there to protect the membrane. Do not install the paper in the channel, as this can lead to clogs in the channel and system. Your membranes may be protected with white paper or blue paper.



Membrane packaging, open along long axis to avoid cutting membrane

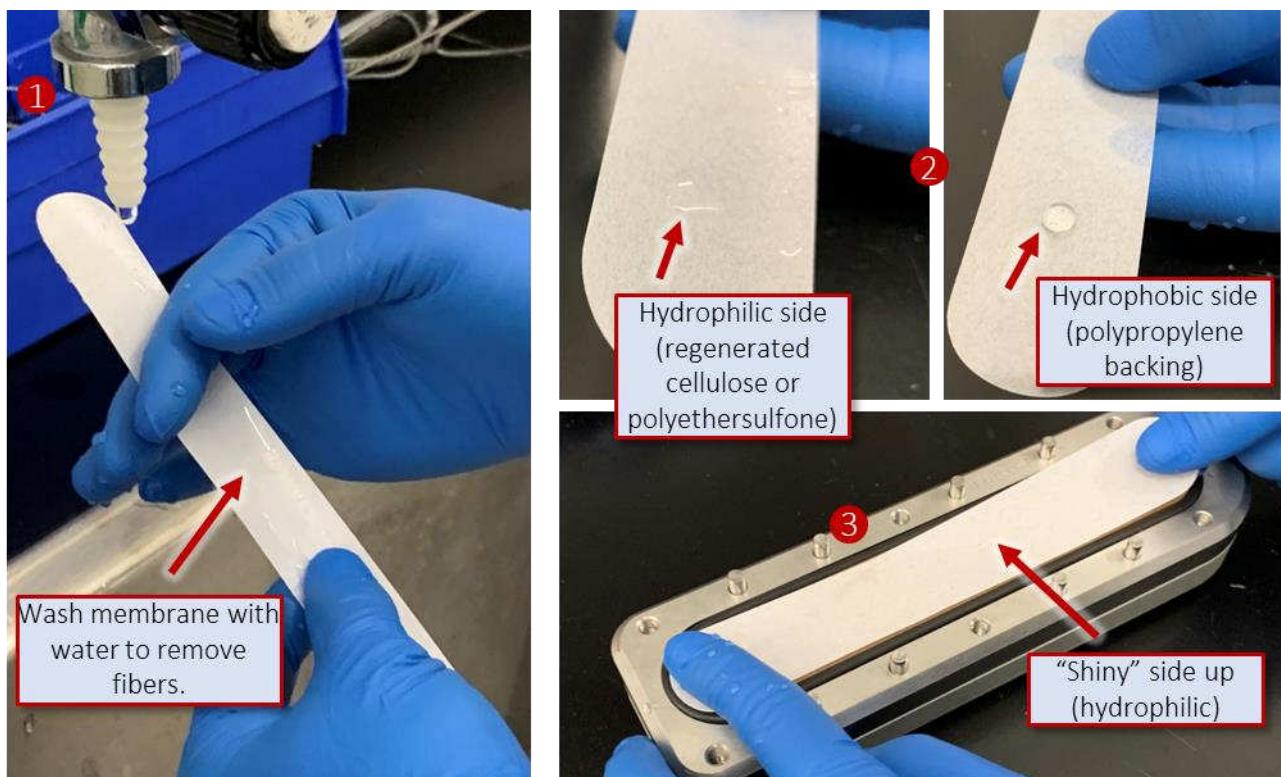


Membrane with hydrophilic side and a hydrophobic side
Paper spacers (Do not install!)

NOTE: Avoid aggressively rubbing the surface with your nail or in a manner than can damage the critical membrane surface. The membrane should be rinsed with water and gently handled.

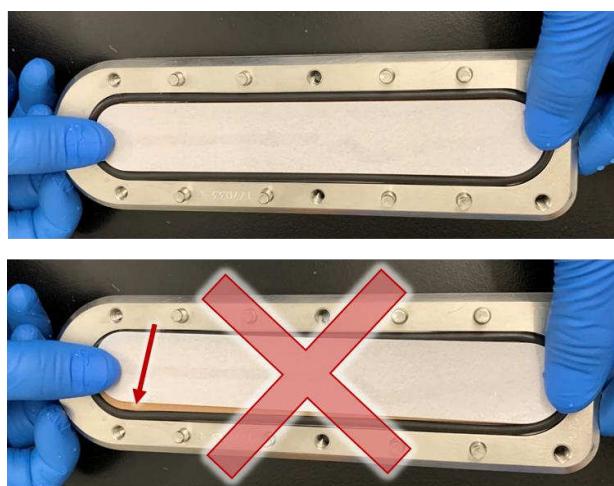
12. Once the membrane is separated from the two paper spacers (which can be discarded), the membrane should be rinsed thoroughly with water to remove fibers and dust that can clog downstream detectors. If you are using custom-cut membranes not from Wyatt Technology, you may consider soaking the membranes in water to help fully remove fibers. The membrane consists of two sides, the porous membrane material and usually a polypropylene backing to give the membrane some structure. The membrane material needs to form the accumulation wall in the channel. As a result, the membrane should be installed with the hydrophilic “shiny” side facing up, and the hydrophobic matte side, or polypropylene backing, facing the frit.

NOTE: The membrane may curl up slightly, which can also be used to assess the orientation (U-shaped). It is recommended not to allow the membrane to sit outside in air for too long, as if the membrane curls too much, it may not sit flat on the frit.

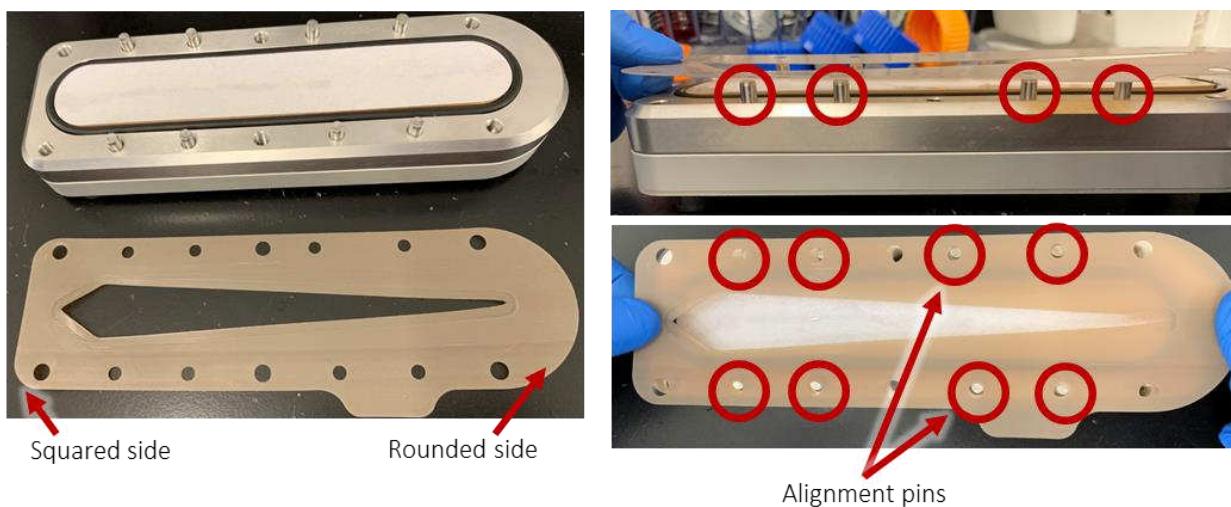


NOTE: When assembling a channel for organic solvent applications (i.e., THF, toluene, etc.), it is recommended to wet the new membrane with water to prevent potential deformation of the membrane during assembly. Then flush with a miscible solvent into the desired chromatography solvent as outlined in the section, [Using Manual Control to Perform a Solvent Exchange with VISION](#). The channel can be directly flushed from water to THF. Changing solvents from water to toluene requires a miscible intermediate solvent such as alcohol.

13. Take great care to ensure the membrane is perfectly centered on the frit. It should cover the frit entirely. A misaligned membrane may cause sample to go around the edge of the membrane and escape the channel through the cross flow waste, potentially contaminating the frit as well.



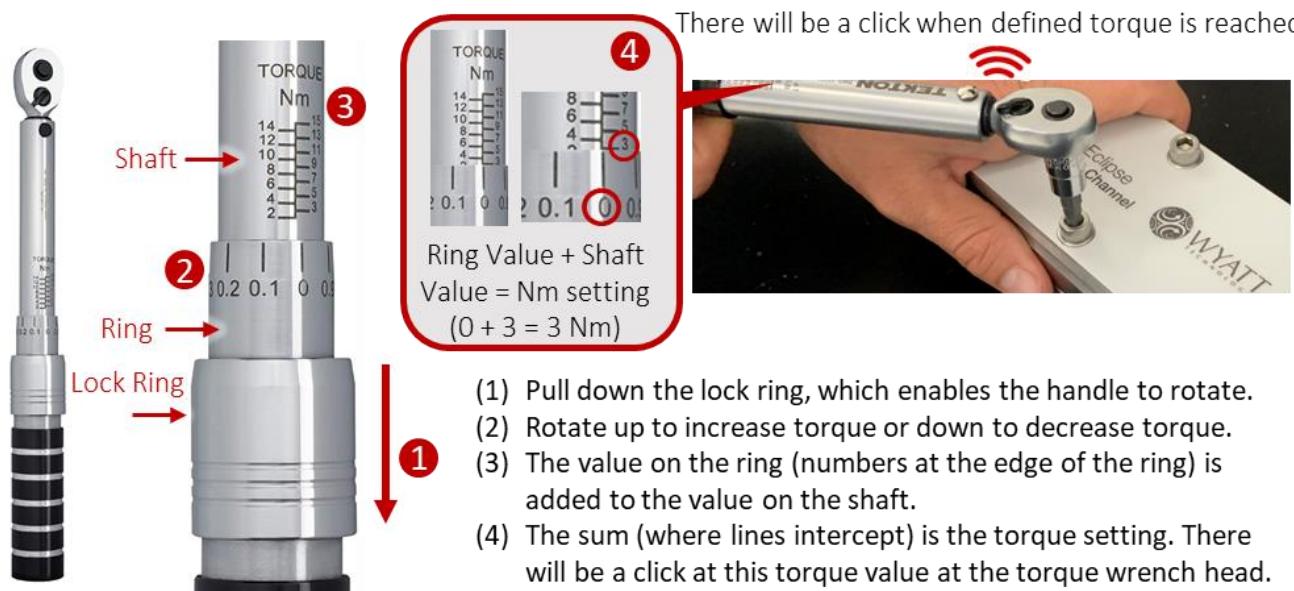
14. Once the membrane is centered, the new laminated spacer (after cleaning the surface and interior edges) can be positioned on top of the membrane (on the bottom block). The spacer has a square end and a rounded end, and should match the channel geometry. There are alignment pins to ensure that the spacer is installed correctly.



15. Avoid re-adjusting the spacer when it is in contact with the membrane, as that may inadvertently shift the membrane positioning. The top block will slide into place along the positioning pins. Avoid rocking the top block to keep the membrane secured.



16. With the newly installed membrane and spacer in place, the top block can now be secured to the bottom block via bolts to seal the channel and create the ribbon for separations based on the channel height. A torque wrench is used in order to uniformly seal the channel and help reduce variation in channel height by applying consistent torque to the bolts. The torque wrench included with the Eclipse hardware kit is either a click-style torque wrench or a digital torque wrench. When the specified torque is reached, the click-style head will make a slight “click” to indicate that you should stop applying torque. For the digital torque wrench, there will be an LCD display and audible alarm. It is important to not over-torque the screws and make many small motions instead of one larger motion to ensure extra torque is not applied and the click of the torque head occurs before the bolt is overtightened.



NOTE: An electronic torque wrench may instead be offered in the Eclipse hardware kit. Instead of a mechanical click, these torque wrenches are set electronically and have an audible alarm when the torque is reached. These will not have the tactile feedback but may reduce accidental over torque.



To operate this torque wrench, please ensure the following:

1. Install battery (use coin at bottom)
2. Press the “C” button to power on the digital torque wrench.
3. The target torque value can be adjusted by using the “up” and “down” arrows. The maximum torque value for an Eclipse Variable Height (NEON-Generation) Channel is 4 Nm.
4. The torque wrench has two modes—a track mode and peak hold mode. We recommend using peak hold mode. Please refer to the Owner’s Manual for switching between modes.
5. Be careful that units are N-m.
6. Will generate an audible alarm and notify you of peak torque applied when you reach max value.

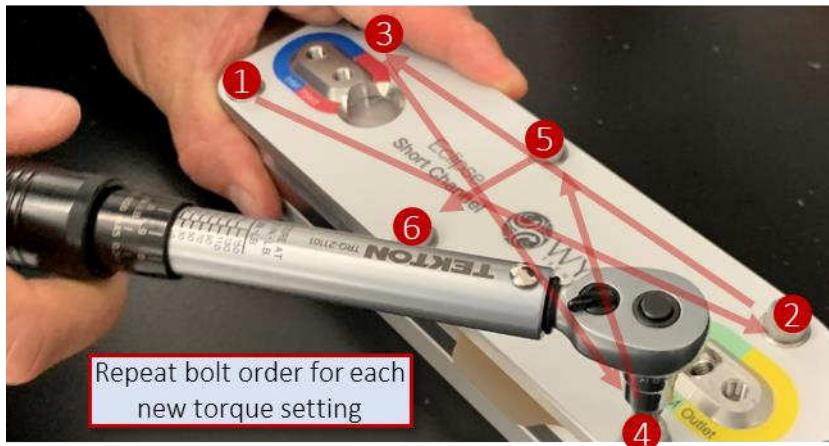
17. It is recommended to first tighten the bolts on the channel by hand initially before using the torque wrench. The torque will be applied from the corner of the channel through the furthest bolt to ensure the best possible seal. The order will vary depending on whether a short channel or a long channel is being used. Refer to the torque order below for each channel type.

Channel + Torque Order	Torque Values	Max Torque
Short Channel (Variable Height) 	<ul style="list-style-type: none"> • Finger-tighten all bolts • 1 Nm all bolts • 2 Nm all bolts • 3 Nm all bolts • 4 Nm all bolts 	4 Nm
Long Channel (Variable Height) 	<ul style="list-style-type: none"> • Finger-tighten all bolts • 1 Nm all bolts • 2 Nm all bolts • 3 Nm all bolts • 4 Nm all bolts 	4 Nm
Dispersion Inlet Channel (Variable Height) 	<ul style="list-style-type: none"> • Finger-tighten all bolts • 1 Nm all bolts • 2 Nm all bolts • 3 Nm all bolts • 4 Nm all bolts 	4 Nm
Semi-Preparative Channel (Variable Height) 	<ul style="list-style-type: none"> • Finger-tighten all bolts • 1 Nm all bolts • 2 Nm all bolts • 3 Nm all bolts • 4 Nm all bolts 	4 Nm
Mobility Channel (Variable Height) 	<ul style="list-style-type: none"> • Finger-tighten all bolts • 1 Nm all bolts • 2 Nm all bolts • 3 Nm all bolts • 4 Nm all bolts 	4 Nm

NOTE: With older channels, it was recommended to start from the middle bolts and work your way outward. Performing a similar torque order for NEON channels has not shown to cause any adverse performance issues. The important thing is to perform a crisscross pattern to ensure even distribution of torque on either side of the channel and to avoid over-torque.

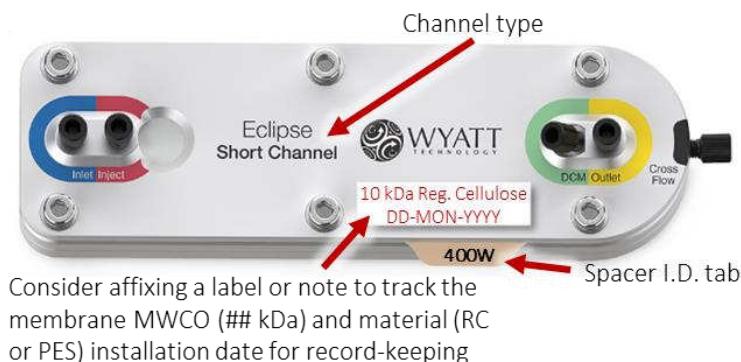
18. This torque order will be applied in cycles with increasing torque. The switch on the torque wrench head will need to be switched so that the bolts can be tightened compared to Step 1 when it was set for loosening. When the switch is to the left (when looking at it with the torque wrench pointed up while holding the handle), it can tighten the bolts. The recommended torques for each cycle are:
- a. Finger-tight across all bolts

- b. 1 Nm across all bolts in the order specified above
- c. 2 Nm across all bolts in the order specified above
- d. 3 Nm across all bolts in the order specified above
- e. 4 Nm across all bolts in the order specified above



NOTE: Once you become familiar with the procedure, you can proceed to follow the crisscross pattern with incremental tightening motions (small torque motions that fall somewhere between finger-tight and 2 Nm for example) as you gradually work each bolt to 2 Nm, then repeat with several crisscross rounds toward 3 Nm, etc. This can help further distribute torque across the entire channel to minimize uneven application of force. The key is many short motions.

19. Once all the bolts have incrementally been tightened to the appropriate torque, the channel is assembled and ready to be installed and flushed. A lint-free paper or microfiber towel can be used to dry the exterior, including between the top and bottom blocks. If the exterior is dried prior to assembly, then it is easier to determine if the channel may be leaking during flushing steps, as opposed to there being residual liquid from assembly.
20. It's important to know the attributes of the channel. VISION has an injection counter to track the number of injections on the membrane; however, it can be helpful to note the type of membrane that is installed and the date it was installed on the channel itself for record-keeping purposes. This can be done with an easy-to-remove label. In the below example channel, a regenerated cellulose (RC) membrane with a 10 kDa molecular weight cutoff (MWCO) was installed with a 400 µm wide laminated spacer.



Replacing the Channel Membrane with Fixed Height Channels

Compared to the assembly of the Variable Height Channels described in the previous section, the Fixed Height Channels do not include a separate spacer for installation. Additionally, they do not need to be assembled with a torque wrench and just need to be assembled with an L-shaped Allen wrench. Additionally, the ceramic frit cannot be removed and cleaned separately from the bottom block. Instead, in situ cleaning or removal of the channel temperature regulator prior to cleaning is recommended.

NOTE: There is no need to use the torque wrench with the Fixed Height Channels. Any steps in the preceding section related to installing the spacer or using the torque wrench do not apply to the Fixed Height Channels. Please refer to this section alone for disassembly and reassembly of your fixed height channel.

The first step for replacing the channel membrane is to bypass the channel in the HPLC + Eclipse setup. Disconnect the channel temperature regulator micro-USB connection cable and then remove the fluid fittings from the channel and connect them to a 5-port union as shown in Figure 16 in the section [Bypassing the Channel for Solvent Exchange or Membrane Installation](#). Installation will be facilitated by the following:

- A clean benchtop as a working surface
- Disposable gloves for handling the membrane and new spacer
- Access to a sink or squirt bottle with DI water for rinsing the membrane
- L-shaped Allen wrench key (included in the Eclipse hardware kit)
- A new membrane
- (Optional) 1% detergent solution and alcohol for additional channel cleaning
- (Optional) Wyatt-supplied flexible O-ring removal pick if cleaning the O-ring

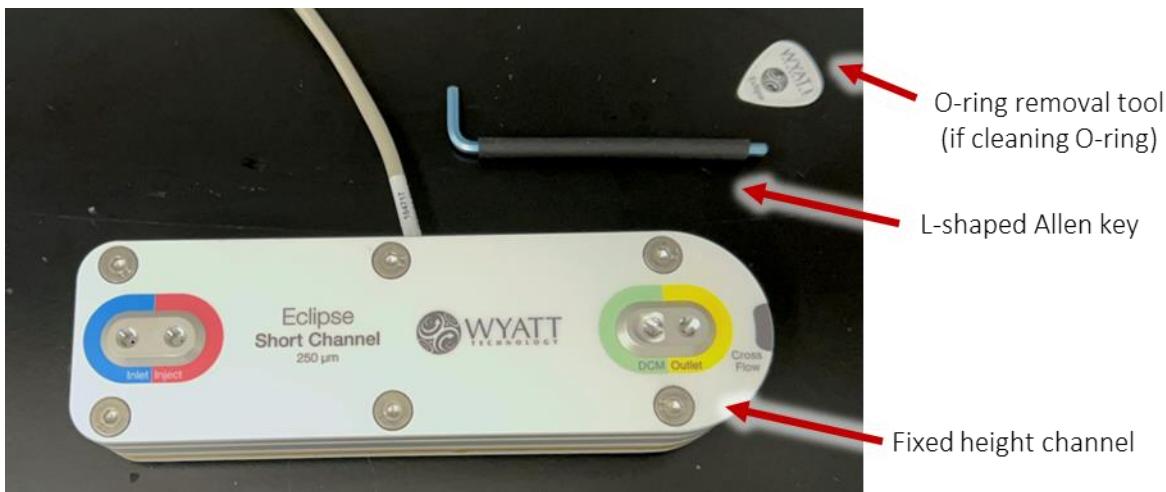
If the channel requires more cleaning due to contaminated frit, it is recommended to perform an in situ cleaning as the frit should not be removed from the bottom block of the channel.

WARNING: Do not remove the ceramic frit in a fixed height channel, as it is precision machined within the bottom block channel to facilitate metal-to-metal assembly of the channel. Removing and re-installing the frit may increase the chance of damage to the frit when the channel is assembled.

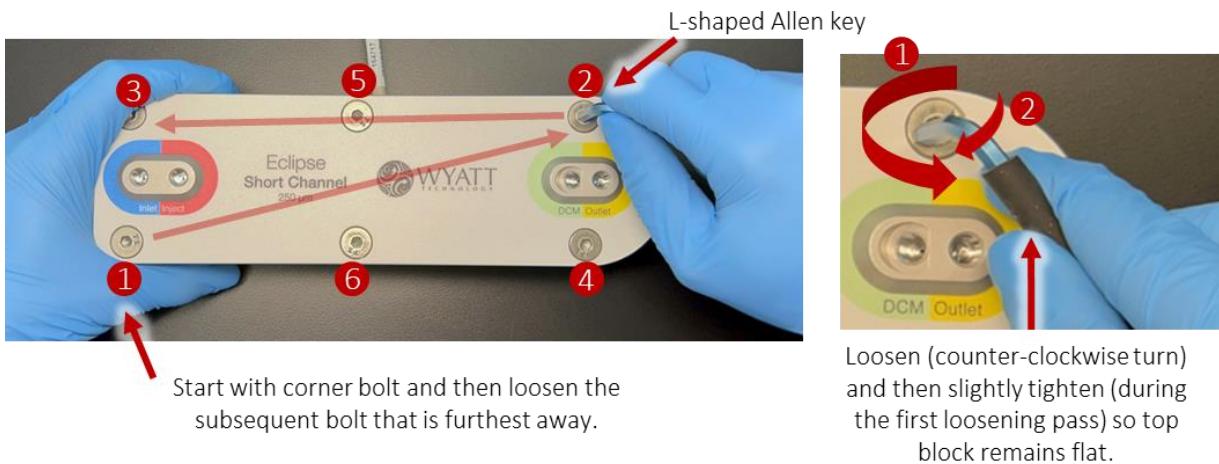
Please follow the instructions below for replacing the channel membrane in a fixed height channel.

1. Place the channel on a flat, clean surface. Either a T-handle or a L-shaped Allen wrench key with the correct-sized driver (4 mm) can be used to loosen the bolts securing the top block of the channel to the bottom block.

NOTE: Early model fixed height channels may have self-guiding flat-head bolts, while newer models may have socket-head bolts with washer. The socket-head bolts are less likely to strip or wear, and make the channel easier to assemble and disassemble. Please contact Wyatt Technology for replacement bolts.



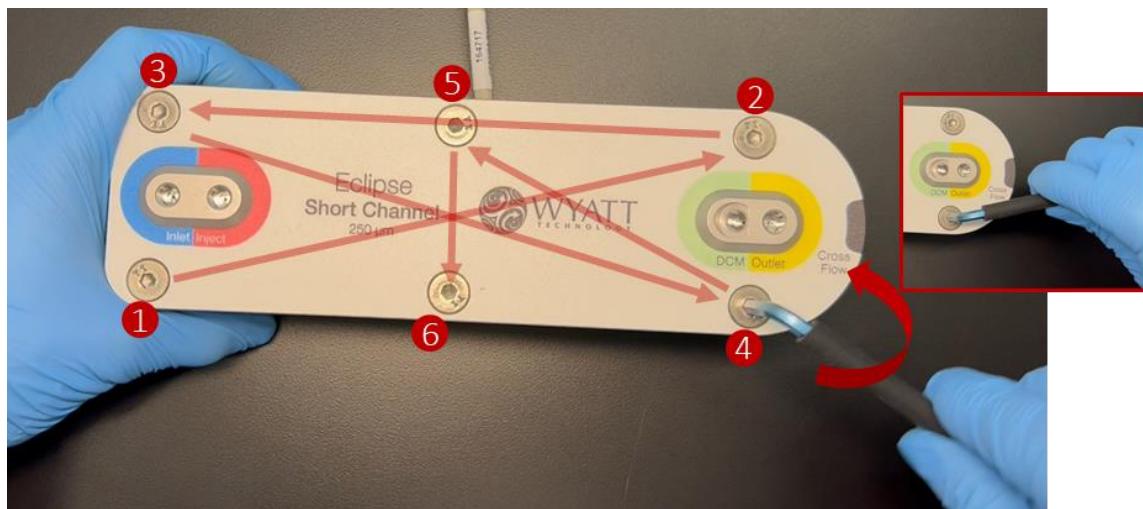
- When loosening the bolts, it is important to go in a crisscross pattern. It is recommended to loosen the bolt but then tighten slightly during the first pass to avoid the top block from tilting, which can make it challenging to remove bolts that haven't yet been loosened.



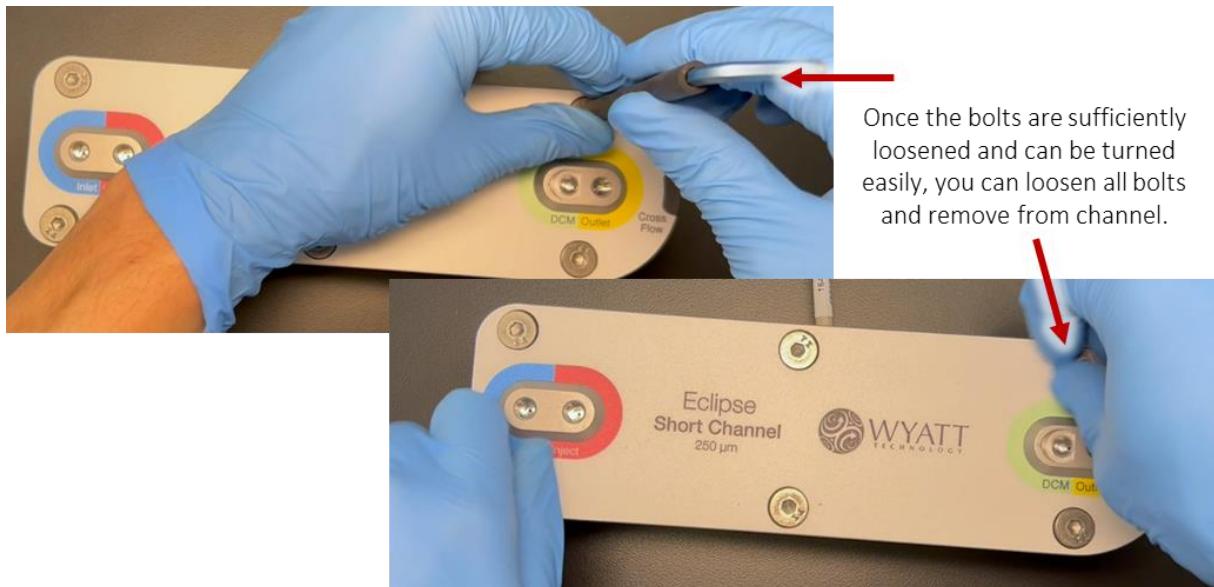
Crisscross pattern for 6-, 8-, and 10-bolt channel designs:



- Once all the bolts have been loosened but then subsequently tightened slightly, you can then proceed in the same crisscross pattern to fully loosen all the bolts, ensuring they are gradually loosened across each bolt—roughly a quarter turn each pass.



- Once loosened completely, the bolts can be removed from the channel using the long part of the L-key for quicker rotation or eventually by hand.

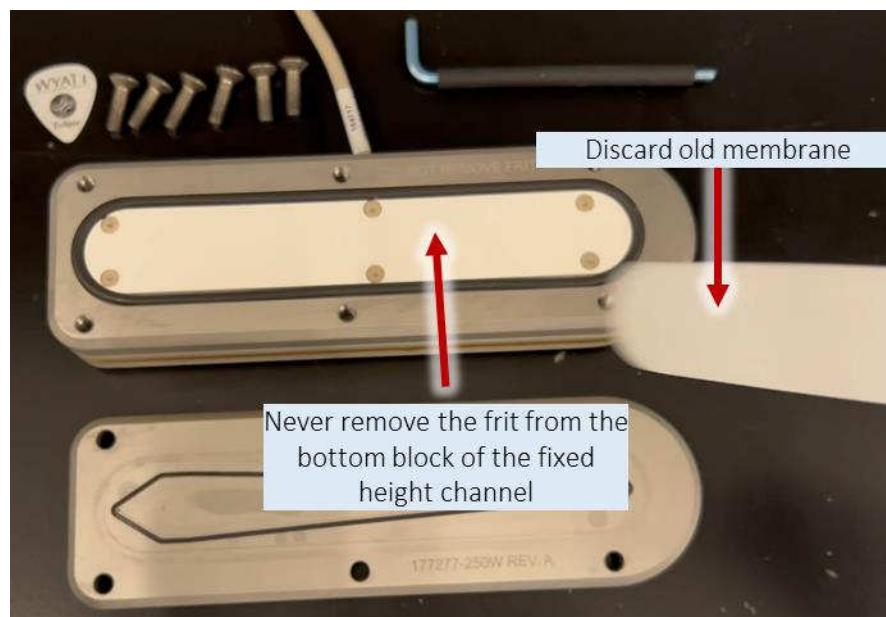


- Set the bolts aside. Avoid washing the bolts or exposing them to liquids, as this can wash away the grease and make it more challenging to reassemble the channel.

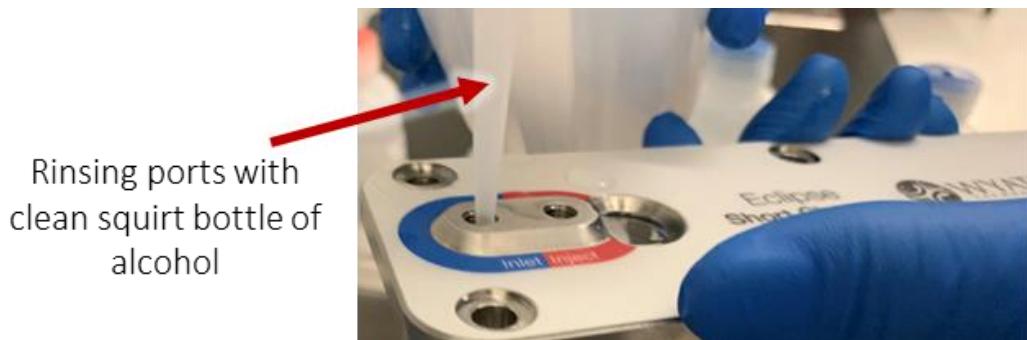


- Remove the top block from the channel and set aside. You can then remove the previous membrane (if membrane was installed) and discard it. Membranes should never be re-used as the spacer is imprinted on the membrane and it does not reliably seal or perform well when re-used. With the fixed height channel,

there will be an O-ring on both the top and bottom blocks, as well as a ceramic frit. Never remove the ceramic frit from the bottom block of the fixed height channel.



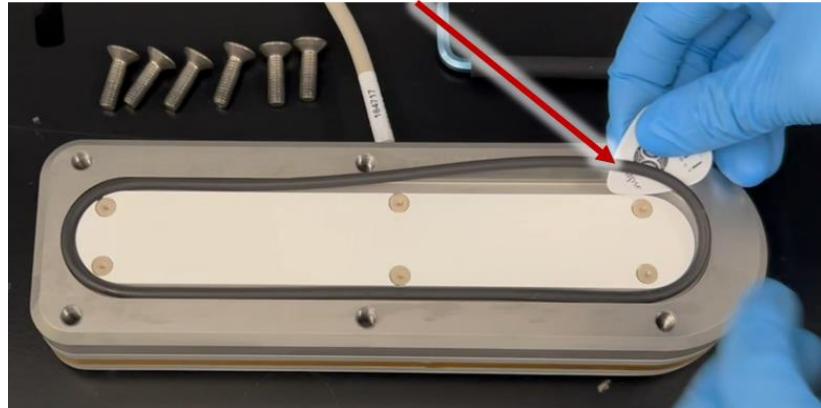
7. A squirt bottle of distilled water or alcohol can be used to rinse the fluid ports on the top block of the channel. The surface of the top block can also be scrubbed with cleaning solution. Additionally, the holes for the bolts should be dried to minimize solvent getting into the threads. This can be done with compressed air or with Kim wipe.



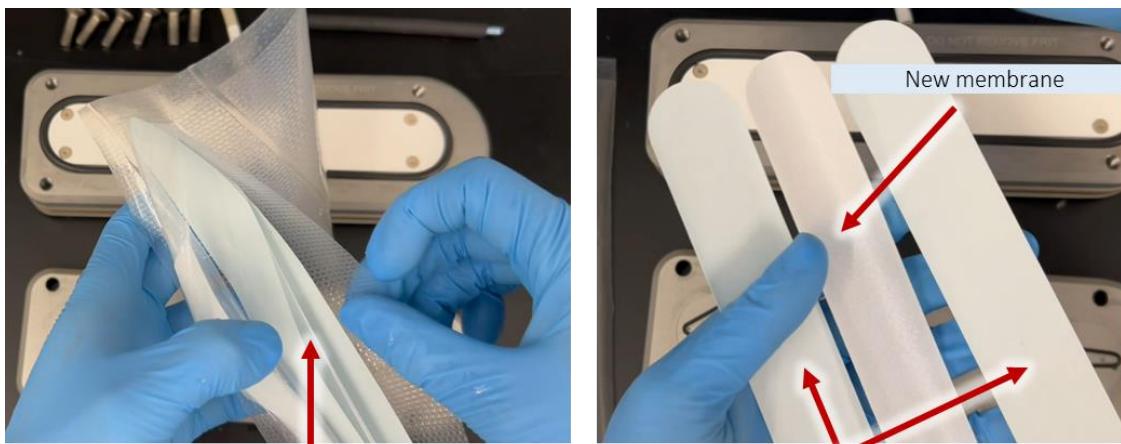
CAUTION: The Kalrez O-ring is an expensive component. Only use the Wyatt O-ring removal pick included in the hardware kit to remove the O-rings inside the channel. Sharp edges or dental picks may damage the O-ring or the channel. Contact Wyatt Technology to purchase replacement O-rings if damaged.

8. (Optional) You may also remove and clean the O-ring if salt deposits or contamination are suspected on the O-ring in the bottom block of the channel. The O-ring can be rinsed under water or with detergent, and then rinsed with copious amounts of water before drying and re-installing around the non-removable frit inside the fixed height channel.

Only use the O-ring removal tool to remove the O-ring when cleaning, as other tools may damage the O-ring.



9. Always wear gloves when handling a new membrane. The new membrane should be removed from the packaging. The membrane consists of a porous material such as regenerated cellulose or polyethersulfone on a polypropylene backing. As part of the membrane cutting process and to protect the membrane, it is sandwiched by two paper spacers.



Membrane is sandwiched between two paper spacers

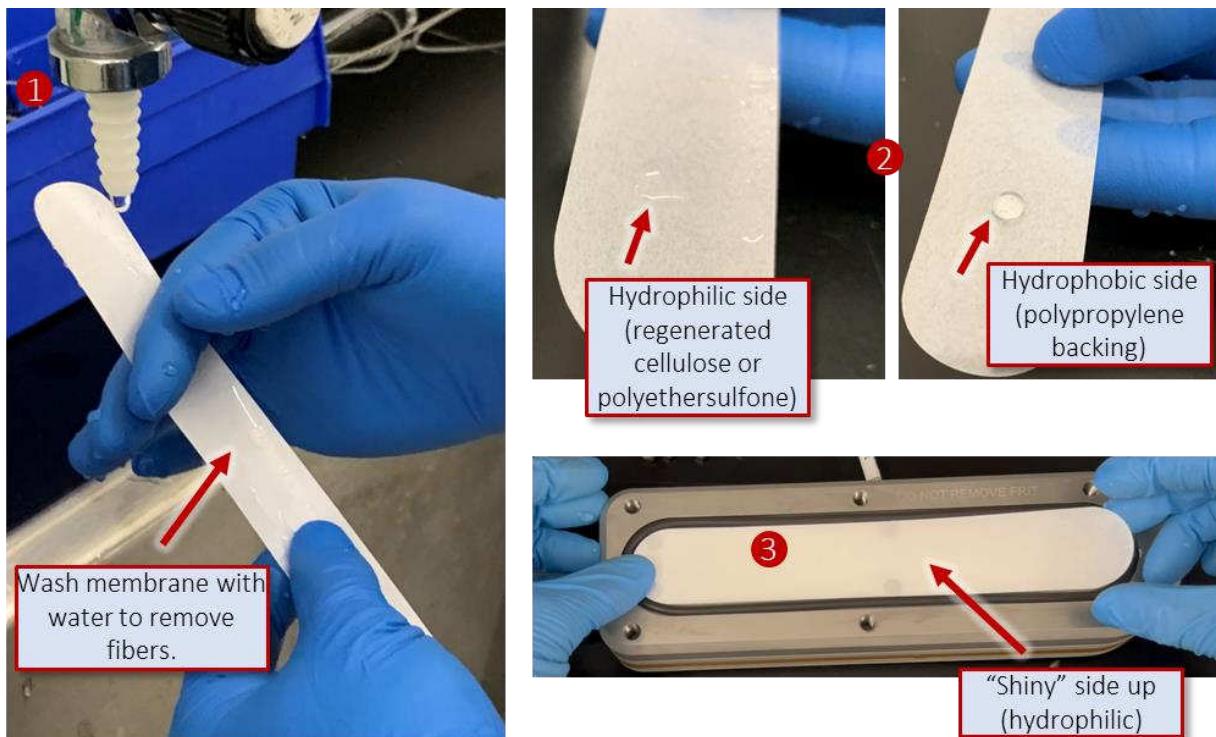
Discard both paper spacers when removing the membrane from the packaging.

CAUTION: The paper spacers sandwiching the membrane are there to protect the membrane. Do not install the paper in the channel, as this can lead to clogs in the channel and system. Your membranes may be protected with either white or blue cleanroom paper.

10. Once the membrane is separated from the two paper spacers (which can be discarded), the membrane should be rinsed thoroughly with water to remove fibers and dust that can clog downstream detectors. If you are using custom-cut membranes not from Wyatt Technology, you may consider soaking the

membranes in water to help fully remove fibers. The membrane consists of two sides, the porous membrane material and usually a polypropylene backing to give the membrane some structure. The membrane material needs to form the accumulation wall in the channel. As a result, the membrane should be installed with the hydrophilic “shiny” side facing up, and the hydrophobic matte side, or polypropylene backing, facing the frit.

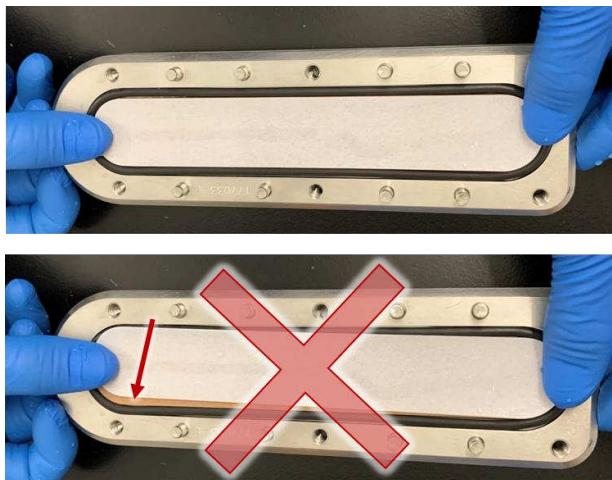
NOTE: Avoid aggressively rubbing the surface with your nail or in a manner than can damage the critical membrane surface. The membrane should be rinsed with water and gently handled.



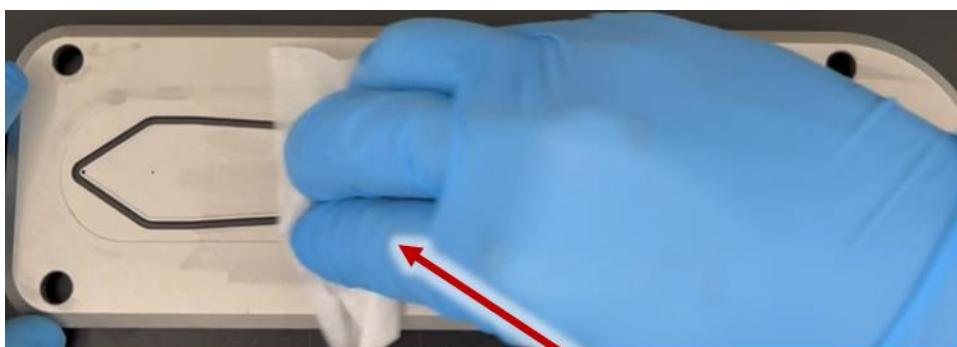
NOTE: The membrane may curl up slightly, which can also be used to assess the orientation (U-shaped). It is recommended not to allow the membrane to sit outside in air for too long, as if the membrane curls too much, it may not sit flat on the frit.

11. Take great care to ensure the membrane is perfectly centered on the frit. It should cover the frit entirely. A misaligned membrane may cause sample to go around the edge of the membrane and escape the channel through the cross flow waste, potentially contaminating the frit as well. Once positioned, the membrane may stick to the frit, which can help with assembly. If the membrane is not sticking to the frit, you can wet the frit with water to help with adhesion.

NOTE: When assembling a channel for organic solvent applications (i.e., THF, toluene, etc.), it is recommended to wet the new membrane with water to prevent potential deformation of the membrane during assembly. Then flush with a miscible solvent into the desired chromatography solvent as outlined in the section, [Using Manual Control to Perform a Solvent Exchange with VISION](#). The channel can be directly flushed from water to THF. Changing solvents from water to toluene requires a miscible intermediate solvent such as alcohol.

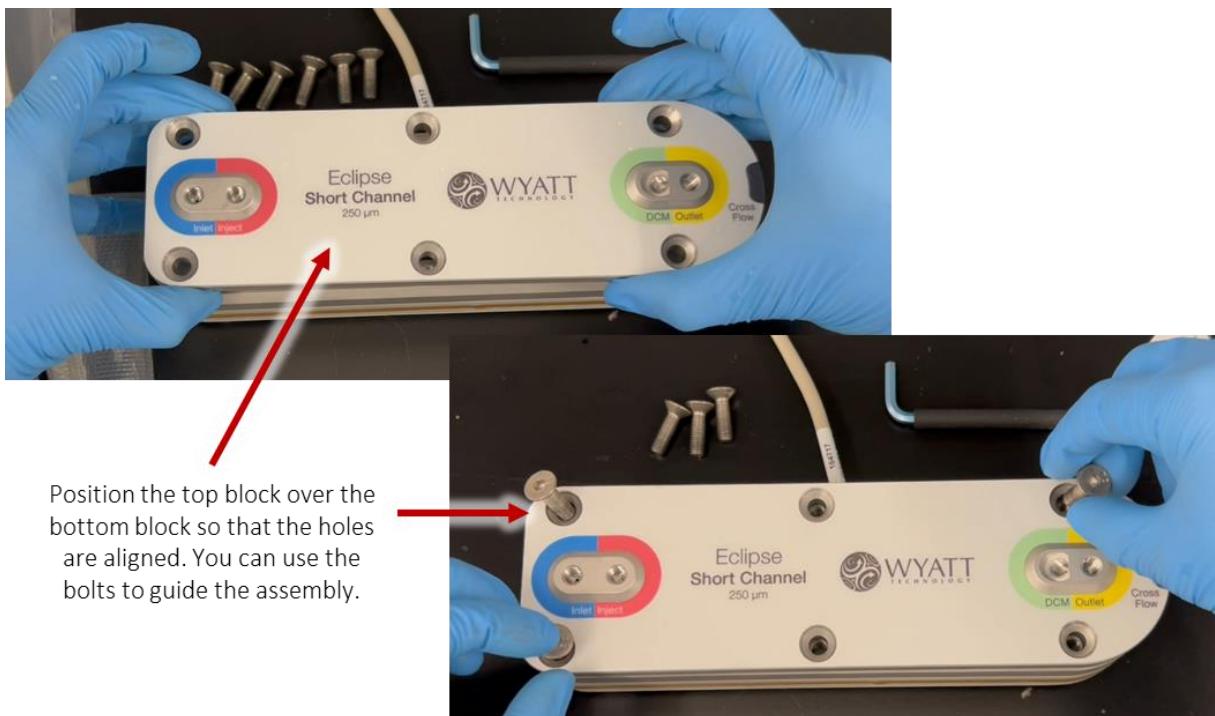


12. Dry the top and bottom blocks outside of the O-ring after installing the membrane. This can help to avoid accidental liquid leaks that may trigger the channel leak alarm in Eclipse instruments with that functionality and prevent liquid from getting into the threads of the bolts, which can wash away lubricants.



Dry the top and bottom blocks with a paper tissue or other low-shedding paper or cloth.

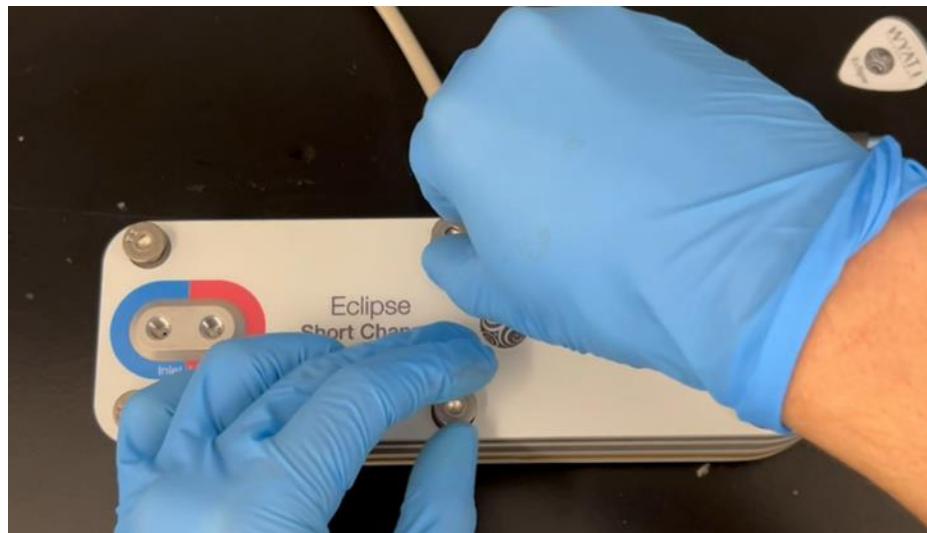
13. With the fixed height channels, there are no alignment pins between the top and bottom block. Instead the bolts can be used to guide the top block onto the bottom block, or the block can be positioned carefully such that the holes are fully visible through the top block. Avoid moving or dragging the top block when assembling, as this can shift the membrane. It can be helpful to wet the ceramic frit with water before placing the membrane to help the membrane adhere in place when placing the top block



14. Begin with a counter-clockwise rotation to help the bolts catch the threads on the bottom block of the channel, which will ensure the bolts can be installed smoothly.



15. Once all the bolts are aligned with the threads, you can begin to finger-tighten all the bolts with a clockwise rotation until it is difficult to grip the bolts with your fingers. At this stage, you can switch between bolts frequently in a crisscross pattern and gradually tighten each in turn until the bolts are no longer easy to rotate by hand.



16. Afterwards, you can switch to the L-key to proceed with tightening each bolt gradually until the bolts become flush with the top block of the channel but can continue to be rotated. Be sure to tighten in a crisscross pattern as shown below for your channel type:



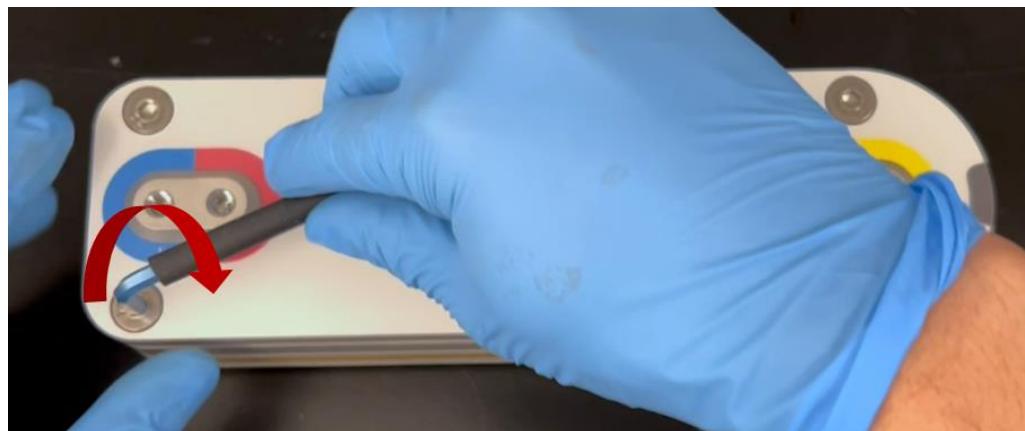
17. Proceed with tightening each bolt with a quarter-turn in a crisscross pattern until you begin to feel the initial resistance of the O-ring (the L-key does not freely or easily rotate).

L-key

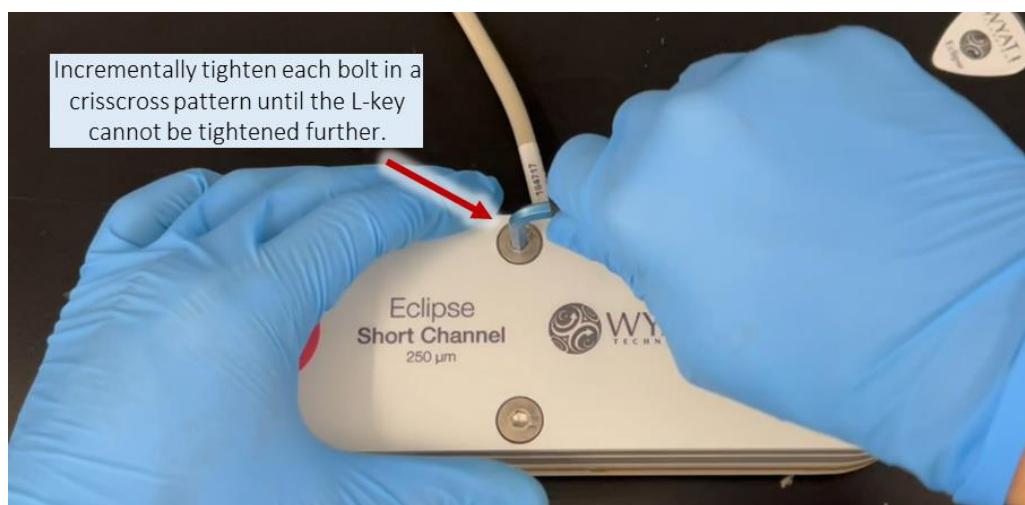
Use the L-key to tighten the bolts in a crisscross pattern until flush with the top block.



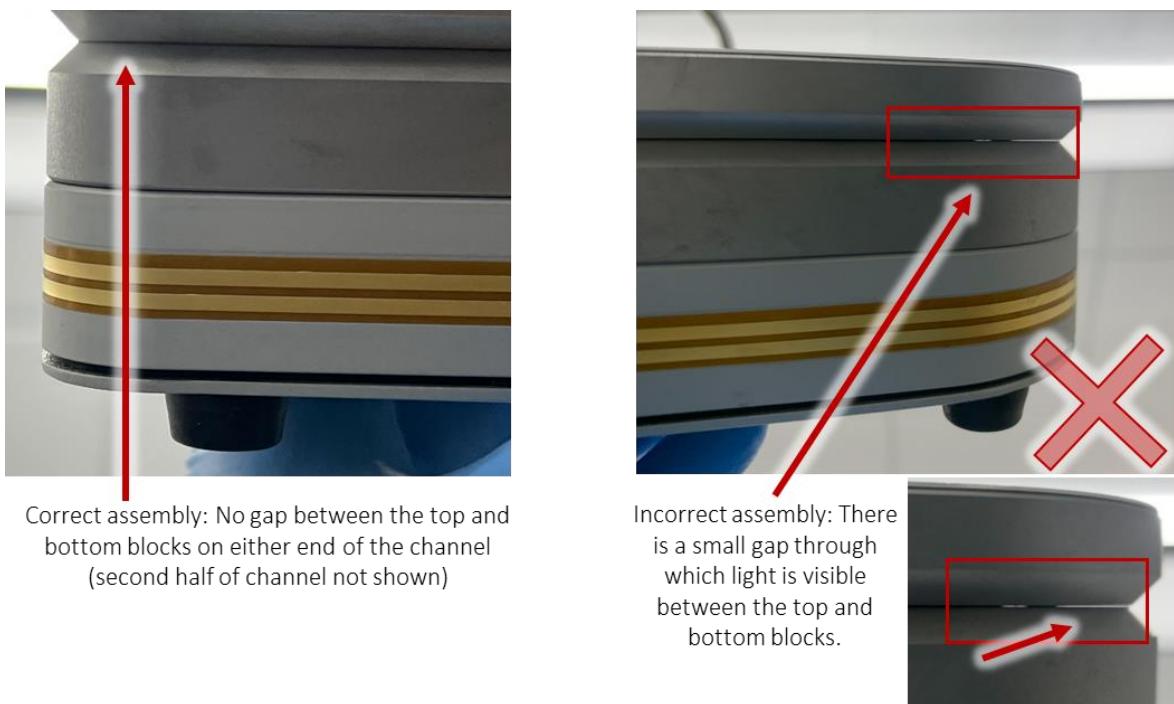
18. Once the bolts are flush with the top block, you can begin the final tightening process. In a crisscross pattern starting with a bolt in the corner, proceed to tighten each bolt with roughly a quarter turn while using the required force needed to rotate with the L-key as a guide. The goal is ultimately to apply uniform torque across the channel bolts while tightening incrementally until you reach a hard stop.



19. It may take up to 10 – 15 cycles of incrementally tighter quarter turns before the bolts are not able to be tightened anymore with the L-key. The amount of cycles will depend on the O-ring compression and how tight the bolts were initially. Ultimately, the top block and bottom block will make metal-to-metal contact, so there is no risk to overtightening with the L-key and you will eventually feel a hard stop.



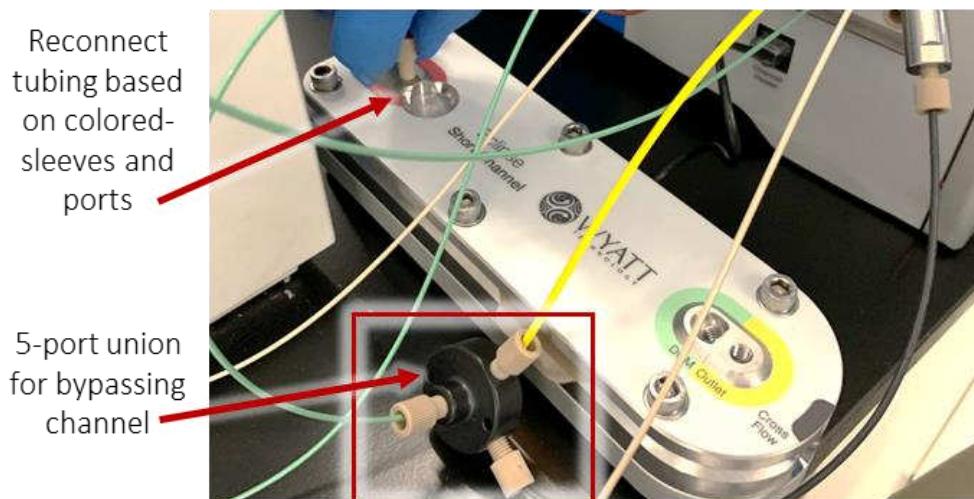
20. Perform one final pass to ensure none of the bolts can be tightened further. If some can be tightened more than a quarter turn, proceed in a crisscross pattern until you hit a hard stop for each bolt. A hard stop is when the L-key cannot physically be turned further because of metal-to-metal contact between the top and bottom block.
21. You can hold the channel up to a light and visually inspect the interface between the top and bottom blocks. A correctly assembled channel will *not* have a gap between the top and bottom blocks.



22. If a gap is present, the bolts may be able to be tightened further until no gap is visible.

Re-Installing the Assembled Channel on the Eclipse

Once the membrane and/or spacer (if using Variable Height Channel) has been replaced, the channel can now be re-installed on the Eclipse. Remove the fittings from the 5-port union and re-connect them to the channel. The tubing is color-coded with insulating sleeves on the PEEK tubing. Additionally, the side panel is color-coded to match the channel, so that the appropriate connections are made. Wyatt offers replacement channel tubing kits with sleeves; however, tubing can be replaced per the PEEK I.D. specifications in Table 1 and the sleeves may be re-used. An excerpt is provided below:

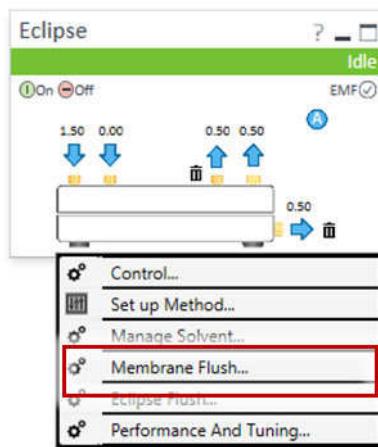


	Eclipse Side Panel	Module	Tubing (1/16" O.D.)	Notes
HPLC	Non-SEC switching models do not have “Channel Inject.”	Port 6 for standard Agilent Autosampler Rheodyne	Tan (0.010" I.D.), Yellow (0.007" I.D.), or Red/Pink (0.005" I.D.) PEEK	In non-SEC Eclipse models, connect directly from Port 6 (Agilent) to channel inject (color-coded red).
Channel (Super flangeless)	Channel Inlet	Super flangeless ferrule into Channel Inlet	Green (0.030" I.D.) PEEK	Color-coded blue sleeve
	Channel Inject (SEC switch models)	Super flangeless ferrule into Channel Inject	Tan (0.010" I.D.) PEEK	Color-coded red sleeve (from Autosampler if not SEC switching model)
	Channel DCM (DCM Models)	Super flangeless ferrule into Channel DCM	Green (0.030" I.D.) PEEK	Color-coded green sleeve
	Channel Outlet	Super flangeless ferrule into Channel Outlet	Tan (0.010" I.D.) PEEK	Color-coded yellow sleeve
	Channel Cross Flow	Super flangeless ferrule into Channel Cross Flow	Green (0.030" I.D.) PEEK	Color-coded black sleeve

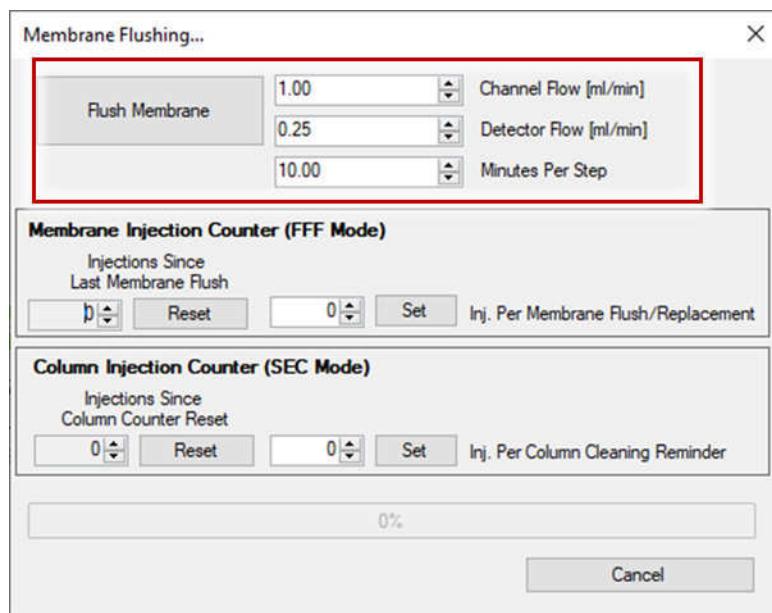
Once the fluid connections are re-established, the membrane can be flushed. This procedure will vary depending on whether your Eclipse model has the DCM option. If it does have the DCM option, proceed to [Flushing Membrane with Eclipse + DCM models \(WECD, WECDS\)](#). If it does not have the DCM option proceed to [Flushing Membrane without Eclipse + DCM Model \(WEC, WECS\)](#).

Flushing Membrane with Eclipse + DCM models (WECD, WECDS)

- With a **Channel DCM** port, the Eclipse can automate membrane flushing in VISION. As a result, the membrane can be flushed through the DCM flow path to remove particulates and fibers that can clog the downstream detectors.
- Right-click on the Eclipse module to access the “Membrane Flush” option.



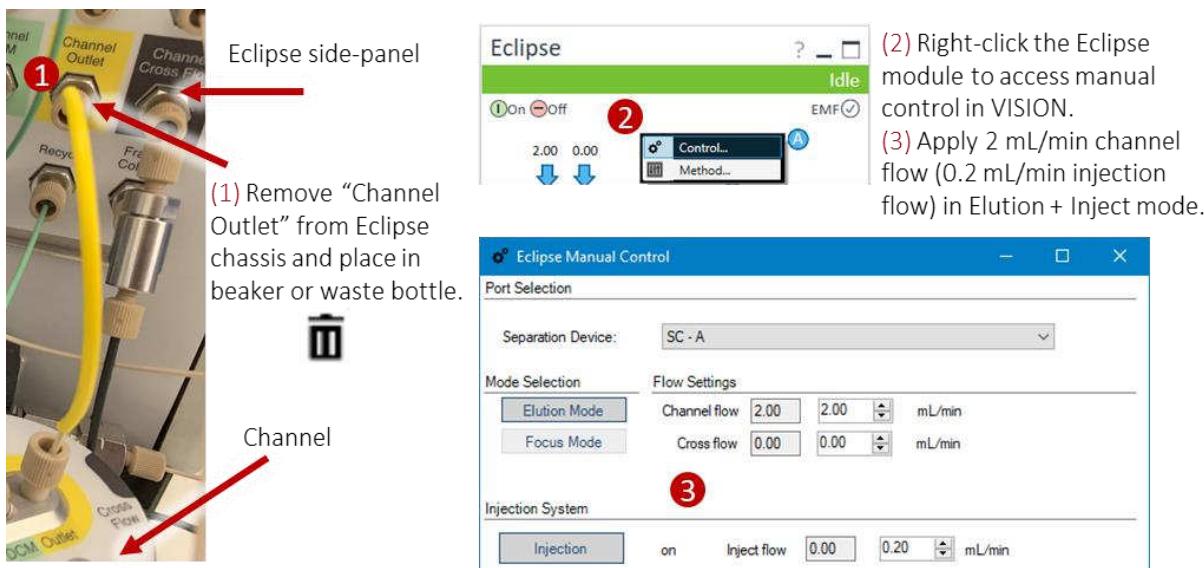
- In the Membrane Flush window, one of the options will be **Flush Membrane**. The default settings will apply 1 mL/min channel flow and 0.25 mL/min detector flow across three 10-minute steps. This will flush the membrane for 30 minutes total. During the flushing, the Eclipse will be in a Not Ready state.



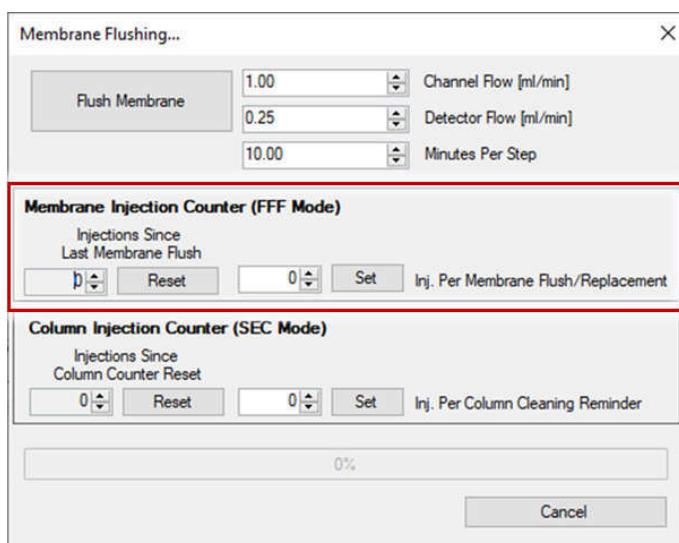
4. After the flushing step is complete, the membrane injection counter will reset to 0. This is a counter that increases with each injection to help generate protocols for when to replace the membrane (for example, after 100 injections). There are individual counters for FFF and SEC injections.
5. Now that the membrane is flushed, you can proceed to the section, [Conditioning the Membrane](#).

Flushing Membrane without Eclipse + DCM Model (WEC, WECS)

1. Without a **Channel DCM** port, the automatic flushing option is not available in VISION. As a result, it is necessary to manually flush the membrane to remove particulates and fibers that can clog the downstream detectors.
2. If the channel has been reconnected to the Eclipse chassis, the tubing entering the Eclipse via the channel outlet side-panel bulkhead union should be disconnected and placed in a beaker or waste container. This will enable flow from the channel to exit via the **channel outlet** tubing, but instead of going into the Eclipse, it will drain into a beaker or waste container. This will avoid clogging the Eclipse and downstream detectors.



3. Under the Manual Control window in VISION, apply a channel flow of 2.0 mL/min and injection flow of 0.2 mL/min with the Eclipse in Elution + Injection mode. Rinse the membrane for at least 10 – 15 minutes to flush out membrane fibers, dust, and air bubbles.
4. Reduce the channel flow rate to 1 mL/min without cross flow and reconnect the channel outlet from the beaker or waste container back to the Eclipse chassis at the channel outlet port.
5. Turn off injection flow (Elution mode only) and while maintaining 1 mL/min channel flow, increase the cross flow to 3 mL/min. Allow the channel to flush for at least 10 – 15 minutes, or until the system pressures stabilize and are constant.
6. After the membrane has been flushed, you can navigate to the “Membrane Flush” window by right-clicking on the Eclipse module. Here you can reset the injection counter to 0 for your newly installed membrane. This is a counter that increases with each injection to help generate protocols for when to replace the membrane. The value is displayed on the front panel of the Eclipse. There are individual counters for FFF and SEC injections.



Now that the membrane is flushed, you can condition the membrane. Proceed to [VISION RUN Operation Workflow](#) to learn how to create methods and sequences for conditioning the membrane. If you already have a method, proceed to the section, [Conditioning the Membrane](#).

Conditioning the Membrane

A one-time injection is made such that your sample itself (if not sample-limited) or a sample of similar chemical composition to your samples is overloaded onto the channel in order to bind any active sites on the new membrane surface. This defined binding or covering layer can help improve mass recovery and peak shape.

This requires experience loading, creating, or editing a method, followed by running a sample injection via sequence. If you are familiar with VISION RUN, you can proceed to the section, [Methods for Membrane Conditioning](#), where some advice for conditioning membranes is provided.

However, if you are not comfortable with navigating and using VISION RUN, the next section, [VISION RUN Operation Workflow](#), will provide a foundation for creating methods and running sequences. Once you understand the workflow, you'll be able to employ different methods to perform experiments.

Membrane Installation Quick Reference Sheet for Variable Height Channels

Replacing the Variable Height Channel Membrane

Supplies:

- Clean bench, wear gloves
- DI water (sink or squeeze bottle)
- Torque wrench
- New membrane
- New laminated spacer
- 1% detergent solution and/or alcohol for additional channel cleaning

- 1** Replace channel with 5-port union. Take to clean bench near sink and remove top block with hex driver or torque wrench.

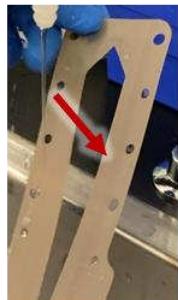


- 2** Remove top block, wash surface with soap, water, and/or alcohol. Clean ports or O-ring as needed.

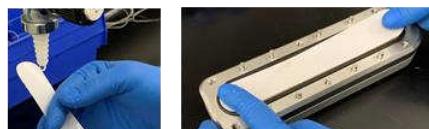


- 3** Remove spacer and discard membrane. Clean new (laminated spacers) or re-usable (Mylar) spacer with water, especially the inner edge.

Note: Channel heater is not waterproof. Avoid soaking/rinsing bottom block itself.



- 4** Rinse new membrane (discard paper in packaging) to remove fibers and install centered on the supporting frit with **shiny side (wetted side) up**.



- 5** The spacer has alignment pin holes to help with orientation, line up square side and round side with channel. Install the spacer and then place the top block on top, securing it with the alignment pins. Avoid shifting the membrane!



- 6** Set the torque wrench to 1 Nm and secure the bolts (following a crisscross pattern) across the channel. **Repeat** for 2 Nm and then **again** for 3 Nm and 4 Nm to fully assemble the channel. There will be a “click” when the defined torque is reached—make small motions to avoid over-torqueing!



- 7** Reconnect the channel to the Eclipse. The tubing sleeves are color-coded for convenience. Flushing steps varies by model:

Non-DCM: Disconnect **Channel Outlet** from Eclipse chassis (not channel) and flush to beaker with 2 mL/min channel flow in Elution + Injection (0.2 mL/min). Stop flow, reconnect **Channel Outlet**. Then Focus + Inject with the addition of 1.5 mL/min cross flow.

DCM Option: Right-click Eclipse widget in VISION, select “Flush Membrane,” and allow the system to automatically flush the membrane.

- 8** Run a conditioning run with a standard of similar chemical composition.

Replace membrane every 2-4 weeks, or when elevated noise is observed. Also replace when sample type is changed.

Figure 44. Quick Channel Membrane Installation Guide for Variable Height Channels

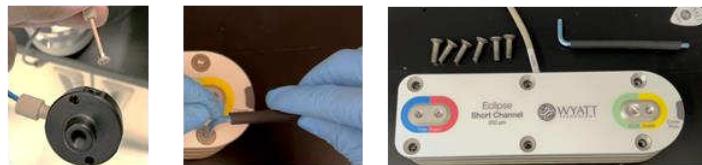
Membrane Installation Quick Reference Sheet for Fixed Height Channels

Replacing the Fixed Height Channel Membrane

Supplies:

- Clean bench, wear gloves
- DI water (sink or squeeze bottle)
- Torque wrench
- New membrane
- New laminated spacer
- 1% detergent solution and/or alcohol for additional channel cleaning

- 1** Replace channel with 5-port union. Take to clean bench near and remove top block with L-key, incrementally loosen bolts.



- 2** Remove top block, wash surface with soap, water, and/or alcohol. Clean ports or O-ring as needed.

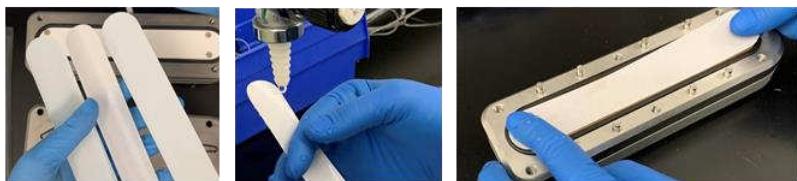


- 3** Discard old membrane.

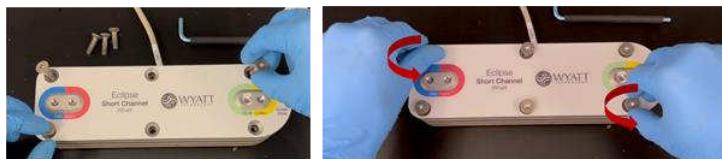
Note: Do not remove the channel frit.

Note: Channel heater is not waterproof. Avoid soaking/rinsing bottom block itself.

- 4** Rinse new membrane (discard two papers in packaging) to remove fibers and install centered on the supporting frit with *shiny side (wetted side) up*.



- 5** Position the top block over the bottom block so the holes are aligned. You can use the bolts to guide the assembly. Start with counter-clockwise rotation to ensure bolts catch the threads.



- 6** Use the L-key to tighten the bolts (following a crisscross pattern) across the channel. Repeat with quarter rotations until the bolts reach a physical stop and the L-key cannot be turned further. The fixed height channel has a metal-to-metal assembly. Ensure there is no visible gap between the top and bottom blocks.



- 7** Reconnect the channel to the Eclipse. The tubing sleeves are color-coded for convenience. Flushing steps varies by model:

Non-DCM: Disconnect **Channel Outlet** from Eclipse chassis (not channel) and flush to beaker with 2 mL/min channel flow in Elution + Injection (0.2 mL/min). Stop flow, reconnect **Channel Outlet**. Then Focus + Inject with the addition of 1.5 mL/min cross flow.

DCM Option: Right-click Eclipse widget in VISION, select “Flush Membrane,” and allow the system to automatically flush the membrane.

- 8** Run a conditioning run with a standard of similar chemical composition.

Replace membrane every 2-4 weeks, or when elevated noise is observed. Also replace when sample type is changed.

Figure 45. Quick Channel Membrane Installation Guide for Fixed Height Channels

VISION RUN Operation Workflow

This section will walk you through monitoring your system, creating a method, and creating a sequence for data acquisition. If you have a method already created, then you can skip ahead to running a sequence ([Creating and Running a Sequence](#)), which includes routine operations and workflow. For configuring VISION, please refer to the section, [VISION RUN Configuration](#), or consult the VISION manual.

Instrument Status

The instrument status field consists of the dashboard and a status bar. The dashboard is depicted in forms of panels (or widgets) for each module in the installed configuration, while each panel or widget provides detailed information of the respective device and its current status.

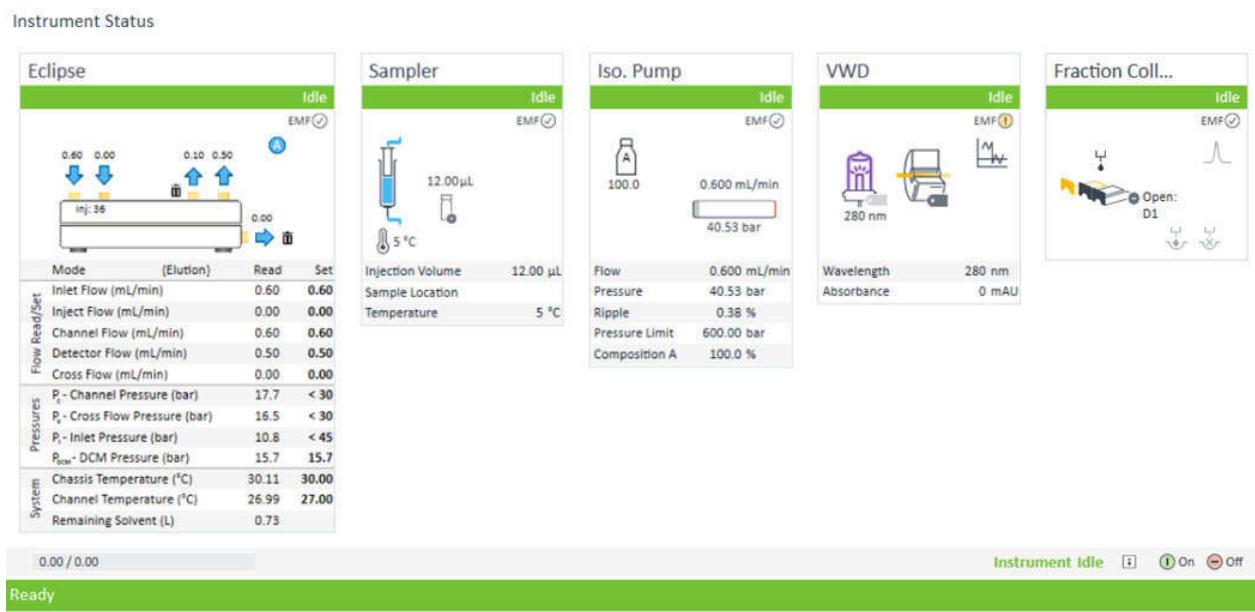


Figure 46. The VISION RUN dashboard showing the Eclipse and HPLC modules that have been configured.

Fold out or hide more detailed information by clicking the expanded window button in the upper right corner of each panel. Hide the panel by clicking the collapsed window button in the upper right corner. Turn on or off a device, put it in a ready state, or clear an error by using the on or off buttons On Off . These options are illustrated in Figure 47.



Figure 47. Instrument Module Widget for a VWD detector. Details can be expanded or collapsed at the top.

The Eclipse widget displays the instrument status, including flow rates, pressures, system temperature, and mobility settings. The Eclipse status window will show both read and set values, as shown in Figure 48, and will highlight a module parameter in red text when there is a mismatch between the read and set values. The time interval before these errors appear can be defined under More → Eclipse → Hardware Configuration.

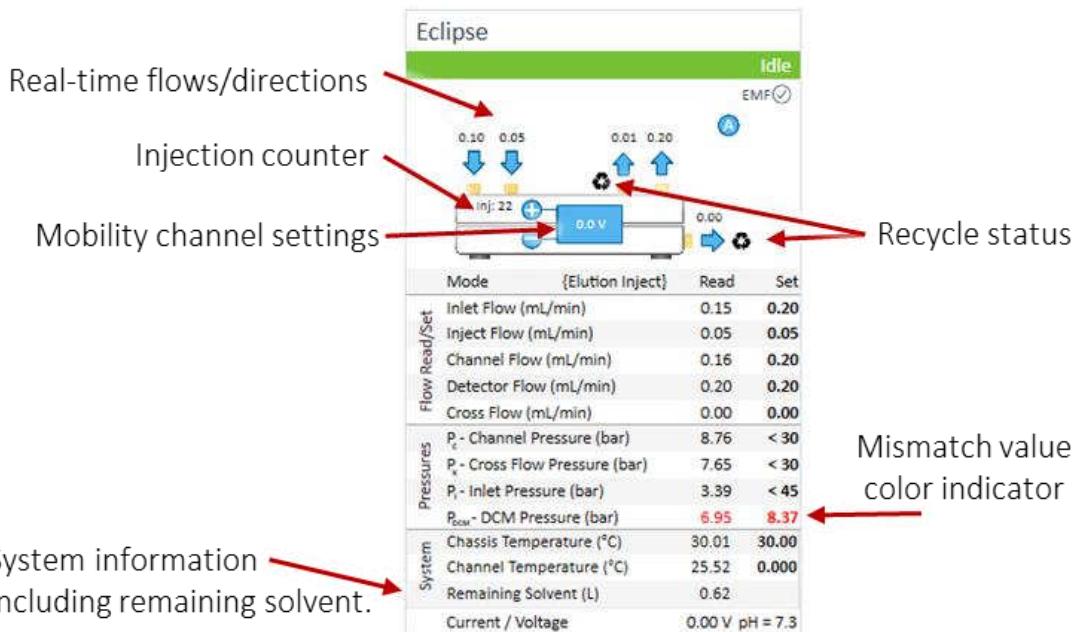


Figure 48. Eclipse expanded widget that displays the current mode, flow rates, pressures, and other system information. Red text indicates a mismatch between read and set values.

Beneath the dashboard, you can turn on or off all the devices, put them all in a ready state, or clear an error by using the on or off button at the status bar as shown in Figure 49.

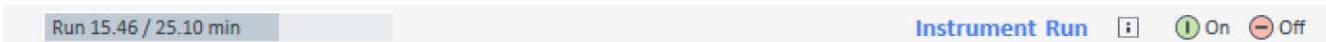


Figure 49. Run status bar beneath the Dashboard.

Click the info button to see detailed hardware and software information of every module in the configuration. Furthermore, the status information shows the runtime of an experiment and the current instrument status. For example, the “Instrument Run” status is displayed during a run as shown in Figure 50. These status indicators are color coded.



Figure 50. Instrument Run Status is indicated by a blue bar.

The system status is shown by the status bar with the corresponding color and label. It will also show the currently loaded method. During a run, the sequence progress, vial position, injection number, and method are shown on the left side of the bar, whereas the progress in time (hours) is displayed on the right. The color status indicators are shown in Table 7.

Table 7. Status Indicator Colors and Descriptions

Module Status	Instrument Status	Description
Offline	Instrument Offline	One or more modules are disconnected.
Standby	Instrument Standby	A module (i.e., HPLC pump) is in standby mode.
Not Ready	Instrument Not Ready	One or more modules are not ready for experiments (e.g., UV lamp is not ignited).
Idle	Instrument Idle	The modules are ready for manual control and experiments.
Prerun	Instrument Prerun	A module is waiting for another module or ASTRA before a run.
Injecting	Instrument Injecting	The instrument is injecting sample.
Run	Instrument Run	The instrument is running an experiment.
Error	Instrument Error	An error has occurred.

In case the instrument is not ready or an error occurred, move the cursor over the device status bar to view information about the cause of the device's condition. An example with an Agilent DAD is shown below in Figure 51.



Figure 51. A “Not Ready” condition for a module. More information can be found by placing the mouse cursor over the module’s status bar.

Right-clicking a user interface module and selecting Control... will allow the Eclipse or HPLC components to be manually controlled. Manual Control will have different functions and capabilities from the Method. In addition to Manual Control, different instrument options are accessible by right-clicking on the modules. Please refer to your HPLC instrument manual for specific options and capabilities for your hardware, including options under More when right-clicking a module. Some recommended control settings are provided in the section, [Eclipse and HPLC Control Settings](#).

Online Plot

The online plot in VISION RUN can be used to monitor signal curves for your Eclipse and HPLC modules, for example the UV signal (mAU) or the Eclipse pressures (bar) as shown in Figure 52. The time range and the signal colors can

be customized. The signals can be toggled on or off on the left-hand side of the interface, and the y-axis units are defined by the currently selected or highlighted signal in the left panel.

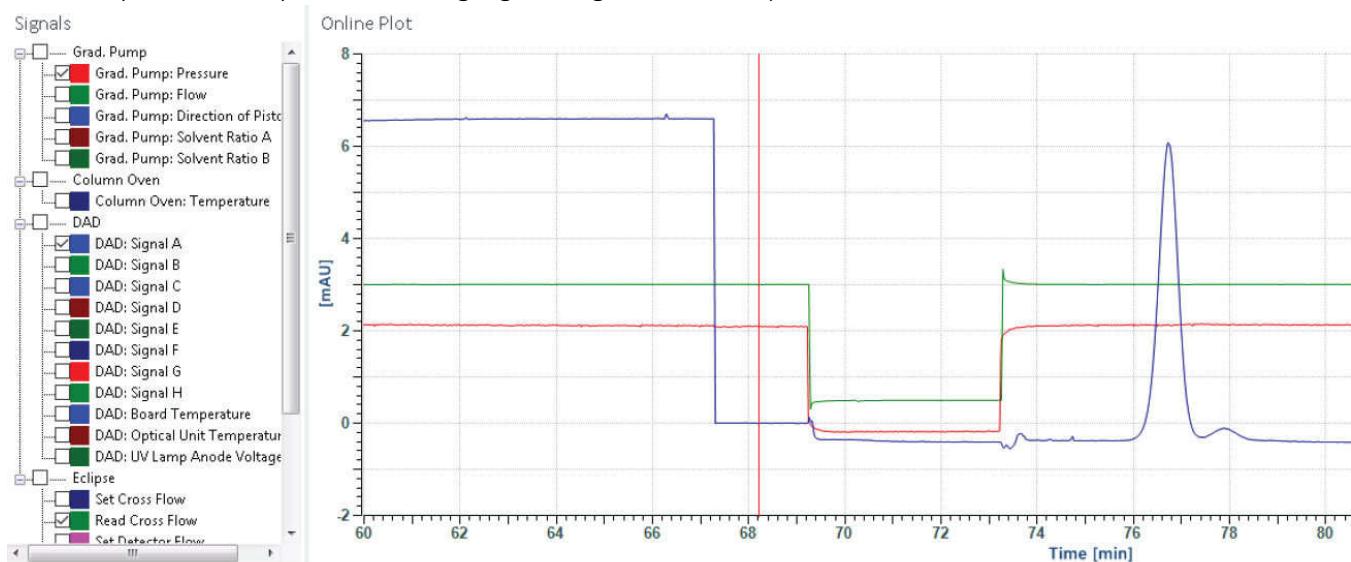


Figure 52. The online plot display in VISION RUN. Signals can be toggled on or off and the currently selected/highlighted signal will determine the y-axis. In this case, the blue signal for the DAD UV signal is selected and defines the y-axis; however, the other signals, like the Eclipse pressures, are visible for relative comparisons. A green vertical line will appear when a sequence row has started and a red line will appear if an abnormality was observed.

Right-clicking on the graph allows you to copy the graph to a clipboard, copy the line values to the clipboard, reset the zoom, or zoom out one level, and the options for plot configuration can be accessed. The plot configuration enables control over the y-axis scale, the time range, and the colors for the various signals or whether they should be displayed or not.

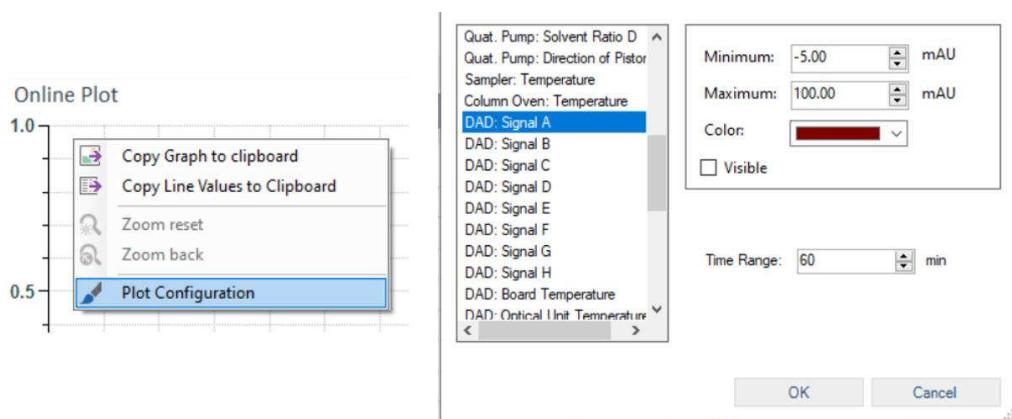


Figure 53. Options for the online plot by right-clicking (left) and after selecting "Plot Configuration" (right).

The online plot will also display information about when an experiment injection has occurred or red lines when there is a mismatch or error detected. An example is shown below in Figure 54.



Figure 54. The online plot will display a green vertical line when an injection has happened, or a red vertical line if there is a flow or pressure mismatch, or other error. Placing the mouse cursor over the line will display more information.

With an overview of the user interface discussed, as well as the key functionality of the manual control window covered in the section, [Eclipse Manual Control Overview](#), the next sections will cover creating a method and creating sequences. From that discussion, example methods for automating solvent exchange, conditioning a membrane, and other common procedures will be explained further.

Creating a Method

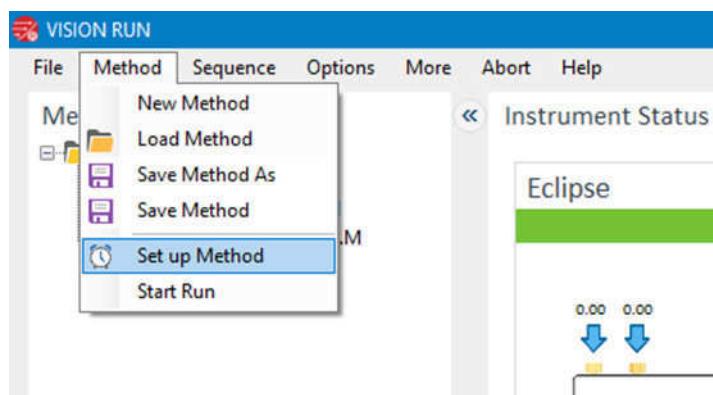
The method applies various instrument settings during the experiment. These methods can be individually edited for each module (i.e., Eclipse, UV Detector, Fraction Collector, etc.). Methods can be saved or loaded. In this section, we will discuss how to create and edit methods. Both example methods and method considerations will be discussed in the section, [VISION RUN Eclipse Methods and Protocols](#). For specific method advice for samples—such as appropriate cross flow settings—please refer to [TN6005 - Eclipse Aqueous Method Development Quick Start Guide](#). Methods for conditioning a membrane will be discussed in a subsequent section, Methods for Membrane Conditioning.

A method can be created in VISION RUN by navigating to the top menu bar, and selecting **Method → New Method**. However, a new method will need to have all the pump, UV, autosampler, and other settings configured when built from a scratch. It can be more convenient to open an existing method, for example one created by Wyatt during the installation and then proceed to edit that method as needed to save as a new method.

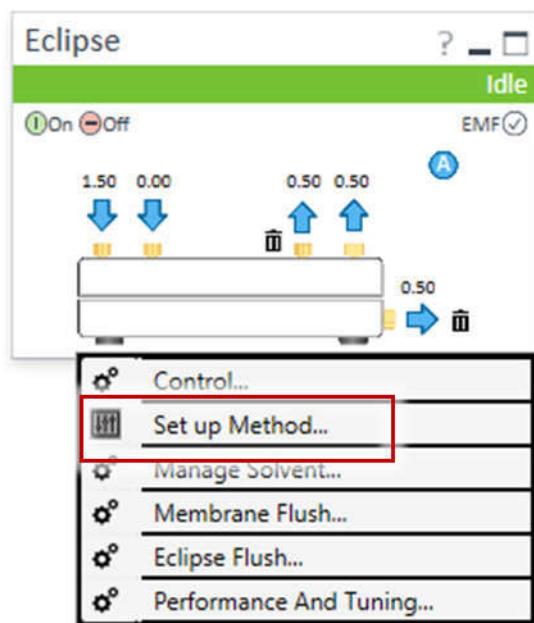
NOTE: Instead of creating a new method, consider opening an existing method and saving it as a new method (**Save Method As**). This way, you can edit the relevant fields without having to re-build the HPLC settings from scratch.

Editing a Method

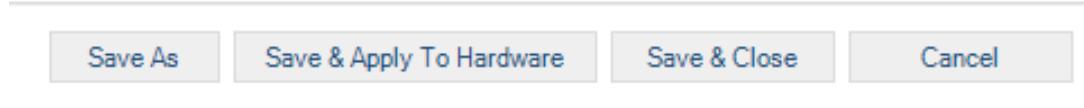
A method is always present in VISION RUN, and may be a blank method upon launching the software or before loading an existing method. Running an experiment in a sequence table requires a method. It is preferable to edit the method using the “Set Up Method” workflow, which will allow you to edits the methods for all hardware present in the configuration. From the top menu bar, navigate to **Method → Set up Method**. A new window will open with all the instruments accessible by tabs where their method parameters can be changed.



Additionally, the same window can be accessed by right-clicking on the Eclipse and selecting “Set up Method.”



When editing methods, there are a number of options for saving and applying hardware settings. With VISION 3.1.2 and later, it is possible to edit methods while sequences are running or when modules are offline. In the method editor window, the bottom of the window contains the following options:



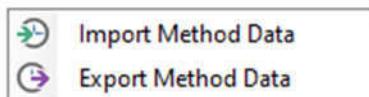
- Save As: The current system method (Eclipse + Agilent method) can be saved under a new name. This strategy can be used when opening a “template” method (i.e., a method will appropriate Agilent HPLC method parameters like UV method settings), and saving a copy to modify the Eclipse method.
- Save & Apply to Hardware: This will save the currently edited method but also applies the method to hardware. You can use this to equilibrate the system after editing a method by applying it to the system. Only select this option if you want to change the current hardware method.

- Save & Close: Saves the currently edited method, but does not apply to hardware, and then closes the method editor window. This is ideal for creating offline methods or editing methods during a sequence, as you can create/edit a method without changing the currently loaded method on the system.
- Cancel: Don't save any changes to currently edited method and close the window.
- Most quick edits can be done with "Save & Close" and then re-open as needed.

In this technical note, the Eclipse method settings will be the discussed. Options for your Agilent HPLC instruments may be found in your manufacturer's hardware manuals. Some recommended settings and tips for instrument connectivity for Agilent HPLC modules can be found in [TN3600 Agilent HPLC Connection Guide for Wyatt Instrumentation](#). For advice on configuring and running an Agilent fraction collector in VISION, please refer to [TN6504 Configuring a Fraction Collector with VISION 2.0](#).

Eclipse Method Editor

The Eclipse method editor window consists of the timetable, flow settings, separation device, focus valve position, solvent & membrane settings, and the flow profile settings graph. This window is shown in Figure 55. In this section, the options and parameters will be explained. Methods can be exported or imported by right-clicking on the window. If a method has been created in VISION DESIGN, you can right-click on the Eclipse method editor window and select **Import Method Data** and load the ECMF (Eclipse method file with extension *.ecmf) generated by VISION DESIGN.



Creating a method time table in VISION DESIGN is discussed in the section [Simulation and Method Refinement](#) in [Typical Workflow with VISION DESIGN](#).

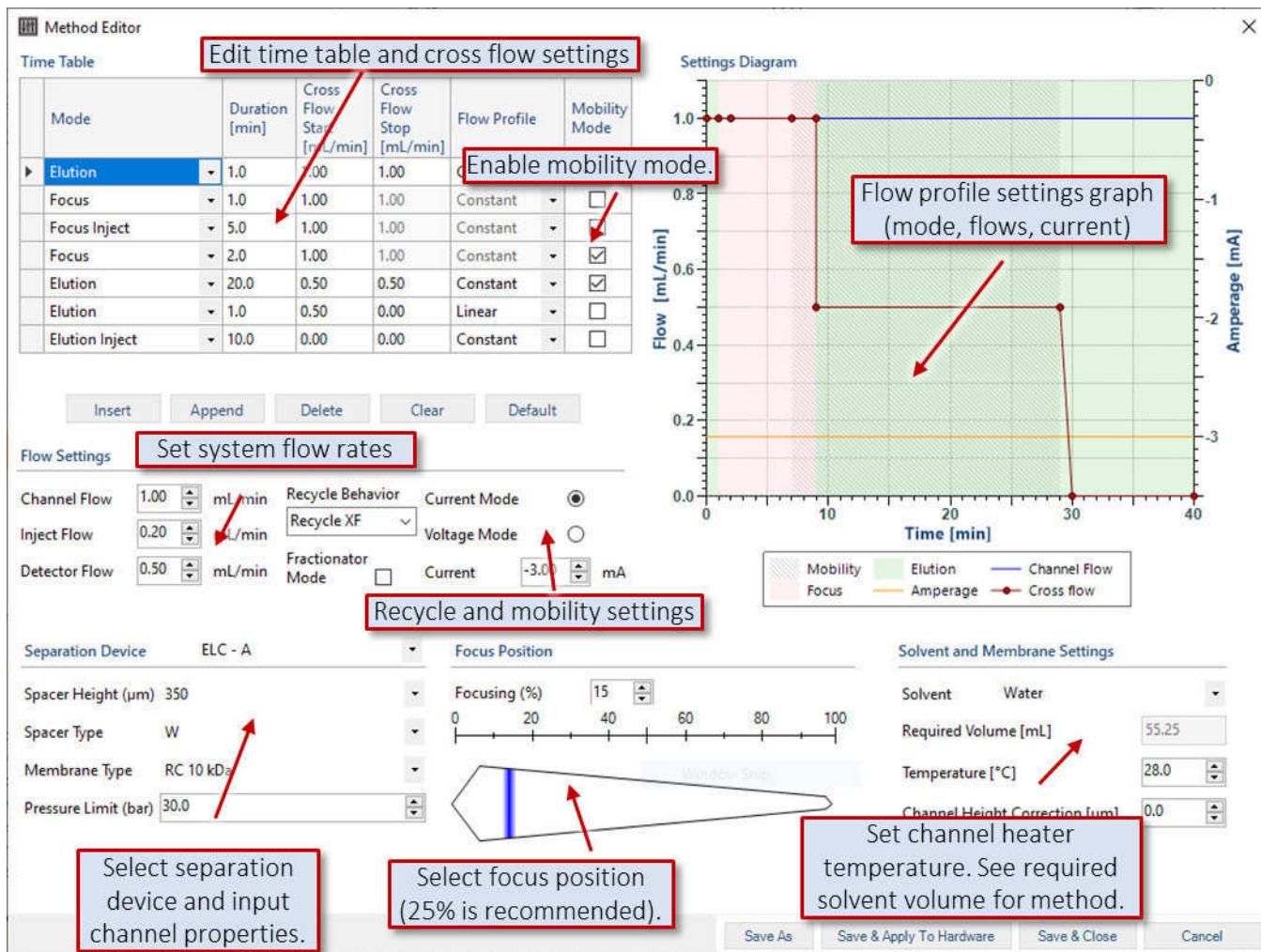


Figure 55. The Eclipse method editor window with time table, flow settings, separation device, focus position, solvent & membrane settings, and settings diagram (flow profile) with focus and elution modes indicated by pink and green respectively and the flow rates for the channel and cross flow pathways indicated.

Eclipse Time Table

Each line in the time table contains information about the mode (i.e., Elution or Focus), the duration of the step, the cross flow (mL/min) at the start of the step and the end of the step, and the flow profile for that cross flow gradient (constant, linear, or exponential). The exponential flow profile can be adjusted by right-clicking on “Exponential” and selecting **Edit slope** if desired.



If you have the Eclipse Mobility module, an additional column for enabling or disabling a current or voltage will be displayed. More information on appropriate methods with the Eclipse Mobility module are discussed in the [Eclipse Mobility Module](#). Individual rows can be inserted, appended, or deleted. The entire time table can be cleared and there is also an option to build a default method table based on the separation device and Eclipse model.

Flow Settings

The Eclipse has several different flows that are defined below:

- **Cross Flow (Configurable):**

- During focus mode, this defines the total focus flow (mL/min) of the two inlets (which come together to focus the sample in a narrow band). During focus mode, a good starting flow rate is 1.5 mL/min but can be decreased if you are focusing a larger sample that diffuses slowly.
 - During elution mode, this is the key parameter that determines the separation. Stronger cross flows will delay elution, more so for larger macromolecules compared to smaller macromolecules. Large macromolecules may not even elute under high cross flows, so typically there is variable cross flow (from high to low) to ensure elution of the sample as it separates. A rapid change in cross flow can help sample elute quickly after separation.
- **Channel Flow** (Configurable): The channel flow describes the total flow across the top of the channel. This determines the parabolic flow profile that will be applied to the sample. The ratio of cross flow to channel flow determines the effectiveness of the separation. This is discussed further in the section, [Appendix B – Basic Theory](#).
 - **Inlet Flow** (Defined by Settings): The combination of cross flow and channel flow determines the total inlet flow, minus the injection flow. This is a measure of the flow entering the channel, which then becomes either outlet flow (to detectors), DCM flow, or cross flow.
 - **Detector Flow** (Configurable): The detector flow describes the total flow exiting the channel outlet to downstream analytical detectors. The ratio of channel flow to detector flow determines the split ratio in the channel, or the degree to which sample is concentrated due to the DCM siphoning off pure mobile phase.
 - **DCM Flow** (Defined by Settings): The difference between the channel flow and the detector flow determines the DCM flow. This is flow that does not enter the analytical detectors and is simply mobile phase that has been removed from the top of the channel (where sample is not located) to concentrate the sample entering the analytical detectors. More theory on the concentration profiles in a channel are provided in [Appendix B – Basic Theory](#). Even when the DCM function is not desired, it is recommended to apply some DCM flow for best detector flow regulation.

While the cross flow profile can be variable, the channel, detector, and inject flow settings are constant and set in the **Flow Settings** field. The detector flow can also be set in the sequence table. The detector flow determines the flow rate through the downstream detectors from the channel outlet. Typical detector flow rates may range from 0.15 mL/min to 1.2 mL/min, depending on the solvent viscosity and system pressures. Lower detector flow rates may be more suitable when higher split ratios are needed (for dilute samples with DCM) or when using inline DLS such as with a WyattQELS embedded DLS detector or DynaPro NanoStar or Mobius with DLS Compatibility Kit in the MALS detector. In the Eclipse, the injection flow can be specified but is typically 0.10 – 0.25 mL/min. The focus position can be set in the focus position section.

[DCM Flow Settings Explanation](#)

The difference between the channel flow and the detector flow determines the DCM flow. The ratio of DCM flow to detector flow determines the split ratio. For example, a channel flow of 1.0 mL/min with a detector flow of 0.25 mL/min means the DCM flow is 0.75 mL/min. The split ratio is therefore $0.75 / 0.25 = 3$. The split ratio plus 1 is the concentration enhancement. A split ratio of 3 provides a 4x enhancement of concentration. This is provided below as an equation:

$$\text{Split Ratio (SR)} = \frac{\text{Channel Flow (}V_c\text{)} - \text{Detector Flow (}V_d\text{)}}{\text{Detector Flow (}V_d\text{)}}$$

$$\text{Concentration Enhancement (#}x\text{)} = \text{Split Ratio (SR)} + 1$$

A comparison of the UV signal increase with different concentration enhancements is provided in Figure 56. A concentration enhancement up to 5x is recommended for minimal loss of resolution. A concentration enhancement greater than 5 begins to exhibit peaks with a loss of resolution. For analytical characterization of aggregates or samples with low concentration, a concentration enhancement between 2x and 5x is recommended. Different split ratios can easily be configured by adjusting the detector flow in the VISION RUN sequence table and so multiple methods do not need to be created. Concentration enhancement above 5x may lose peak resolution and are recommended when collecting samples via fraction collector or when high concentrations are needed over analytical peak resolution.

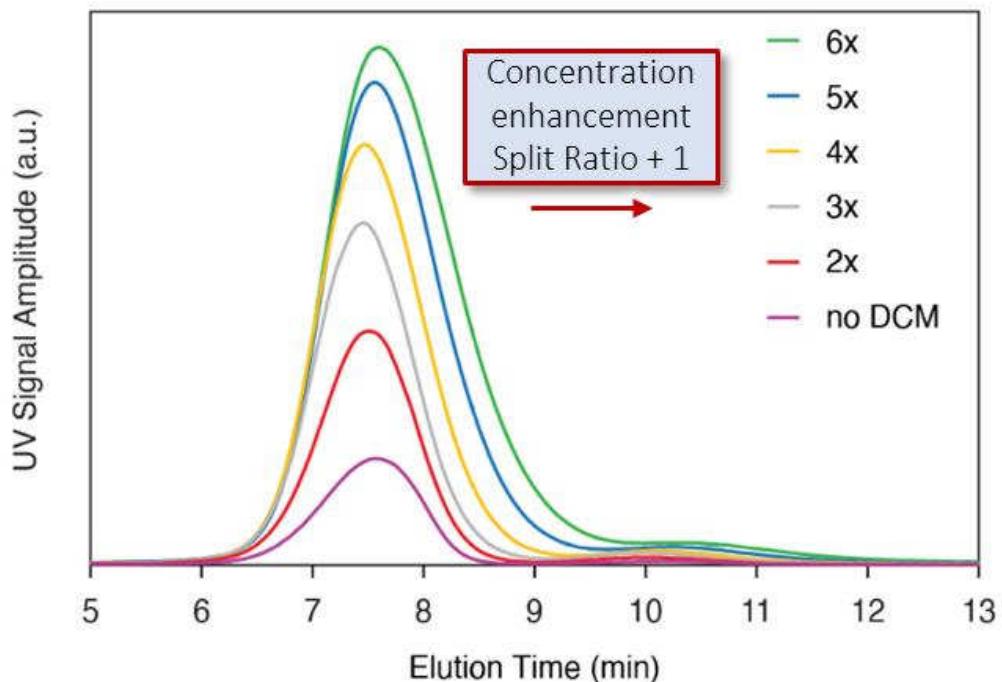


Figure 56. Comparison of BSA protein standard with concentration enhancement ranging from no enhancement to 6x enhancement.

Separation Device

The separation device section enables selection of the separation device and, if using an Eclipse with SEC switching option, which port that separation device is installed on. The options are for an Eclipse channel to be installed on the FFF port and a column to be installed on the SEC port. Available separation devices are summarized in the table below:

Table 8. Abbreviations for separation devices in VISION RUN and their pressure limit

Abbreviation	Separation Device	Pressure Limit
SC	Short Channel	30 bar
LC	Long Channel	30 bar
SP	Semi-Preparative Channel	30 bar
ELC	Electrical Long Channel (For Eclipse Mobility)	30 bar
DP	Dispersion Inlet Channel	30 bar
Column	SEC Mode Device (HPLC Column)	75 bar
No Device	5-port union or no separation device configured	30 bar

When a channel is configured, the flow settings specified in the Eclipse method will automatically apply the appropriate pump time table to the pump method. As a result, there is no need to edit the pump timetable after making changes to the Eclipse timetable. The separation device and the pressure limit will apply to the method and are needed for running methods. The membrane type and spacer height are for informational purposes only but it is recommended to update with the currently installed membrane and spacer height for record-keeping purposes.

When a column is configured, the Eclipse method will automatically apply an Elution + Inject flow mode for SEC separations. This default behavior will apply an isocratic pump flow through the column. A custom pump time table can be created for solvent gradients or flow rate changes by toggling the Pump Behavior Button from **Use Eclipse Method Timetable** to **Use User-Defined Pump Timetable**. The time table, flow settings, and separation device options when a column is configured is shown in Figure 57.

When SEC mode is enabled, a new pressure limit is automatically applied for the column. The pressure limit for SEC mode is 75 bar. For channels, the recommended pressure limit is 30 bar due to the flow controller hardware.

NOTE: The pressure limit when using columns is 75 bar. When using multiple columns or columns with smaller packing beads that add increased backpressure, it may be necessary to reduce the flow rate if you are exceeding the 75 bar limit.

Exceeding the channel or column pressure limit will shut off the pump and stop a sequence that is in progress. As a result, it can be helpful to use the manual control window to assess the pressures of the system at the highest flow rates in a method before starting your sequence.

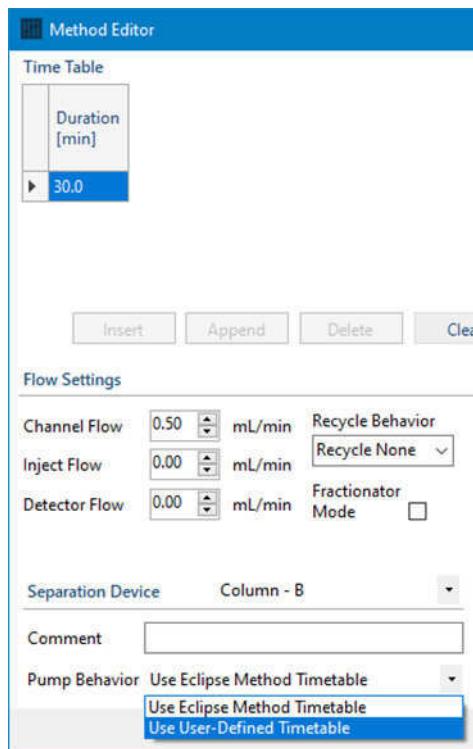


Figure 57. The Eclipse method editor window when a column is configured in SEC mode. Duration for Elution + Inject (default mode for SEC runs) can be specified and the channel flow rate can be set under flow settings. The pump behavior can be set either using "Eclipse Method Timetable" for a single solvent composition or with "User-Defined Timetable" for solvent gradients.

Focus Position

The focus position setting determines where the sample band is formed during focus mode. In the Eclipse, the focus position is controlled by a CoriFlow controller. The focus position is typically set just after the widest portion of the separation space, a few millimeters ahead of the injection port, indicated with a blue line below. A typical value for the short channel is 25%, and for the long channel it is also appropriate to start at 25% of the channel length and decrease as needed if more resolving length is needed.

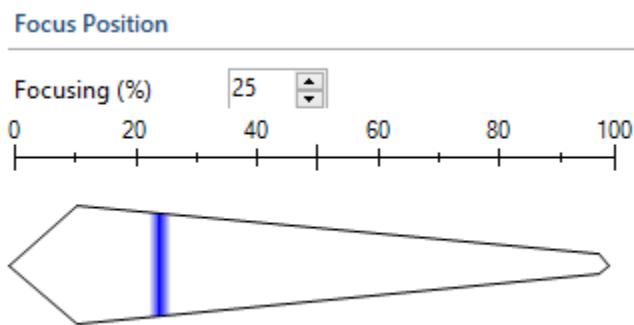
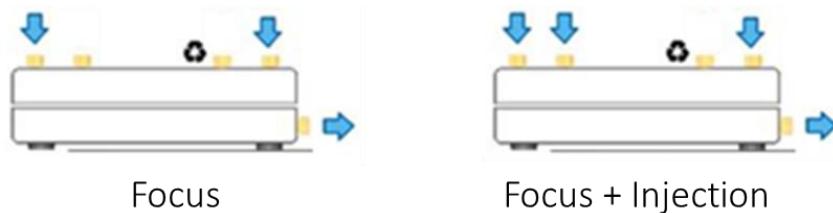


Figure 58. Focus position (in %) for a short channel. Although the focus position (%) depends on the channel geometry, it is recommended to focus just after the injection port, a few millimeters ahead of the widest part of the spacer. In general, it is recommended to focus at 25%.

It is important to note that during focus and focus + inject, factors such as focus position (%), inject flow, and total focus flow (cross flow during focus step), may influence the total flow. The focus position governs the value during Focus, and the system will attempt to achieve as close a value to that as possible during focus + inject, but that may be influenced by these parameters. Let's look at an example. Consider the following two states, focus and focus + injection:



Consider the following Focus step method:

- Focus Position = 25%
- Focus Flow (Cross Flow during Focus step) = 1.0 mL/min
- Inject Flow = 0.2 mL/min

During the focus step, to achieve a focus position of 25%, the inlet port will deliver 0.25 mL/min flow while the outlet port will deliver 0.75 mL/min flow. When the mode switches to focus + inject, you will have 0.2 mL/min inject flow. To achieve a focus position of 25%, the inlet port will now deliver only 0.05 mL/min flow while the outlet port will deliver 0.75 mL/min flow.

You can see now that depending on the settings, and the fact that we cannot have a negative inlet flow, that we may not get the desired focus position during focus + inject, even though we achieve an appropriate focus position during the Focus step. For example, let's consider the follow method:

- Focus position = 20%

- Focus Flow = 0.5 mL/min
- Inject Flow = 0.2 mL/min

In this case, during focus, we achieve 20% focus position by having 0.1 mL/min enter the channel via inlet and 0.4 mL/min enter via the outlet, which gives us a total focus flow of 0.5 mL/min. During focus + inject, we have an injection flow rate of 0.2 mL/min. We cannot have a negative inlet flow, so instead we have 0.0 mL/min via inlet flow. We also cannot exceed our 0.5 mL/min focus flow, so only 0.3 mL/min enters via the outlet. As a result, during focus + inject, our true focus position is 40%. After the focus + inject step is complete, and we return to purely focus without injection, the focus position returns to 20%.

There are a few strategies to consider for these cases:

1. Since the focus position will be correct, and only the focus + inject step will focus further upstream under certain conditions, the second “focus” step should return the sample to the desired focusing position. A longer focus step can be applied to ensure this happens.
2. To avoid no inlet flow during focus + inject, you can either increase the focus position, decrease the inject flow, or increase the focus flow. Making these changes will enable the focus + inject to be closer to the desired focus position of the focus step.
3. If you do consider decreasing the inject flow, ensure your focus + inject step is long enough to fully introduce the sample into the channel. Specifically, ensure you flow for enough volume to flush the entire volume of the sample from the sample loop.

Solvent & Membrane Settings

In this section, various solvent or membrane settings can be specified. Importantly, the channel heater temperature can be set in this panel as shown in Figure 59. The channel heater, or channel temperature regulator, can regulate the temperature from slightly above ambient temperature to 50 °C to improve signal stability and reproducibility. Please contact Wyatt Technology for information on regulating the temperature at sub-ambient temperatures, which would require a HPLC-grade refrigeration chamber/case or cold room.

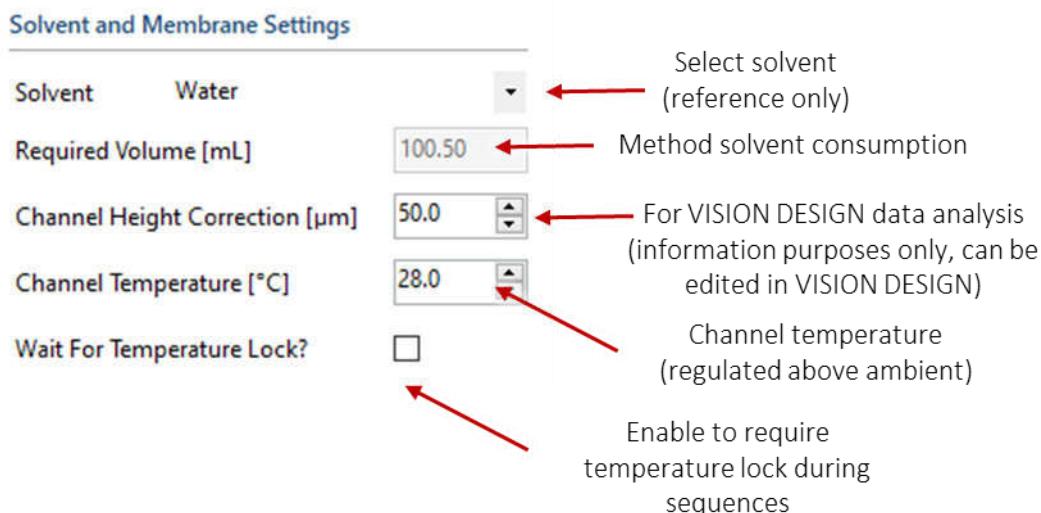


Figure 59. The solvent and membrane settings in the Eclipse method editor window allow the solvent and channel height correction to be stored for information purposes. The solvent consumption and channel heater temperature are set here.

NOTE: It is recommended to set the channel temperature to slightly above ambient in order for the temperature to be regulated.

The solvent can be selected for record-keeping; however, the available solvents are pulled from the VISION DESIGN database. Please refer to the [Solvent Settings & Adding New Solvents](#) in the [VISION DESIGN Operation Workflow](#) section or the [Wyatt Support Center](#) for more information regarding adding solvents to the database. The below is a screenshot from VISION DESIGN where the solvent database is accessible via Settings → Solvents:



Figure 60. Screenshot from VISION DESIGN where the Solvents can be configured under Settings. The solvent database is accessed by VISION RUN in the Eclipse Method Editor.

Settings Diagram

The settings diagram (flow profile) displays the time table in a graph format. The focus and elution modes are indicated by pink and green shades respectively, and the flow rates for the channel and cross flow pathways are indicated. This graph is updated when the time table is adjusted and provides a visual representation of the flow profile. The graph can be copied as a vector graphic and pasted elsewhere by right-clicking the diagram and selecting **Copy graph to clipboard**.

Saving or Loading a Method

From the Method Editor, selecting **Save As**, **Save & Apply to Hardware**, or **Save & Close** will save the method or open the file explorer to specify a file name. When “applied to hardware,” the system will apply the flow rate settings of the first row/line of the time table. Methods are generally saved from the Eclipse method editor window; however, you can also save (required for VISION 3.1.1 or earlier) from the Method dropdown at the top menu bar. To do this, navigate to the Method dropdown from the top menu bar as shown in Figure 61.

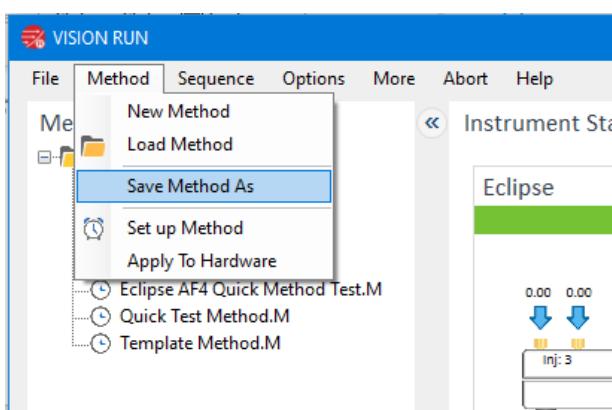


Figure 61. After editing a method, the method must be saved in order to preserve changes and utilize the method in a sequence.

To save the method as a new method, select “Save Method As” and a Windows explorer window will open and the method can be saved as a <file name>.M file. Save changes to an already loaded method by selecting “Save & Close” or “Save & Apply to Hardware” in the method editor.

[Save As](#)[Save & Apply To Hardware](#)[Save & Close](#)[Cancel](#)

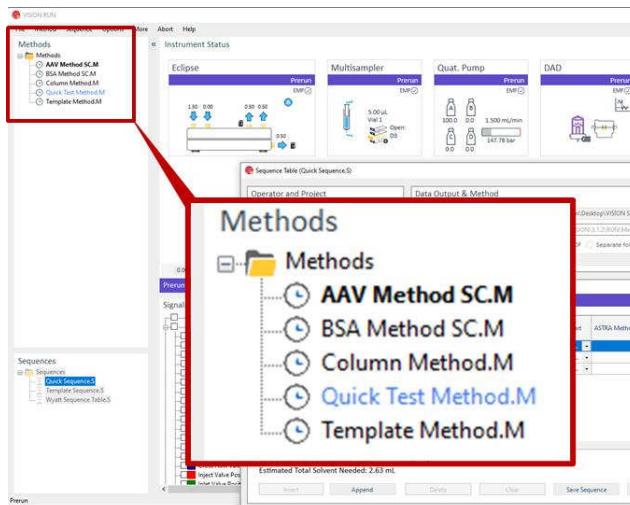
A method can be loaded from the same dropdown, **Method** → **Load Method** or by double-clicking on the methods listed on the left panel of the main VISION RUN screen if the panel is not collapsed (expand with). When loaded, the method will execute the first row or line of the time table and display the current method in the status window above the Online Plot:



And the loaded method will be indicated by a highlighted text in the left panel. The default save location for VISION RUN methods in VISION is under C:\ProgramData\WTC\VISION\<version>\RUN\Method.

NOTE: A method can be locked using the Windows functionality for the file, this is done by navigating to the file in Windows and then enabling “write protection.”

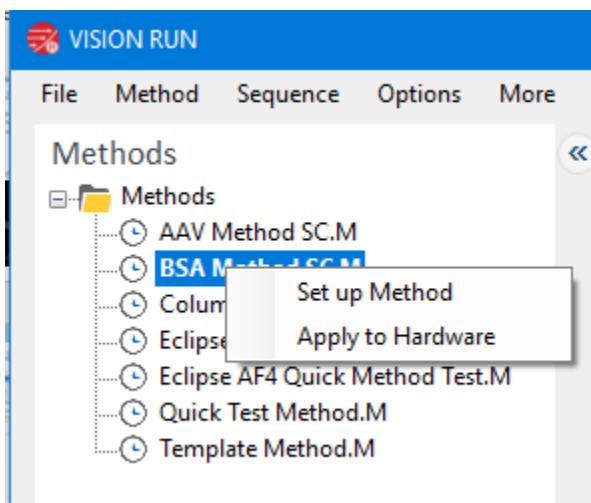
The left panel is designed to help you identify whether a method is running, edited, or available:



Method Panel Colors:

- Black text: All methods currently available, right-click to edit or load.
- **Bold text:** Currently edited method (Set Up Method window that's open)
- **Blue text:** Method is currently being run in the sequence; “bold” edits when saved will apply to the next time that method is run in a sequence.
- If text is both **bold and blue**, that indicates the currently running method is also being edited. Saved changes to the method will apply the next time that method is utilized.

Methods do not need to be loaded onto the hardware prior to running a sequence; however, the method can be applied to the hardware by selecting “Save & Apply to Hardware” in the Method Editor, or by right-clicking on the side panel method that you wish to apply to the hardware.



Please see [VISION RUN Eclipse Methods and Protocols](#) for some recommended Eclipse methods for various conditioning standards, cleaning, etc. In the follow section, method settings for other HPLC components will be discussed.

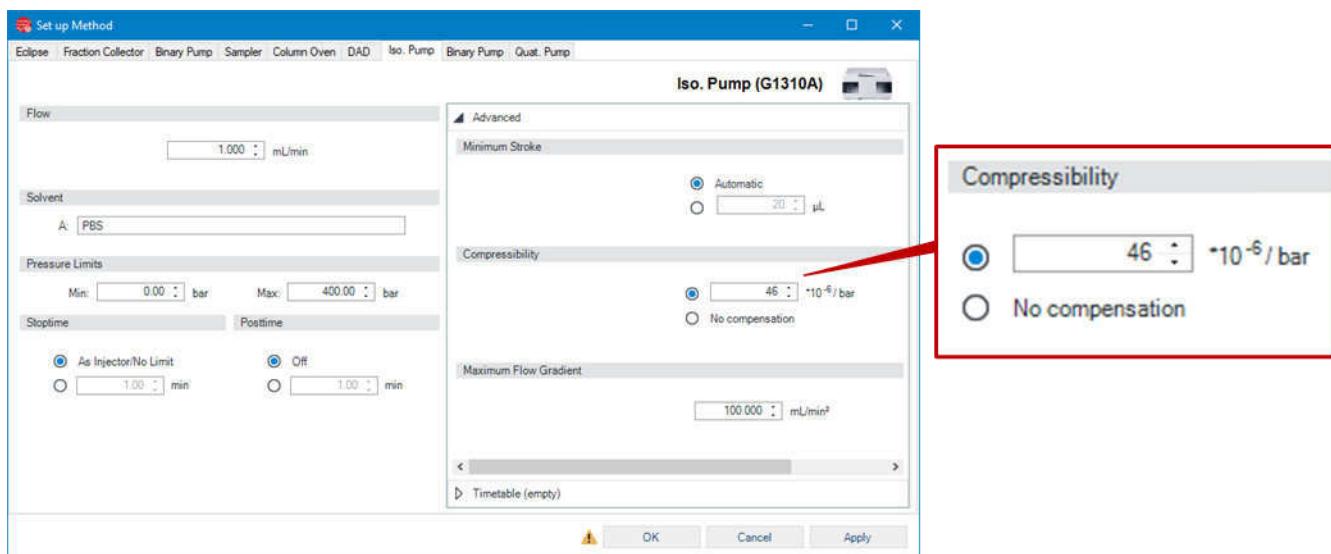
Considerations for some HPLC Instrument Methods

Please consult your Agilent instrument user manual for information on available settings. Some recommended settings for detectors that will have signals collected in ASTRA will be discussed.

HPLC Pump

Settings vary by HPLC pump. VISION RUN applies a total pump flow to the HPLC pump and the Eclipse directs the total flow in different ways to achieve cross flow, injection flow, channel flow, DCM, and detector flow. There are several pump settings to be aware of, including:

- **Bottle Fillings** (right-click on module): This feature should not be used because it will conflict with the Eclipse Intelligent Solvent Management (ISM) system. Please see section, [HPLC Pump Settings](#), for disabling the bottle fillings option.
- **Solvent Reservoir** (under Method settings): When a blank or default method is launched, VISION RUN may default to Solvent Reservoir A (if using a binary or quaternary pump). Take care to ensure when setting flow rates from the Eclipse Manual Control that the Pump Method is pulling from the desired solvent bottle.
- **Advanced Settings – Compressibility**: Under the HPLC Pump method, advanced options like compressibility can be set. Compressibility affects the performance of the pistons and can be optimized per solvent to reduce pump ripple and improve signals, especially for refractive index detectors (RID). Certain Agilent pump modules can automatically set the pump piston compressibility by accessing a database of solvent compressibility values; however, many require an input for the compressibility:



Some compressibility values for solvents are provided by Agilent and summarized in this table below from Agilent (<https://community.agilent.com/docs/DOC-1679>) or in the *CRC Handbook of Chemistry and Physics, 90th Edition*. Solvent purity and/or whether the solvent is degassed prior to pumping may affect these values:

Solvent (pure)	Compressibility (10^{-6} bar)
Water	46
THF	97
Toluene	87
Acetonitrile	115
Chloroform	100
Dichloromethane	99
Ethanol	114
Isopropyl Alcohol	100

Advanced Settings – Maximum Flow Gradient: When using a channel for FFF-MALS separations, it is important to ensure fast flow rate changes by using a high flow gradient (100 mL/min^2) for rapid changes in the cross flow rate. However, when a column is configured in SEC mode, it is important to use slow flow gradients to minimize shock to SEC columns, which are more sensitive to flow rate changes than the Eclipse channel separation devices. The SEC flow gradient options under More → Eclipse → Hardware Configuration will override the values in the advanced method section of the pump for your column's safety.

UV Detectors

Settings vary by detector. It is important in the UV method window to verify the following settings:

- **Wavelength** to be monitored by the UV detector—usually 280 nm for proteins.
- **Acquire Signal without Reference** – ensure this box is selected so the UV data is not modified. It is important that UV data, which is used quantitatively in ASTRA, should not be subtracted by a reference signal, which may lead to incorrect signal intensity.
- Set a bandwidth value of 2 nm (MWD/DAD).

- **Peak width** – Should be set as “>0.1 min (2 s resp. time) (5 Hz).”
- **Analog Output**
 - **Zero Offset** – should be set to 5%
 - **Attenuation** – set it to 1000 mAU if you have chosen the analog output range of 1 V, which results in a UV response of 1 AU/V.

Ensure the appropriate UV settings are configured in your ASTRA method as well. Please refer to the ASTRA User Guide or consult the HPLC Connectivity Technical Notes, such as [TN3600 Agilent HPLC Connection Guide for Wyatt Instrumentation](#).

FLD Detectors

Settings vary by detector. It is important in the FLD method window to verify the following settings:

- **Gain (PMT)** Setting – a value of 12 is a good starting value and can be increased if the sensitivity is too low.
- **Excitation** – a value of 280 nm is typical for proteins; 260 nm is typical for nucleic acids
- **Emission** – a value of 340 – 350 nm is ideal for BSA or other proteins; 320 – 330 nm for nucleic acids

As of May 2022, ASTRA does not natively feature a dedicated FLD configuration. FLD data for informational purposes or for analyzing peak area, can be acquired as an artificial UV signal or generic VI or RI signal.

Fraction Collectors

For advice on configuring and running an Agilent fraction collector in VISION, please refer to [TN6504 Configuring a Fraction Collector with VISION 2.0](#).

Enabling 3D Spectral View in VISION DESIGN

VISION DESIGN has the capacity to display a 3D viewer for spectral data (Figure 62). This allows for data across multiple wavelengths to be viewed simultaneously when performing experiments with a UV-visible diode-array detector (DAD), select variable-wavelength detectors (VWD), and/or fluorescence detectors (FLD).

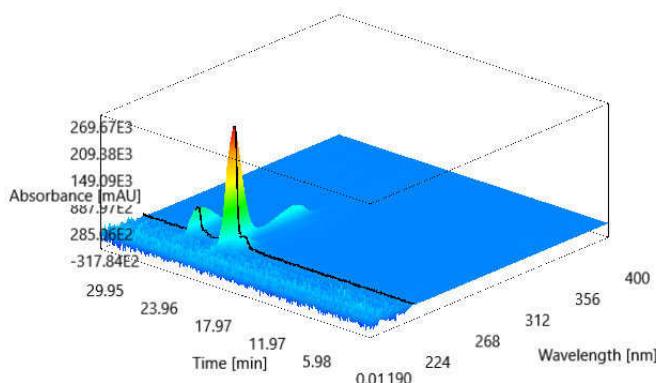


Figure 62. Example of VISION DESIGN’s 3D spectral view, plotting data acquired with a DAD at 190 to 400 nm during an FFF separation of BSA.

DADs allow for continuous acquisition of different UV wavelengths at the same time. On the other hand, VWDs and FLDs can scan a wavelength range in order to acquire spectral data. VISION can digitally read data from both collection strategies to generate 3D plots. This allows for a quick inspection of several data streams and the ability to gauge the appropriate UV or FLD wavelengths for analysis.

NOTE: Some VWDs only allow for spectral scans in stop-flow mode, and thus would not be compatible with 3D spectral data from in-line FFF-MALS experiments. For this reason, DADs are recommended for the best 3D spectral view experience.

To collect data, the detector method in VISION RUN should include the appropriate configuration. Please see Figure 63 below for Agilent method settings.

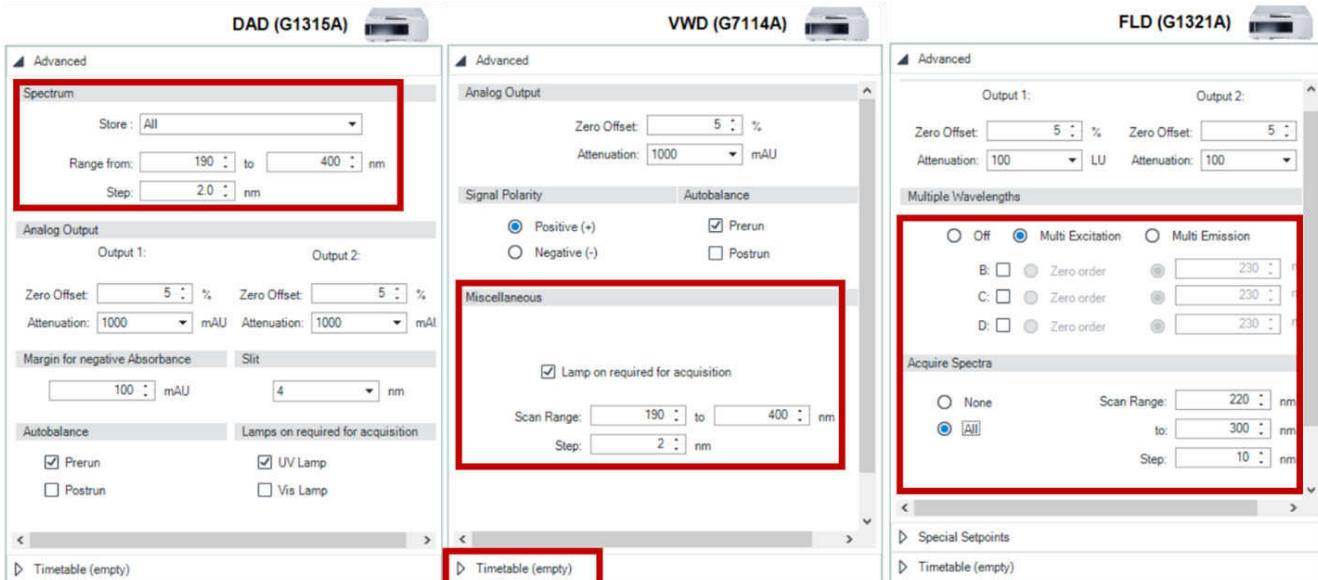


Figure 63. The appropriate method editor settings under the ‘Advanced’ tab in VISION RUN for collecting spectral data for a DAD (left), VWD (middle), or FLD (right) with Agilent detectors. Under the ‘Advanced’ tab in the Method Editor, DAD settings should have “Store: All” enabled and the desired scan range and step selected; VWD settings should select the scan range and step as well but additionally add ‘Take Scan’ to the timetable option as a line in the method; FLD settings should toggle on either ‘Multi Excitation’ or ‘Multi Emission’ and then enable ‘Acquire Spectra: All’ and select the scan range and step.

VISION RUN will then generate a corresponding VISION DESIGN data file with each experiment run, that will include a Spectrum View node for those data. Please refer to the section, [3D Spectral View](#), for additional information on displaying and viewing this data in VISION DESIGN.

Creating and Running a Sequence

Methods are executed via a sequence table to start experiments and acquire data. You can create a new sequence, open a saved sequence, or view the currently loaded sequence table by navigation to the menu bar and selecting “Sequence” as shown in Figure 64.

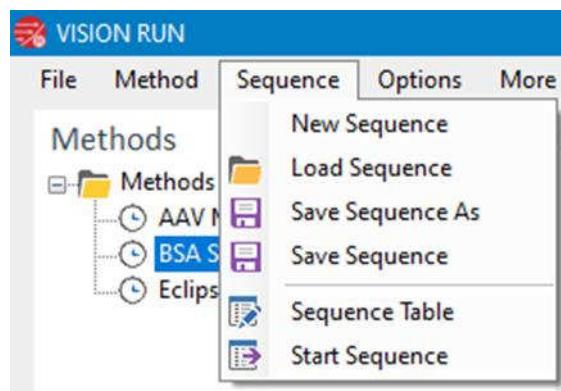


Figure 64. Options for New Sequence, Load Sequence, Save Sequence As, Save Sequence, Sequence Table, and Start Sequence available under the Sequence menu option.

The sequence table, shown in Figure 65, has options for defining the signals that are collected, selecting data output and method folder locations, and editing sequence rows.

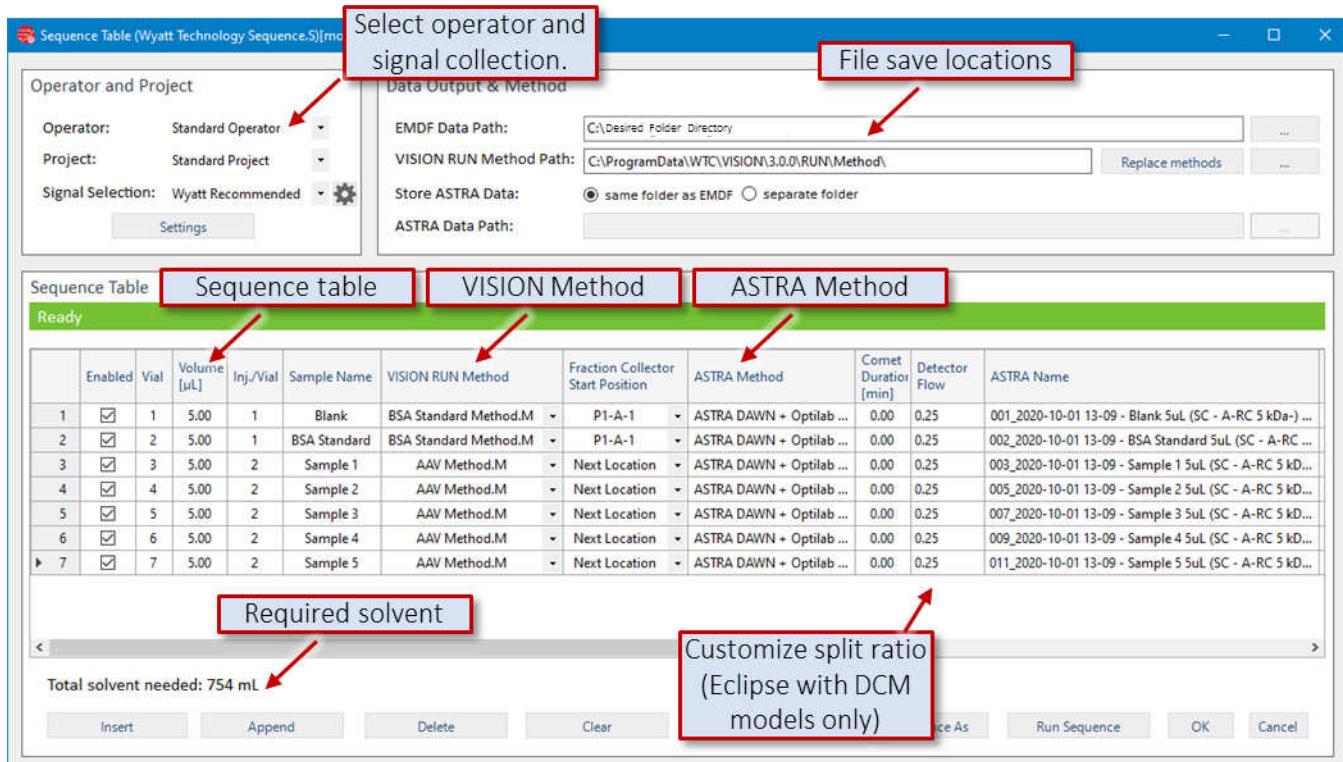
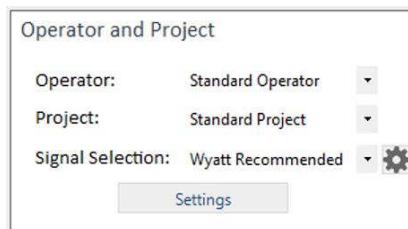


Figure 65. An example of VISION RUN's sequence table

In the following sections, the options for the sequence table will be explained in greater detail.

Operator & Project

This section of the sequence table window allows you to select the operator and the project as well as the signal profile. Access the signal selection window by using the gear button (). Under the "Settings" option, the ASTRA file name, sequence counter options, ASTRA timeout setting, and both Operator and Project list can be edited. Both will be explored below.



Signal Selection Window

The gear button () will open the Signal Selection window as shown in Figure 66.

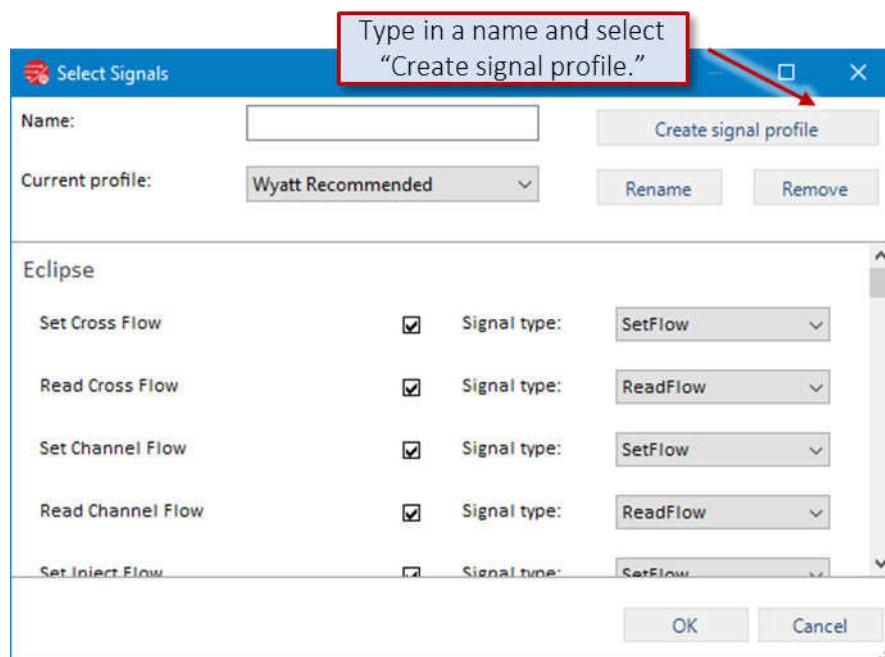


Figure 66. The Signal Selection window. To create a profile, type a name in the Name field and then select “Create signal profile.” To edit a profile, select from the “Current profile” dropdown. The signals for both HPLC modules and the Eclipse will be displayed.

Before performing method refinement and analyzing your experiments with VISION DESIGN, it is necessary to create a signal selection profile in VISION RUN. The selection of the different signals can be saved in different profiles. For every measured sequence an Eclipse method data file (file extension is *.emdf) will be created with these signal selection profiles. To create a profile, type the name in the Name field and then select “Create signal profile.” To edit a profile, select from the “Current profile” dropdown. The signals for both HPLC modules and the Eclipse will be displayed. For troubleshooting purposes, it is recommended to collect at least all of the Eclipse flows and pressures. Collecting the digital UV, RID, or FLD signals is valuable for viewing those respective signals in VISION DESIGN, and can be used for method refinement.

NOTE: For troubleshooting, please collect as many signals as possible and send the EMDF to Wyatt Technology Support.

Sequence Settings Window

Selecting the “Settings” button opens the Sequence Settings window where the ASTRA file name can be customized, the sequence counter can be reset, and the operators and projects defined. This window is shown in Figure 67.

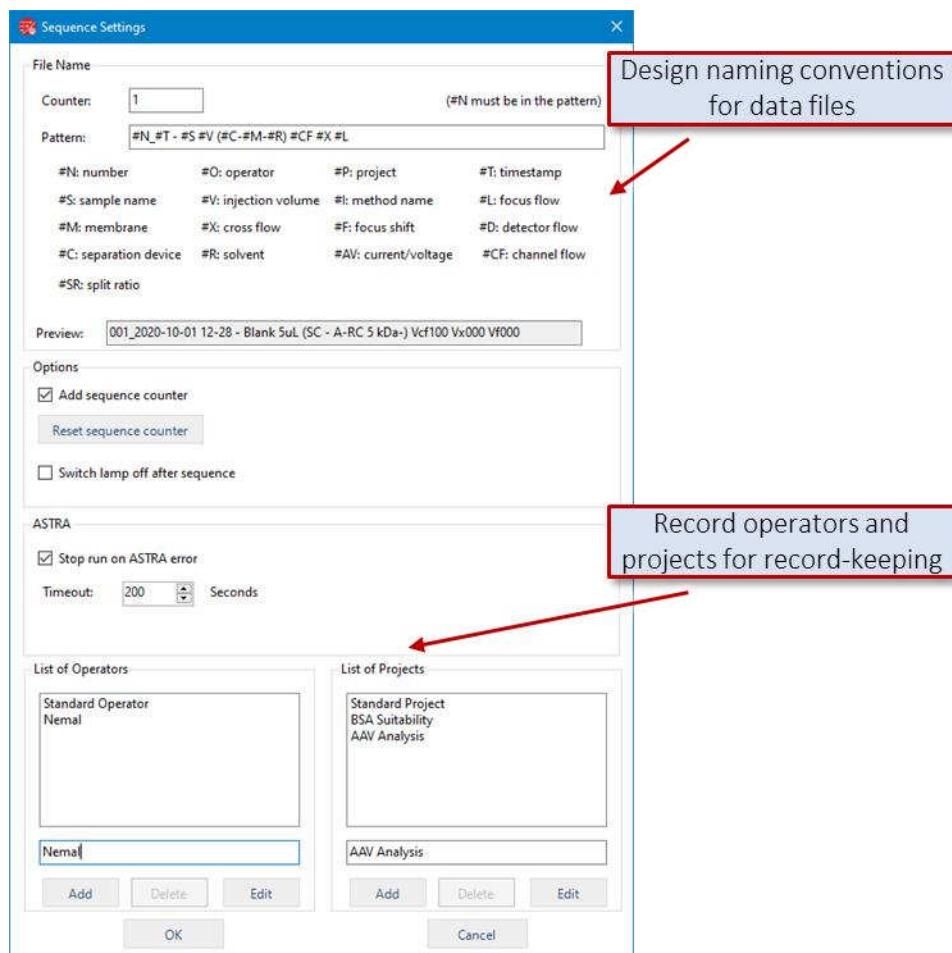


Figure 67. The Sequence Settings window enables customization of the ASTRA file name, the ability to set the sequence counter, and defined the operators and projects. The UV lamp can also be configured to switch off under this window.

The ASTRA file name can be customized by using the various pound sign or hash labels to generate a string of information referred to as a pattern. The #N for number is required but the rest of the string can be customized. The #N refers to the counter, which can be set to a value and increases by an increment of one with each injection. The counter can be reset under “Options” and the UV lamp can be turned off after the sequence. The counter can be customized as needed for convenience. An ASTRA counter of 100 can be set so that ASTRA files are in the 100 format with the VISION DESIGN files starting with 1.

VISION RUN controls ASTRA through an SDK in the background. By selecting the check box, the sequence will stop if an error in ASTRA occurs. The HPLC lamps (UV, FLD) can also be configured to turn off at the end of the sequence from this window.

At the bottom you can configure the lists of operators and projects by adding, editing or deleting entries. These can be used to organize your VISION DESIGN data files and for record-keeping.

Data Output & Method

In this section, the storage folders for the resulting experimental data files can be specified and the method path can be edited. The EMDF (*.emdf) for VISION DESIGN can have a save location specified and the ASTRA data files can either be stored in the same folder or in a different location. If the VISION DESIGN and ASTRA save locations are selected separately, but have the same save location, they will generate files in that folder without subfolders.

If the VISION RUN method path is changed and methods are already specified in the sequence table, it is necessary to select the [Replace Methods](#) button in order to reset the chosen methods and pull them from a different folder.

Sequence Table

The sequence table, shown in Figure 65, displays the current instrument status of the HPLC and Eclipse and the rows. The sequence table view displays the instrument status and the sample rows. Each row can be enabled or disabled, the vial position can be selected, the number of injections per vial (each creating an experiment) can be specified, the sample name can be defined, the VISION RUN and ASTRA method can be selected, and other experiment parameters can be defined.

Sequence rows can be added or deleted, and the options at the bottom of the window include:

- [Insert](#): Creates a new row above the selected row.
- [Append](#): Adds a new row at the end of the sequence.
- [Delete](#): Removes the currently highlighted row.
- [Clear](#): Resets the sequence table and removes all entries.
- [Save Sequence](#): Saves the sequence after it has been modified. The window title will display “[modified]” affixed to the end of the sequence name when unsaved changes are present.
- [Save Sequence As](#): Opens dialog window in order to save a new sequence file based on this table. Saves the sequence after it has been modified.
- [Run Sequence](#): This will run the currently defined sequence table.
- [OK](#): Confirms the changes and closes the sequence table window.
- [Cancel](#): Discards all changes and closes the sequence table window.

You can right-click on the row to have additional options for replacing all cells, or replace upward or downward as shown in Figure 68.

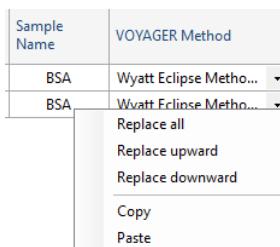


Figure 68. Right-clicking on a cell in the VISION RUN sequence table will enable the ability to replace the all other cells with the currently selected cell or just rows above or below the selected well.

Within a sequence row, several options are available to prepare your experiments and method. The following columns can be defined in the sequence table:

- [Enabled](#): Checkbox to select or disable a row in a sequence. As long as the last line of a sequence has not been run, rows can be added to a sequence during a run so it can be recommended to include a disabled row at the end of a sequence table.
- [Vial](#): The vial position can be specified. Refer to your Agilent HPLC manual to find vial position labeling instructions. This could be Vial 1, P1-A1, or some other labeling scheme.

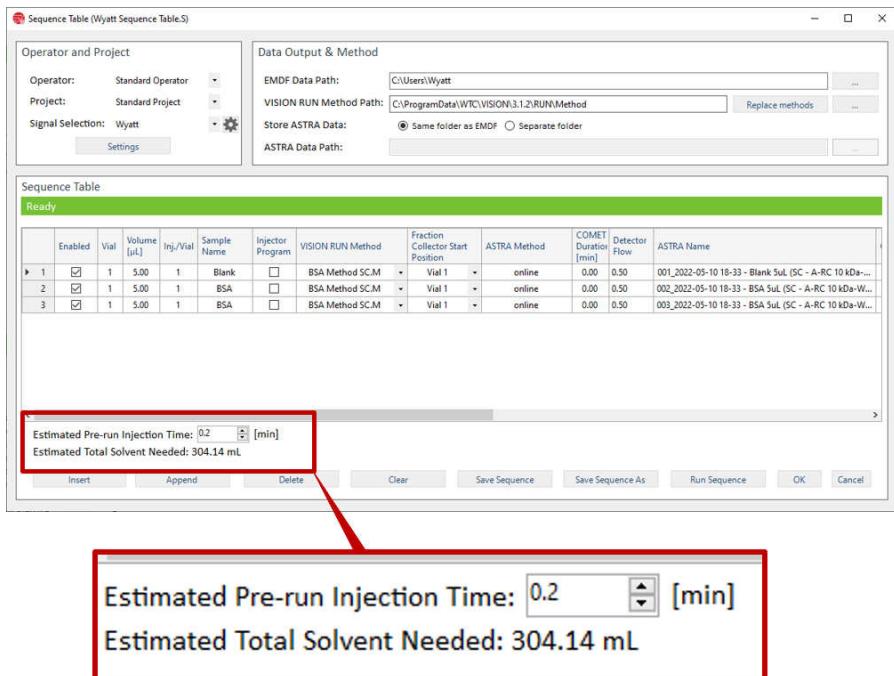
- **Volume (μL):** The injection volume from that vial. The upper limit is determined by the sample loop that is installed on your sampler. The analytical head, metering device, and multi-draw settings can be configured for the sampler in the Instrument Configuration prior to launching VISION RUN.
- **Inj./Vial:** The number of injections per vial. This will define how many times a particular sequence row will be run as replicates.
- **Sample Name:** Determines the sample identifier that is used in the ASTRA experiment file name and the VISION DESIGN data file name.
- **Injector Program:** The Agilent sampler, multisampler, or autosampler can be configured to run a program. Only one injector program can be used at a time and multiple programs cannot be saved, but the injector program can be enabled to run prior to an injection by enabling this option in the sequence table.
- **VISION RUN Method:** Select the desired method from the specified VISION RUN Method Path from the Data Output & Method section. Select a new folder location and “Replace Methods” to pull methods from a new folder.
- **ASTRA Method:** Select the desired ASTRA method from the ASTRA system folder. VISION RUN will automatically modify the ASTRA collection time to match the VISION RUN method. However, any COMET duration included in the ASTRA method basic collection will need to be accounted for in the VISION RUN sequence.
- **COMET Duration (min):** Should be specified to match the current COMET duration in the ASTRA method. VISION RUN will wait this additional time after an experiment to allow ASTRA to execute a COMET cleaning command. To avoid conflicts due to forgetting this time value, it is recommended to not perform COMET as part of the **Basic Collection** settings in your ASTRA method. Run the COMET separately after the sequence if possible.
- **Detector Flow:** The channel flow is defined by the method, but different split ratios can be achieved by modifying the detector flow. These flow rates are applied automatically to the method. VISION will automatically apply the correct flow rate to ASTRA when it generates ASTRA data files. Since alignment and band broadening are based on inter-detector volume and not time, there is not a need to adjust the alignment parameters though it can be worth checking at different split ratios.
- **ASTRA Name:** Determined by the “Settings” definitions under Operator and Project.
- **Fraction Collector Start Position:** Only displayed if a fraction collector is configured. Whether the fraction collector is enabled or disabled, a valid fraction collector vial position will need to be specified.
- **Sample Parameters:** Sample parameters such as concentration (mg/mL), dn/dc (mL/g), UV extinction coefficient ($\text{mL}/(\text{mg}\cdot\text{cm})$), and A2 ($\text{mol}\cdot\text{mL}/\text{g}^2$) can be specified and will be added to the ASTRA sample configuration.

At the bottom of the sequence table, there is an “Estimated Total Solvent Needed” field that reports the estimated solvent that is required to complete the sequence. For each method, VISION RUN calculates the estimated solvent consumed based on the method timetable. At the sequence-level, VISION RUN calculates the estimated solvent needed based on the sequence table methods.

While this can be a good estimate, this strategy does not factor in the time the HPLC is in pre-injection mode. For example, the time needed to draw the sample, or time needed to execute injector program functions. During these times, the solvent consumed will be determined by the flow rates specified in the first row of the Eclipse method and the time needed to complete pre-injection.

A more accurate solvent consumption estimated can be provided by including a time for the “Estimated Pre-run Injection Time” which does not need to be precise, but should provide a rough estimate how long pre-injection may be based on injection volumes and draw speeds, and other HPLC functions like injector program. Based on the time input here, the estimated total solvent needed will be adjusted.

Ultimately, this ensures a minimal sufficient quantity of solvent may be prepared in order for the sequence to run to completion. After observing the solvent requirements, ensure enough solvent is present and the current solvent quantity is defined in the Eclipse Intelligent Solvent Management System.



Using Excel to Build VISION RUN Sequence Tables

To assist with building long sequence tables, the sequence table can be highlighted, then copied (Ctrl + C) and pasted into Microsoft Excel or another spreadsheet to transfer all the columns. In Excel, sequence tables can be customized and then copied and pasted (Ctrl + V) back into VISION RUN to streamline sequence table generation. This process is illustrated in Figure 69.

It is important to copy the sequence table from VISION RUN first, in order to ensure the correct columns and number of columns are copied over.

1 Sequence Table Ready **Highlight and press Ctrl + C to copy the sequence table columns**

1	Enabled	Vial	Volume [µL]	Inj./Vial	Sample Name	VISION RUN Method	Fraction Collector Start Position	ASTRA Method	Comet Duration [min]	Detector Flow	ASTRA Name
► 1	<input checked="" type="checkbox"/>		5.00	1		AAV Method.M	P1-A-1	ASTRA DAWN + ...	0.00	0.50	001_2020-10-01 13-17 - 5uL (SC - A-RC 5 kDa-) Vcf10...

2 Build a sequence table as you'd like in Excel (Headings need to match VISION RUN sequence table)

A	B	C	D	E	F	G	H	I	J	K	
1	Enabled	Vial	Volume	Inj/Vial	Sample	VISION RUN Method	Fraction	ASTRA Method	COM	Detect	ASTRA Name
2	TRUE	2	5	1		AAV Method.M	Next	ASTRA DAWN + Optilab Method	0	0.5	001_2020-10-01 13-17 - 5uL (SC - A-RC 5 kDa-) Vcf10...
3	TRUE	2	5	2	Sample 2	AAV Method.M	Next	ASTRA DAWN + Optilab Method	0	0.5	
4	TRUE	3	5	2	Sample 3	AAV Method.M	Next	ASTRA DAWN + Optilab Method	0	0.5	
5	TRUE	4	5	2	Sample 4		Next	ASTRA DAWN + Optilab Method	0	0.5	
6	TRUE	5	5	2	Sample 5		Next	ASTRA DAWN + Optilab Method	0	0.5	
7	TRUE	6	5	2	Sample 6		Next	ASTRA DAWN + Optilab Method	0	0.5	
8	TRUE	7	5	2	Sample 7		Next		0	0.5	
9	TRUE	8	5	2	Sample 8	AAV Method.M	Next		0	0.5	
10	TRUE	9	5	2	Sample 9	AAV Method.M	Next		0	0.5	

3 Highlight sequence table (e.g. select corner)

4 Press Ctrl + V to paste the Excel spreadsheet information into the sequence table

5 Fill in other missing fields if not included in Excel
ASTRA Names will auto-populate based on naming convention (settings).

1	Enabled	Vial	Volume [µL]	Inj./Vial	Sample Name	VISION RUN Method	Fraction Collector Start Position	ASTRA Method	Comet Duration [min]	Detector Flow	ASTRA Name
► 1	<input checked="" type="checkbox"/>	2	5.00	1		AAV Method.M	▼ Next Location	ASTRA DAWN + Optilab ...	0.00	0.50	001_2020-10-01 13-30 - 5uL (SC - A-RC 5 kDa-) Vcf10...
2	<input checked="" type="checkbox"/>	2	5.00	2	Sample 2	AAV Method.M	▼ Next Location	ASTRA DAWN + Optilab ...	0.00	0.50	002_2020-10-01 13-30 - Sample 2 5uL (SC - A-RC 5 kDa...)
3	<input checked="" type="checkbox"/>	3	5.00	2	Sample 3	AAV Method.M	▼ Next Location	ASTRA DAWN + Optilab ...	0.00	0.50	004_2020-10-01 13-30 - Sample 3 5uL (SC - A-RC 5 kDa...)
4	<input checked="" type="checkbox"/>	4	5.00	2	Sample 4		▼ Next Location	ASTRA DAWN + Optilab ...	0.00	0.00	006_2020-10-01 13-30 - Sample 4 5uL (#C-#M-#R) #C...
5	<input checked="" type="checkbox"/>	5	5.00	2	Sample 5		▼ Next Location	ASTRA DAWN + Optilab ...	0.00	0.00	008_2020-10-01 13-30 - Sample 5 5uL (#C-#M-#R) #C...
6	<input checked="" type="checkbox"/>	6	5.00	2	Sample 6		▼ Next Location		0.00	0.00	010_2020-10-01 13-30 - Sample 6 5uL (#C-#M-#R) #C...
7	<input checked="" type="checkbox"/>	7	5.00	2	Sample 7		▼ Next Location		0.00	0.00	012_2020-10-01 13-30 - Sample 7 5uL (#C-#M-#R) #C...
8	<input checked="" type="checkbox"/>	8	5.00	2	Sample 8	AAV Method.M	▼ Next Location		0.00	0.50	014_2020-10-01 13-30 - Sample 8 5uL (SC - A-RC 5 kD...
9	<input checked="" type="checkbox"/>	9	5.00	2	Sample 9	AAV Method.M	▼ Next Location		0.00	0.50	016_2020-10-01 13-30 - Sample 9 5uL (SC - A-RC 5 kD...

Figure 69. The columns for a VISION RUN sequence table can be copied over to a spreadsheet, like Excel, and built using convenient spreadsheet tools and then re-imported into VISION RUN.

Saving and Starting a Sequence

After all sequence parameters have been defined and confirmed, the sequence can be saved by selecting [Save Sequence](#) in the Sequence Table window. Save the sequence as a new sequence by selecting [Sequence As](#). A Windows file explorer window will open and the sequence can be saved under a desired name with a *.S file extension.

You can run a sequence by either selecting the [Run Sequence](#) button in the sequence table or by selecting [Sequence](#) from the top menu bar and selecting [Start Sequence](#). When a sequence is started, the device will prepare for the run and the instrument status may change to “ASTRA Prerun” and then “Prerun” once communication with ASTRA

has been established. An “Injecting” status indicator will display during injection and then the system status should change to “Instrument Run.” A vertical green line will appear on the online plot when an experiment has started.

During a run, the sequence table options for operator/project and data output & method will be grayed out and cannot be changed; however, the sequence table can still be edited. An example of the sequence table during a run is shown in Figure 70.

Sequence Table						
Run (20% done)		1	1/1	SC BSA QC 25.M	⌚ 00:25 h 02:05 h	
	Enabled	Vial	Volume [µL]	Inj./Vial	Sample Name	VOYAGER Method
1	<input checked="" type="checkbox"/>	1	0.00	1	Blank	SC BSA QC 25.M
► 2	<input checked="" type="checkbox"/>	1	15.00	1	BSA 2 g L-1	SC BSA QC 25.M
3	<input checked="" type="checkbox"/>	1	2.50	3	BSA 2 g L-1	SC BSA QC 25.M

Figure 70. The sequence table when running experiments. The status bar shows the sequence time elapsed and total time, as both a percentage on the left and in hours on the right, with the vial position, injection number, and currently loaded method shown in the middle. The current sequence row is indicated in blue and completed sequence rows are highlighted with gray.

During a sequence run, the sequence table can be edited. Cells that can be edited include the injections/vial (Inj./Vial), and the Enabled, Vial, Volume, Sample Name, Methods, and other sample constants when the row has yet to be run. Lines can also be inserted, appended, and deleted as long as the final row has not started. Select the “Save Sequence” button to save all changes and update the parameters of the sequence status bar.

Aborting a Sequence

A sequence can be stopped or manually aborted by selecting the [Abort Sequence](#) button in the sequence table or by selecting [Abort](#) from the menu bar. The sequence will be automatically aborted when an error occurs, such as a detected leak or an overpressure.

VISION RUN Eclipse Methods and Protocols

In this section, some method guidelines will be explored for various Eclipse procedures. These methods can be applied and run via sequences as described in the preceding sections, [Creating a Method](#) and [Creating and Running a Sequence](#). These include running a conditioning run to bind active sites on the channel membrane, automating the solvent flush which can be used for solvent exchange or cleaning, and will cover some general guidelines for building methods for your samples.

Method for Automating Solvent Exchanges

The solvent exchange protocols described in the section, [Using Manual Control to Perform a Solvent Exchange with VISION](#), can be partially automated by creating a VISION RUN method. The flushing method can also be run as part of a routine to flush the system when the mobile phase is refreshed or to ensure stagnant flow is not present in the SEC port (with SEC switching option). The key principles of the flushing method are:

- Flush all four modes (Elution, Elution + Inject, Focus, Focus + Inject) with at least 10 mL volume each.
- Cycle through modes to clear residual solvent in Rheodynes.
- Relatively low flow rates to minimize risk of overpressures from solvent mixing.
- Combine with injections of mobile phase to help flush needle and sample loop (large volumes matching the sample loop size (i.e., 100 – 900 µL) across multiple vials can help)
- Replace the channel with a 5-port union to minimize total volume requirements
- Ensure the correct solvent reservoir (determined by pump module method) is selected.

One thing to note is that the degasser and pump have a significant volume that will need to be exchanged. As a result, the method can be run multiple times in a sequence or for longer durations.

Solvent Exchange/Flush Method

This method can be performed with an injection (volume of the sample loop) to flush the autosampler. It will consume about 130 mL of solvent. This can be used to solvent exchange the entire system; however, if just the Eclipse needs to be solvent exchange, the steps can be shortened such that 70 – 100 mL of solvent is used.

NOTE: If you are using organic solvents, it is recommended to install the channel after rinsing with water, flush to water, then exchange solvents to your organic mobile phase. Pure water can be directly exchanged into THF, whereas intermediate pure alcohol can be used if switching to toluene.

You may elect to do a slow solvent exchange overnight or a more rapid solvent exchange depending on your needs. When there is a significant viscosity difference between solvents, you can use lower flow rates for longer periods of time when automatically exchanging solvents. Below is an example of a general-use solvent exchange, provided in Figure 71.

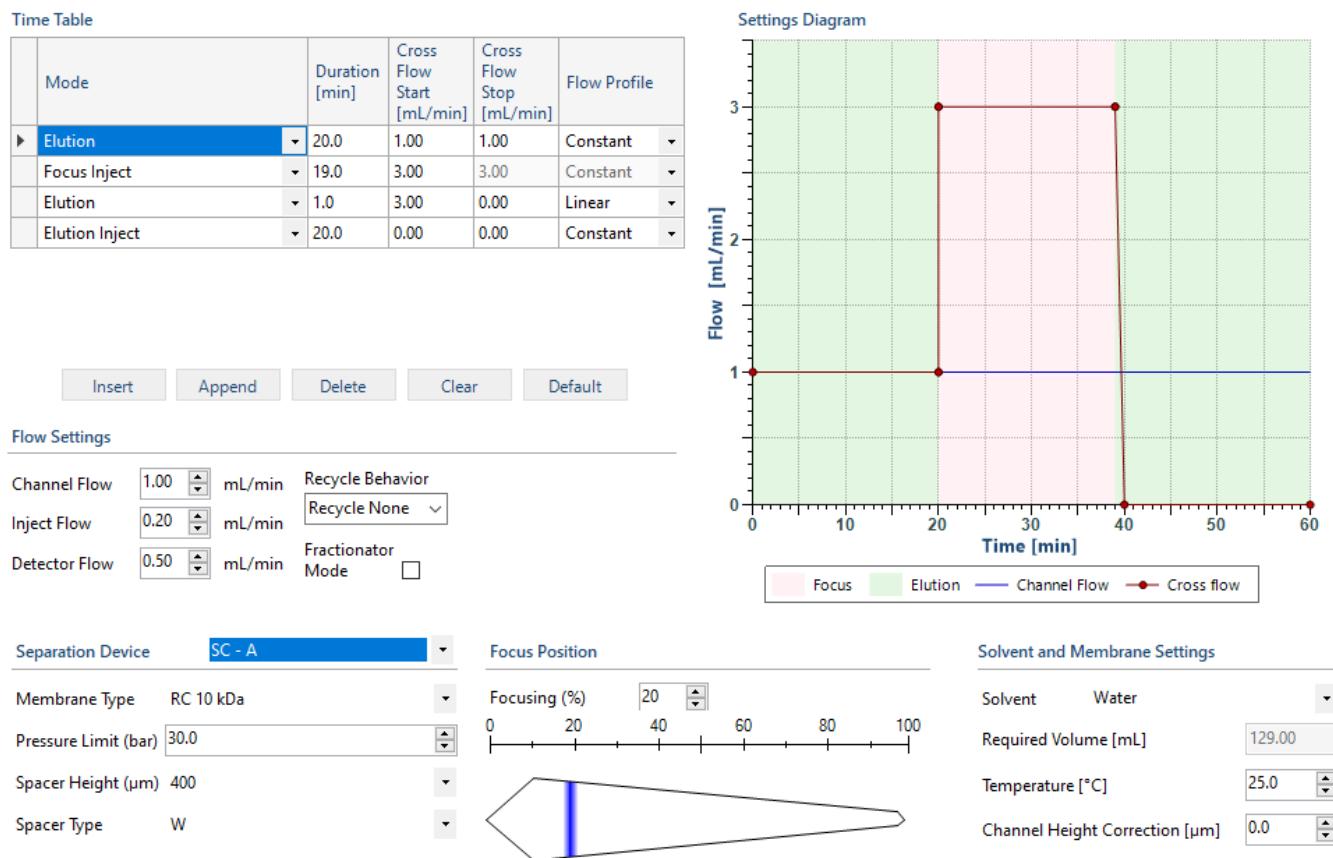


Figure 71. Solvent exchange method for VISION RUN

For Eclipse with SEC option, this will only flush the Eclipse system and Port A. For flushing Port B, the column port, the column can be disconnected and replaced with a short bridge of PEEK tubing and the following SEC flush can be run, provided in Figure 72. This method assumes the Eclipse has already been solvent exchanged and only the SEC port pathways need flushing.

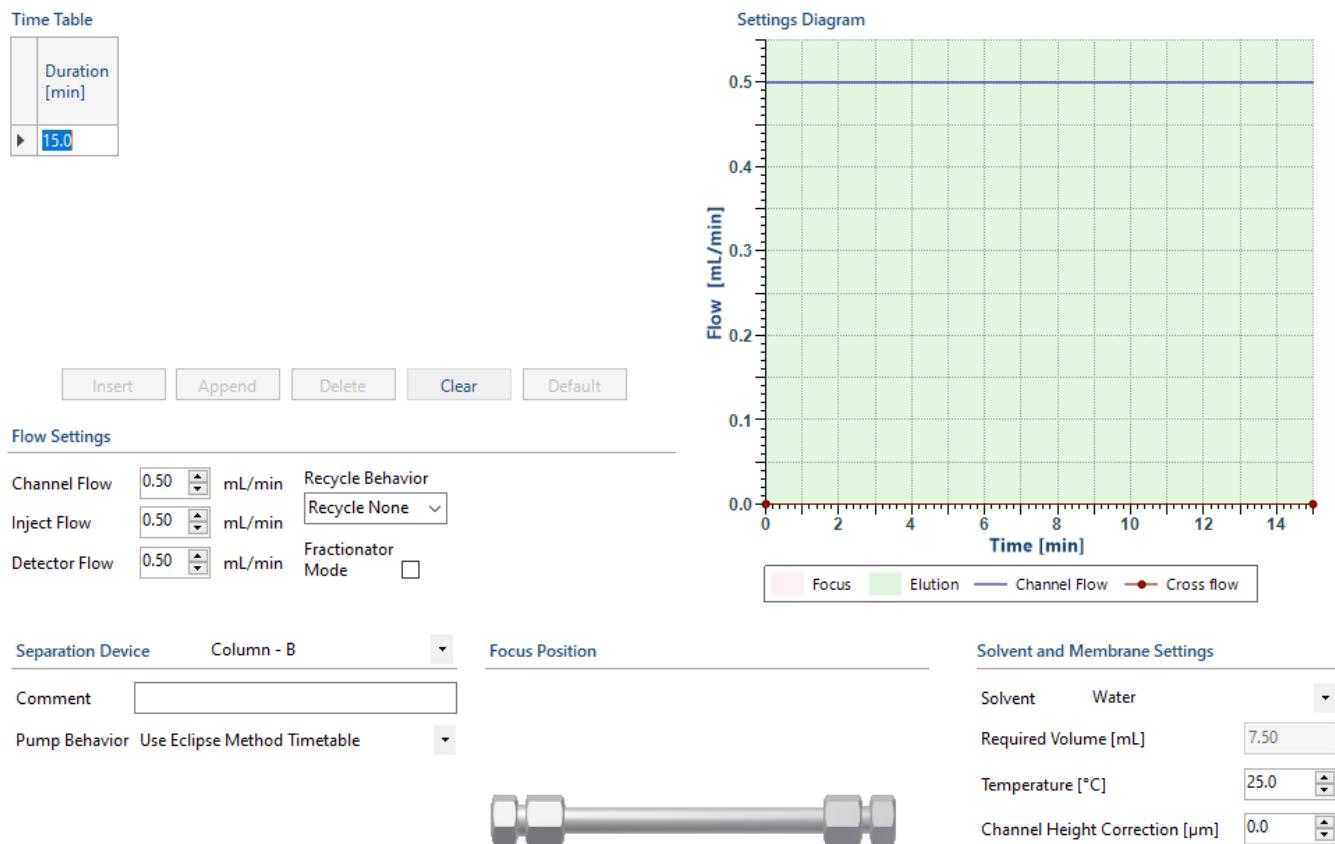


Figure 72. SEC mode solvent exchange after the initial Eclipse solvent exchange of FFF mode is complete.

Once the Eclipse, Port A, and Port B are flushed, it may be desired to flush the Recycle and Valves. The following method in Figure 73 can be run next, with the Recycle Behavior set to Recycle, to complete the solvent exchange.

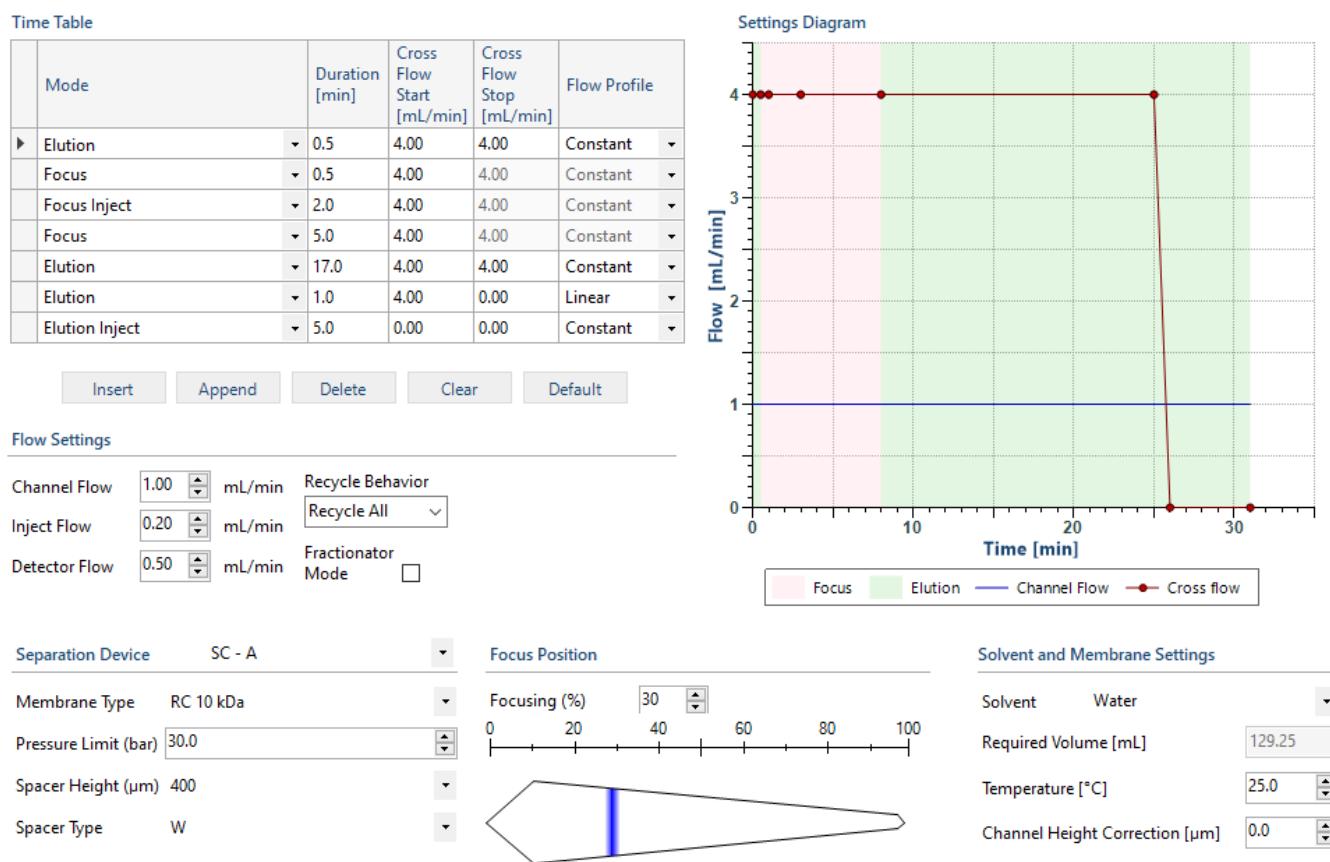


Figure 73. Recycle and valve flush after the Eclipse solvent flushes are complete.

During the main solvent exchange described above, it is recommended to not recycle the solvent to avoid introducing contaminants that are flushed from the system back into the solvent reservoir. Recycle mode can be used for the last method. It is also recommended to purge the RI detector.

An alternative solvent exchange will cycle the valves repeatedly to both help clean out the existing solvent and can be run repeatedly overnight at low flow rates with injections to also clean the autosampler. Although more detailed cleaning instructions are provided in [Eclipse Cleaning Guide](#) section, this example method provided in Figure 74 can be used for solvent exchange or cleaning.

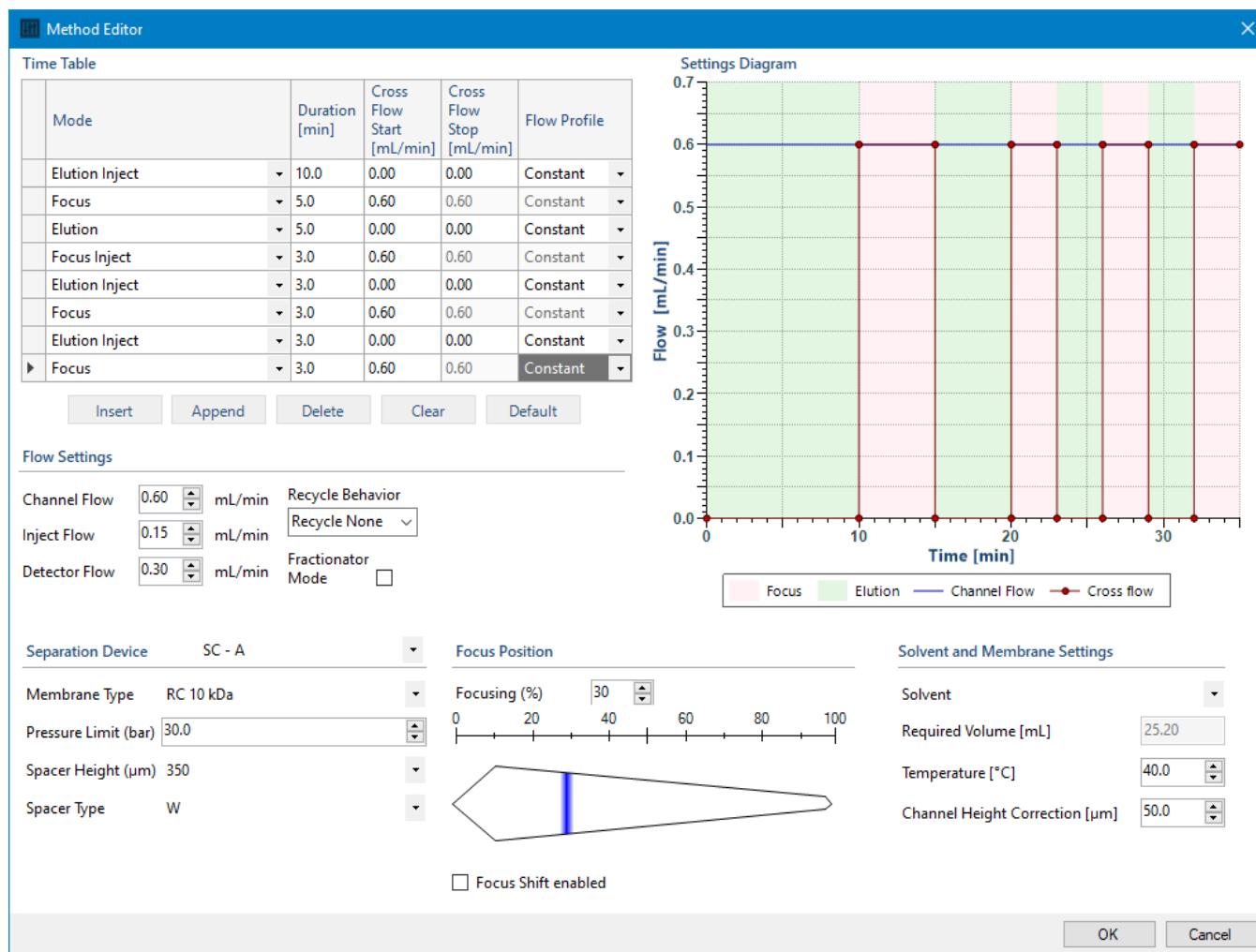


Figure 74. Slow overnight solvent exchange. This method can be run multiple times (at least 4 – 5 times) with injections to help flush the entire system and clean as well.

Methods for Membrane Conditioning

This step is performed after installation of a new membrane, as described in the section, [Eclipse Channel Overview and Membrane Installation](#). The goal of a membrane conditioning step is to ensure the sample itself (if you are not sample-limited) or a sample of similar chemical composition is overloaded onto the channel in order to bind any active sites on the new membrane surface. This defined covering layer can help improve mass recovery and peak shape. In the upcoming sections, example conditioning methods with BSA or PSL will be discussed.

Example Conditioning Methods with BSA or PSL

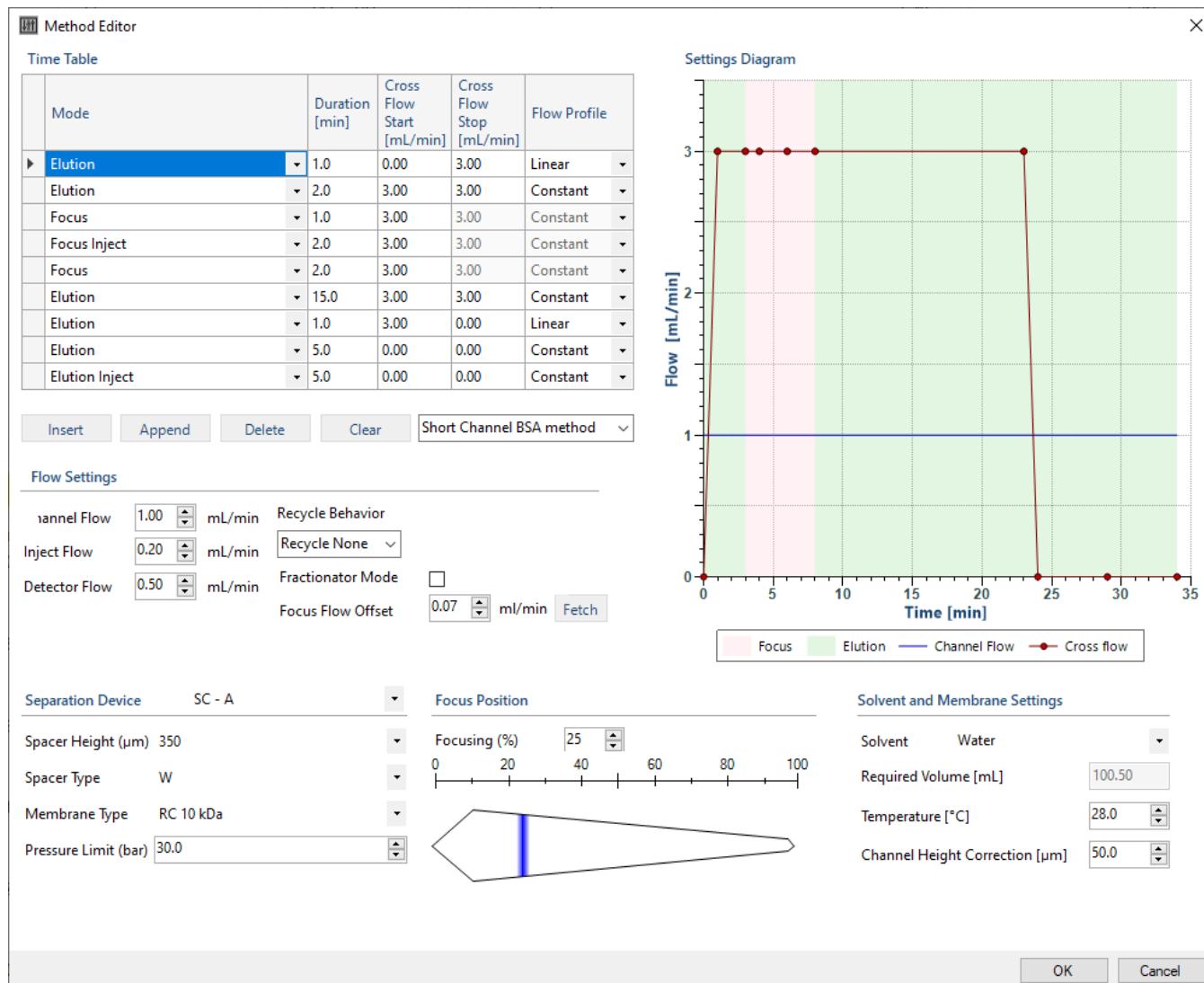
If proteins will be studied, it is recommended to condition the membrane with BSA. A 2 mg/mL solution of BSA with a 50 µL injection (the goal is to overload the channel with 60 – 120 µg of total sample loading) using a mobile phase of PBS or 50 – 100 mM NaNO₃ is an appropriate quantity. This loading applies to both short and long channel dimensions, whereas typical analytical loadings are 10 – 40 µg. Short channel and long channel methods are similar; however, the long channel due to its larger surface area requires higher cross flow to achieve the same fluid flux through the membrane.

NOTE: The examples provided in this section are a starting point and can be adjusted as needed. The broad flexibility of FFF-MALS enables multiple strategies to be successful. Some typical modifications include the following:

- The initial elution step can be extended by a few minutes to improve baseline signals if the baseline will be drawn from that initial step; however, the baseline can also be drawn in the main elution step after the switch from focus.
 - A detector flow rate can be increased. FFF-MALS separations take advantage of the ratio of cross flow to channel flow.
-

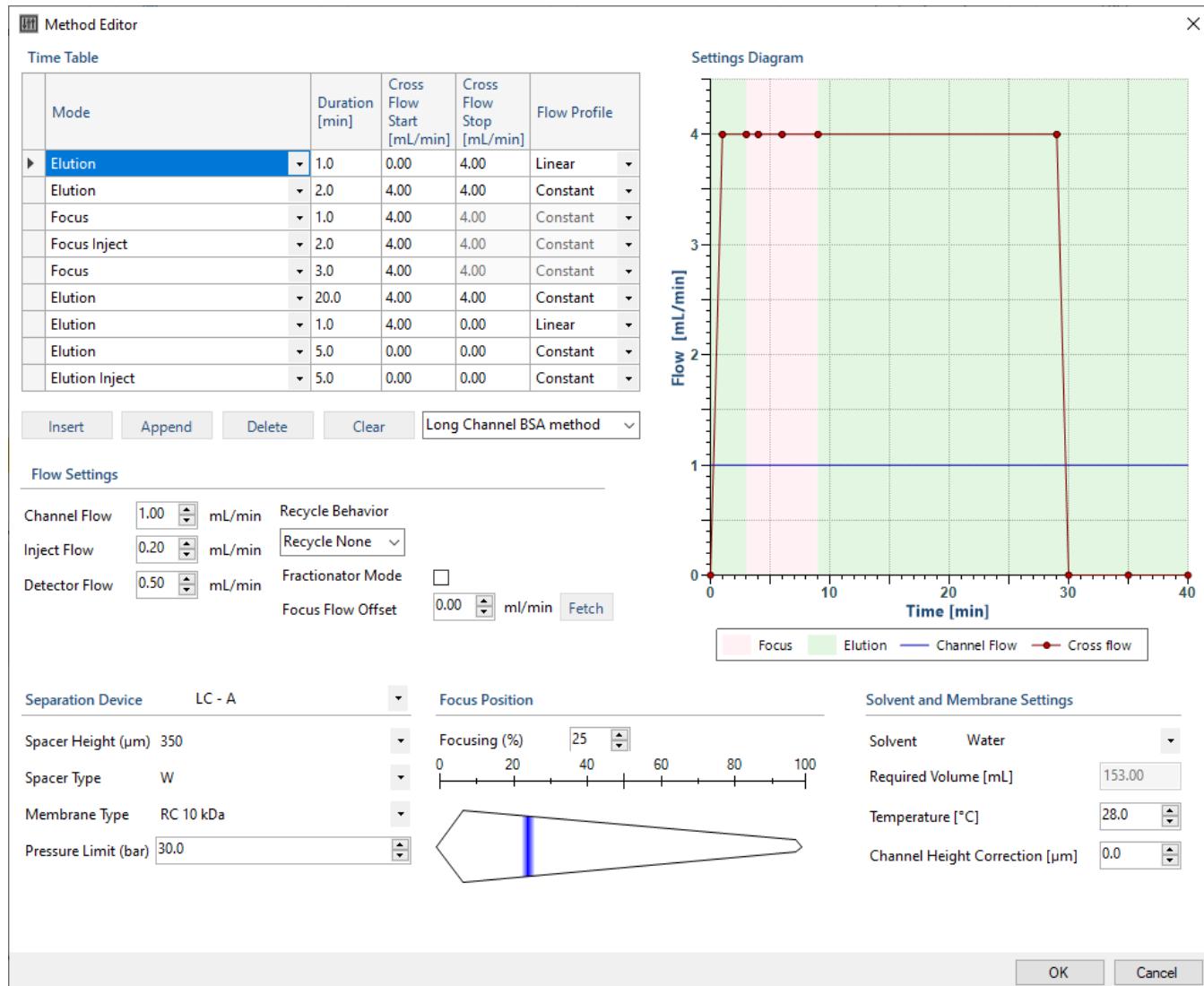
BSA Short Channel Method

An example short channel method (with 350 or 400 µm spacer) is provided below. When performing the experiment with the DCM option, you may consider a split ratio of 1:1. Without DCM, you may consider a detector flow of 0.5 – 1.0 mL/min. For conditioning, a 60 – 120 µg BSA loading can be injected; however typical analytical loadings are 10 – 40 µg.



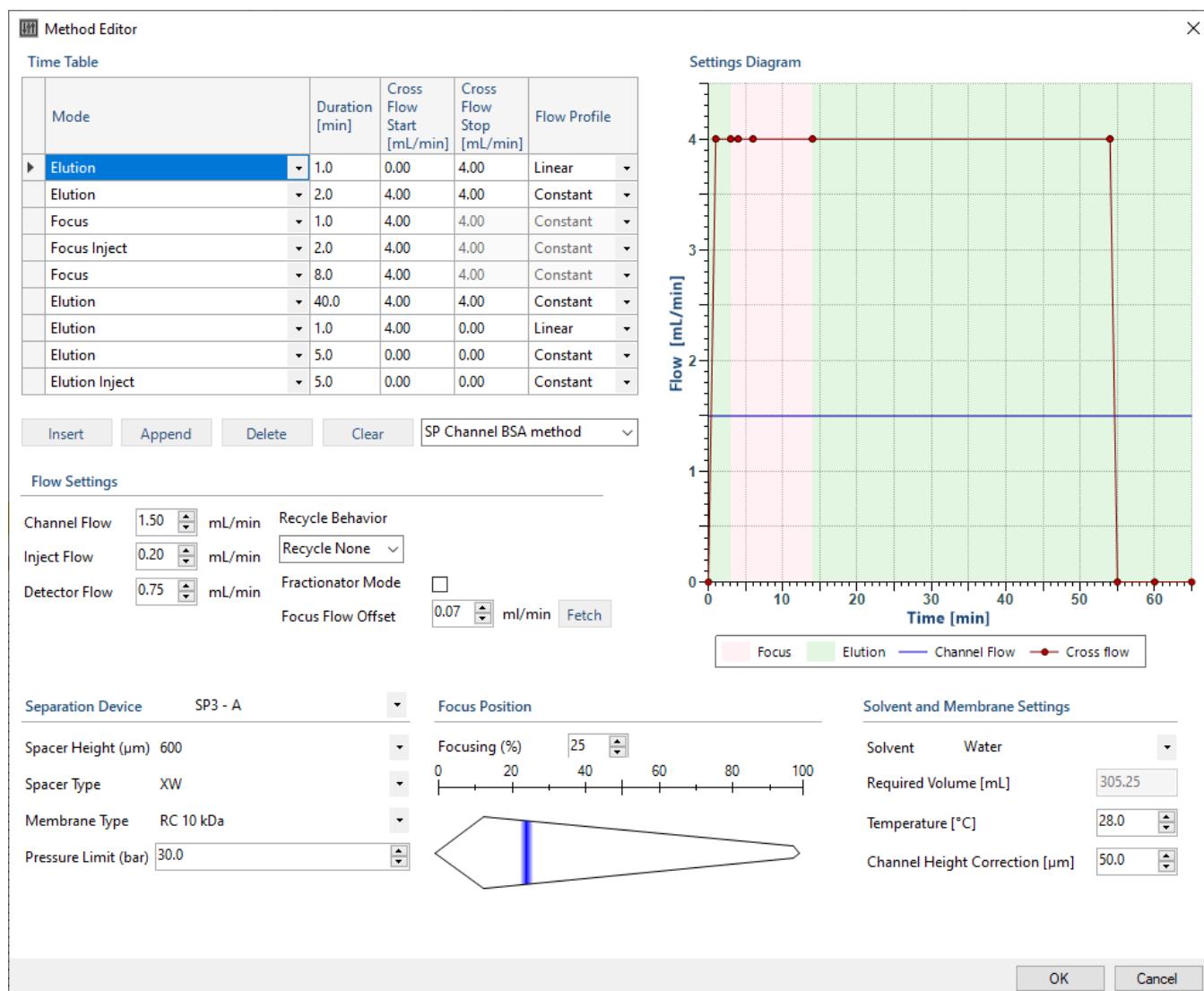
BSA Long Channel Method

An example long channel method (400 µm spacer) is provided below. When performing the experiment with the DCM option, you may consider a split ratio of 1:1. Without DCM, you may consider a detector flow of 0.5 – 1.0 mL/min. For conditioning, a 60 – 120 µg BSA loading can be injected; however typical analytical loadings are 10 – 40 µg.



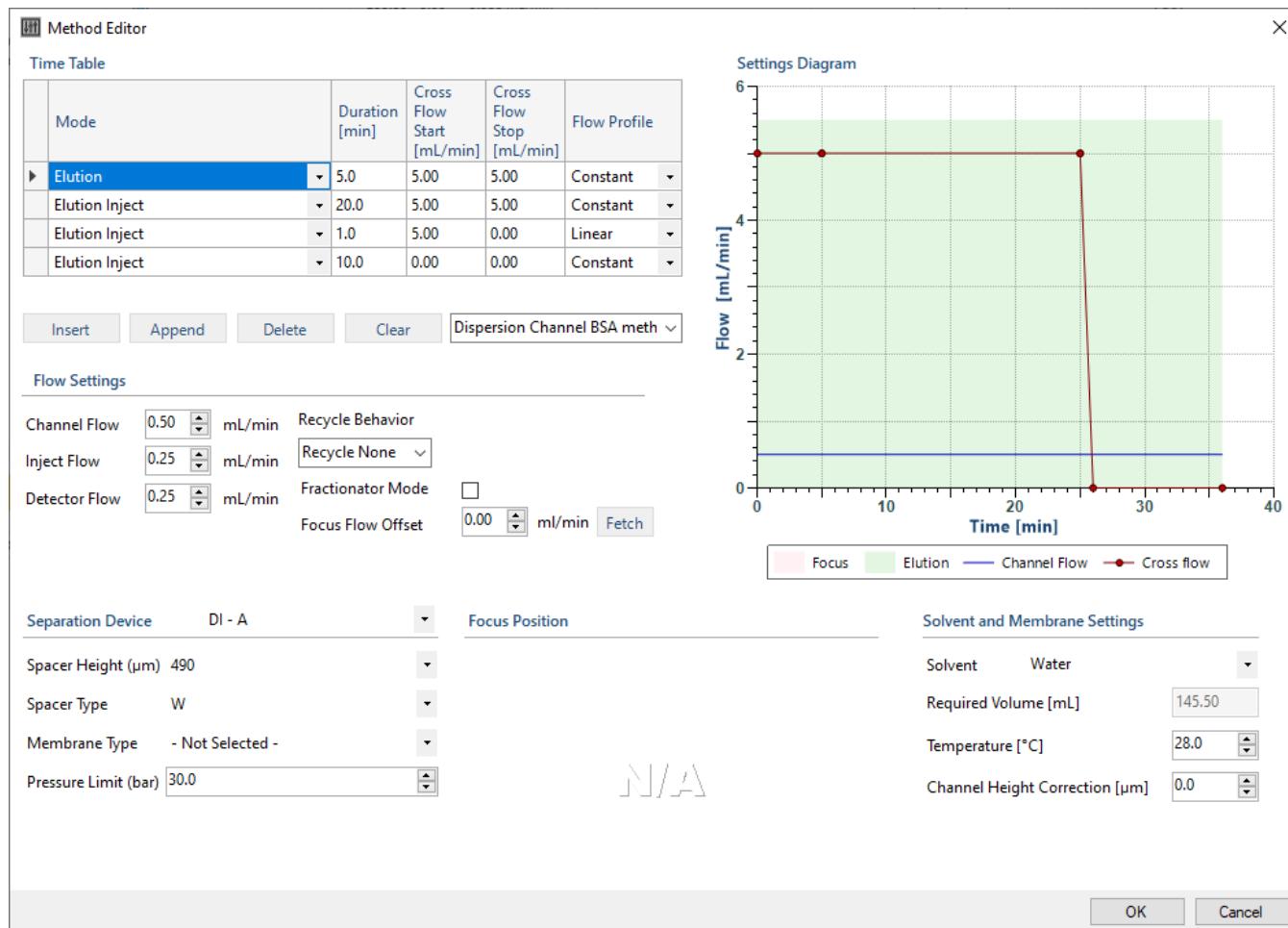
BSA Semi-Preparative Channel Method

An example semi-prep channel method (650 µm spacer) is provided below. When performing the experiment with the DCM option, you may consider a split ratio of 1:1. Without DCM, you may consider a detector flow of 0.5 – 1.0 mL/min. For conditioning, a 120 – 240 µg BSA loading can be injected; however typical SP channel loadings are 20 – 5000 µg.



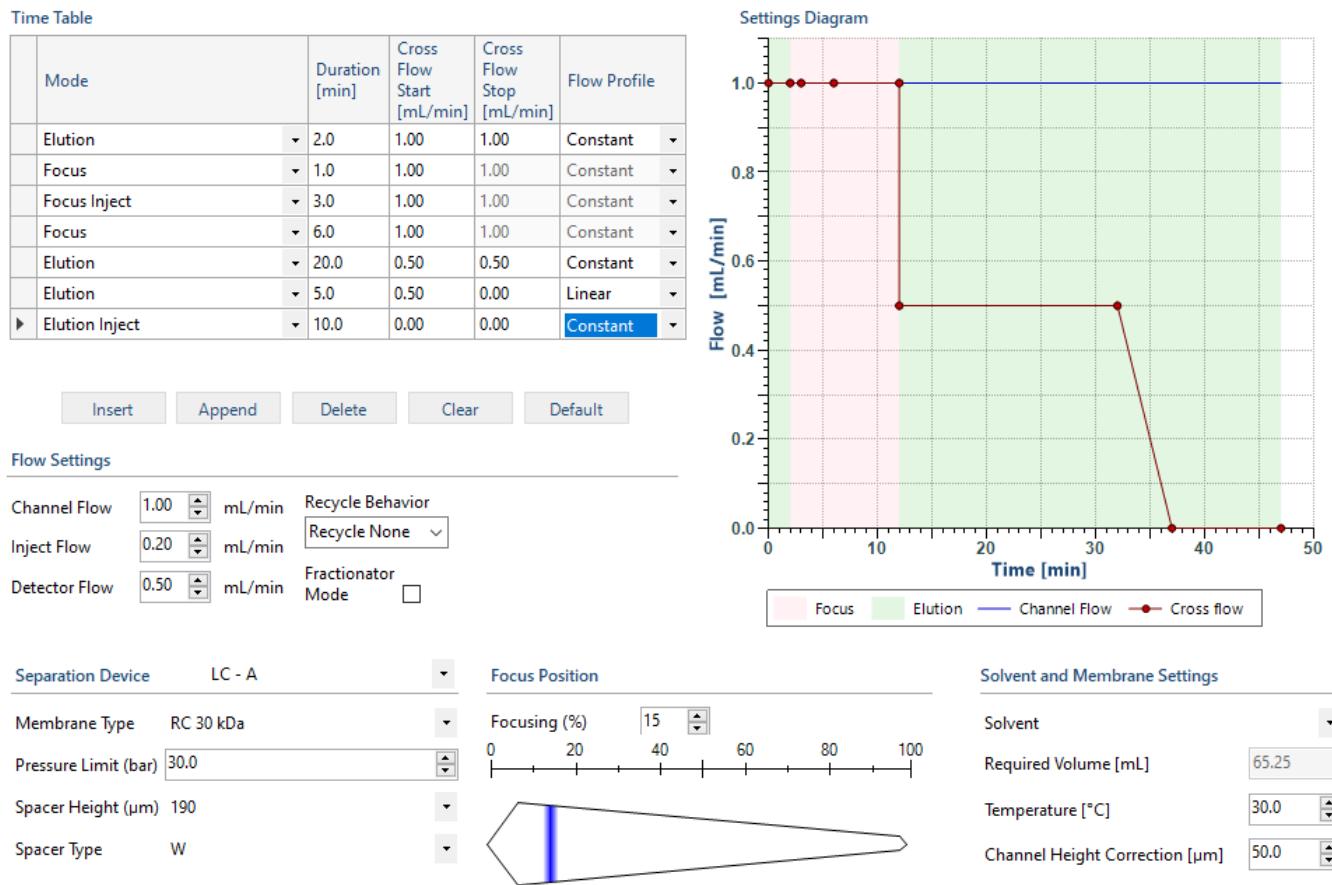
BSA Dispersion Inlet Channel Method

An example dispersion inlet channel method (650 µm spacer) is provided below. When performing the experiment with the DCM option, you may consider a split ratio of 1:1. Without DCM, you may consider a detector flow of 0.5 – 1.0 mL/min. For conditioning, a 60 – 120 µg BSA loading can be injected; however typical analytical loadings are 10 – 40 µg.



PSL Long Channel Method

An example of a long channel method with a 25 nm radius (50 nm diameter) Thermo Scientific™ 3050A Nanospheres™ PSL bead is provided below:



For RC (regenerated cellulose) membranes: We recommend 0.1% FL-70 as the mobile phase.

For PES (polyethersulfone) membranes: We recommend 400 ppm SDS with 50 mM salt (either PBS or NaCl); the salt can help shield the PSL from membrane interactions since PES has sulfone groups and slightly negative surface potential and if the PSL has a negative surface, might interact.

Conditioning Method Considerations

Other methods for other samples may vary. VISION DESIGN can be used to generate a starting method for your conditioning samples if you know the estimated size. In general, the ratio of cross flow to channel flow influences FFF-MALS separations. Keep this in mind when adjusting the DCM ratios or deciding on flow conditions. In the next section, some overall guidelines will be discussed for method creation.

Method Creation Guidelines

Methods generally consist of the following flows to achieve certain goals:

1. Initial Elution Step: Equilibrates pressures
2. Initial Focus Step: Stabilizes focus flow
3. Focus + Inject: Transports sample to the channel
4. Focus: Focuses sample further without disruption from inject flow
5. Elution: May consist of multiple Elution steps. This is the key separation method.
6. Elution with zero cross flow: This clears the membrane of any residual sample that was retained
7. Elution + Inject: Flushes the autosampler and system in preparation for next run

Initial Sample Screening

An initial screening method with a cross flow gradient can be used to determine at which point the sample emerges from the channel. Subsequent methods are refined around the observations. The amount and duration of cross flow depend on the size and type of the sample being evaluated. The following parameters are recommended:

- The 400 μm spacer and 10 kDa regenerated cellulose (RC) membrane are applicable for most protein and polymer applications. Refer to Table 5 for guidelines on selecting a membrane.
- Try to use an orthogonal method's mobile phase, such as the SEC mobile phase or formulation buffer. Low salts are generally recommended. For example, 50 mM phosphate buffer with very little if any sodium chloride (do not use chloride salts with Mobility).
- Use an initial channel flow of 1.0 mL/min. Start with a fast cross flow gradient, such as 3.0 – 0 mL/min over 10 – 20 minutes for smaller proteins or macromolecules. For particles or large macromolecules, you can screen with a lower starting cross flow, for example 1 or 0.5 mL/min – 0 mL/min.
- Larger macromolecules may benefit from lower focus flows as well.
- If coming from an orthogonal SEC method, typical loading amounts in FFF are usually less than those used in SEC, depending on the cross flow applied. You can inject different amounts to assess overloading. Higher loadings may be needed for aggregate screening, or higher DCM flows can be used to improve sensitivity.
- Screen your samples first before starting the optimization process.

Determine optimal flow conditions and injection amount based on the results from fast screening. An example screening method is provided below:

Time Table

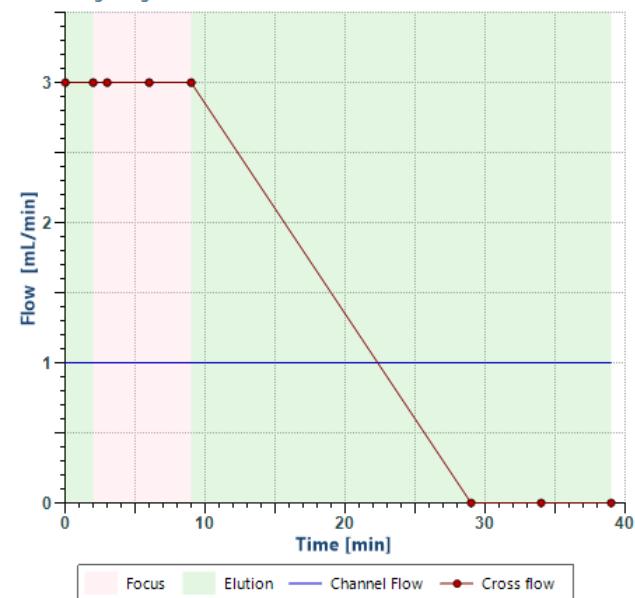
Mode	Duration [min]	Cross Flow Start [mL/min]	Cross Flow Stop [mL/min]	Flow Profile
Elution	2.0	3.00	3.00	Constant
Focus	1.0	3.00	3.00	Constant
Focus Inject	3.0	3.00	3.00	Constant
Focus	3.0	3.00	3.00	Constant
Elution	20.0	3.00	0.00	Linear
Elution	5.0	0.00	0.00	Constant
▶ Elution Inject	5.0	0.00	0.00	Constant

[Insert](#) [Append](#) [Delete](#) [Clear](#) [Default](#)

Flow Settings

Channel Flow	1.00 <input type="button" value="▲"/> <input type="button" value="▼"/> mL/min	Recycle Behavior
Inject Flow	0.20 <input type="button" value="▲"/> <input type="button" value="▼"/> mL/min	Recycle None <input type="button" value="▼"/>
Detector Flow	0.50 <input type="button" value="▲"/> <input type="button" value="▼"/> mL/min	Fractionator Mode <input type="checkbox"/>

Settings Diagram



Separation Device	SC - A	<input type="button" value="▼"/>
Spacer Height (µm)	350	<input type="button" value="▼"/>
Spacer Type	W	<input type="button" value="▼"/>
Membrane Type	RC 10 kDa	<input type="button" value="▼"/>
Pressure Limit (bar)	30.0	<input type="button" value="▼"/>

Focus Position	
Focusing (%)	20 <input type="button" value="▲"/> <input type="button" value="▼"/>
0 20 40 60 80 100	<input type="button" value="▼"/>

Solvent and Membrane Settings

Solvent	<input type="button" value="▼"/>
Required Volume [mL]	92.50 <input type="button" value="▼"/>
Temperature [°C]	30.0 <input type="button" value="▼"/>
Channel Height Correction [µm]	50.0 <input type="button" value="▼"/>

Fraction Collector Considerations

Please refer to TN6504 – Configuring Fraction Collectors with VISION 2. Although written with VISION 2 in mind, the resource contains much applicable information for VISION 3.

Configuration

When connected through CAN cables and with compatible firmware, the Agilent instruments should display on the right side of the configuration window after successfully using Auto Configure. Fraction collectors that were already installed on another system may need to be “cold started,” otherwise they may not appear after the Auto Configuration. Please contact Agilent or see their literature for information about cold starting a module.

Selecting a module and pressing “Configure” will allow you to further configure the modules, which include setting certain options. The fraction collector module may display information about linked modules and well plates as shown below in Figure 75.

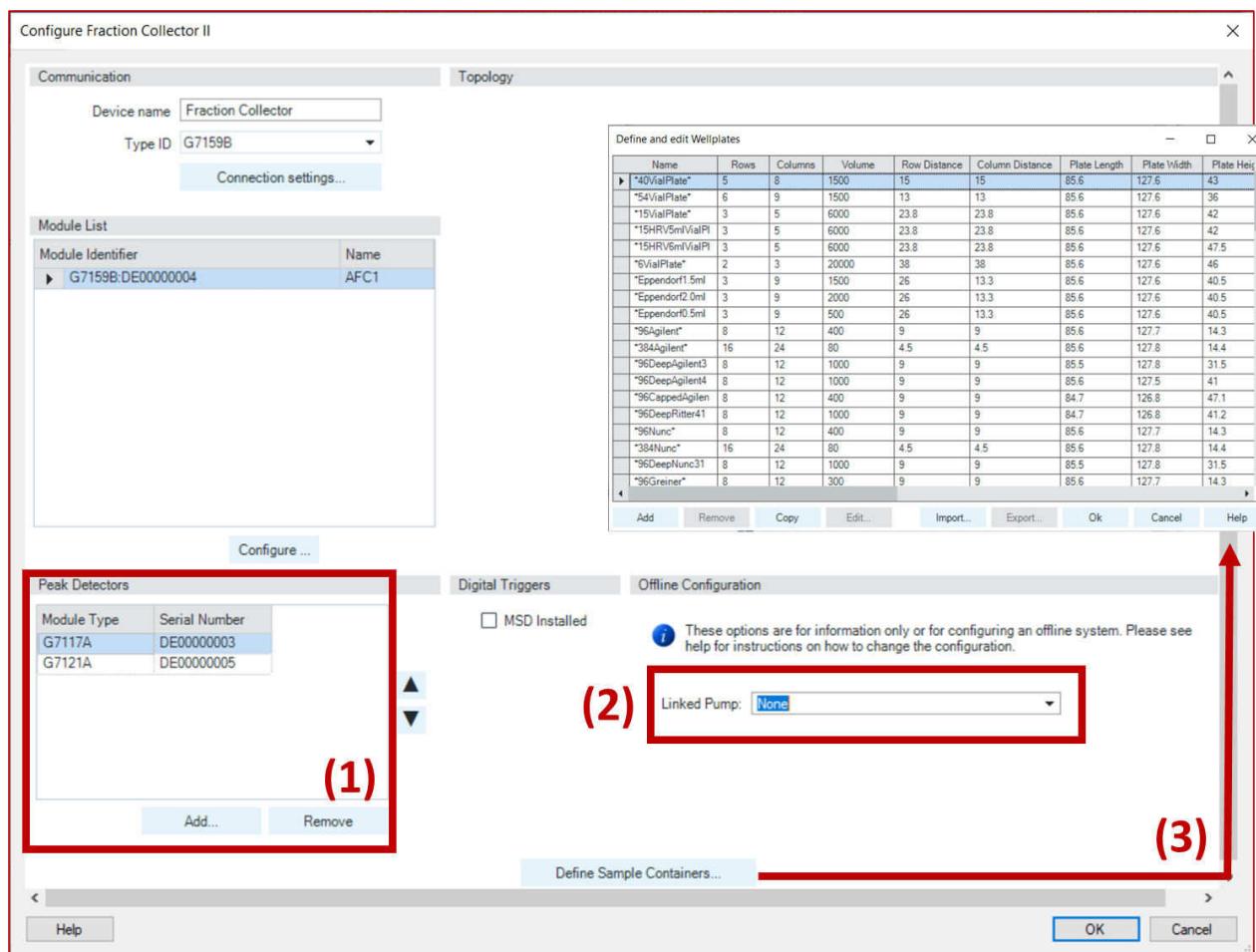


Figure 75. Example configuration window for a fraction collector module. Options may not be available in all models. (1) With a compatible UV or FLD detector linked, VISION RUN is compatible with peak-trigger fraction collections. Currently MALS or analog detector signals are not compatible with VISION RUN peak triggers; (2) Linked Pump option, which should be set to “None” if possible to allow a custom detector flow to be set. This will be further explained in the section entitled Error! Reference source not found. below. Lastly, (3) allows you to view the sample container formats for your vial tray or well plate and add custom formats.

In addition to the HPLC, the Eclipse should be added to the configuration. When launched, VISION RUN should display all the modules, including the Eclipse and fraction collector, as shown below:



Figure 76. Eclipse and Agilent HPLC Modules displayed in VISION RUN

Module Options

The user interface, graphics, and dashboard panel will be familiar to those who have used Agilent software as the capabilities and functions are drawn from the Agilent instrument control framework (ICF). For the most detailed

information, please refer to the appropriate Agilent user manual for your fraction collector. Some functions that are important for use with the Wyatt Eclipse will be covered. The fraction collector module (Figure 77) provides information about the fraction collector needle position, the current fraction mode, the status, the temperature (if applicable), and the ability to start and stop manual fraction collections, which can only be toggled on and off during runs.

By right-clicking on the fraction collector module, options for configuring the module will be displayed. These include viewing the method, resetting the fraction volumes, rinsing the instrument, etc.

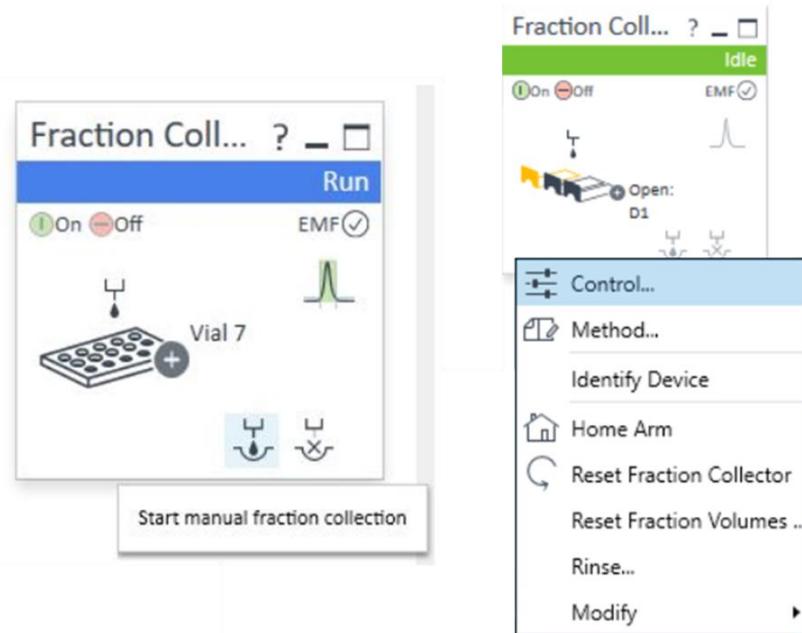


Figure 77. Fraction collector module in Run Mode (left) and in Idle Mode (right). This UI element will display information about the fraction collector and can be used to access settings. In Run Mode, fractions can be started and stopped manually by pressing the buttons in the lower right of the module (if available). Right-click on the fraction collector module to view the Method, reset fraction volumes, rinse, and other actions.

The options for creating a method, modifying parameters, etc. that are most relevant for running FFF experiments will be explained in the subsequent sections. The option to “Reset Fraction Volumes...” is useful for resetting the vial volumes when vials are emptied from the fraction collector. Otherwise, the system may skip vials that were emptied because it will assume that the vial is full.

Creating Fraction Collector Methods

In order to operate the fraction collector, a method needs to be created. The majority of the settings are controlled in the fraction collector method, which will be discussed shortly. If using an Eclipse with DCM option, there is a dedicated fraction collector on the side-panel of the Eclipse as shown in **Error! Reference source not found..**



Eclipse with DCM + SEC (WECDS)

Figure 78. Fraction collector port on side-panel of Eclipse with DCM option.

In order to enable flow to exit that port (instead of going to waste), the Eclipse has to have fractionator mode enabled in the Eclipse method. This can be toggled on in the Eclipse method editor window as shown in **Error! Reference source not found..**

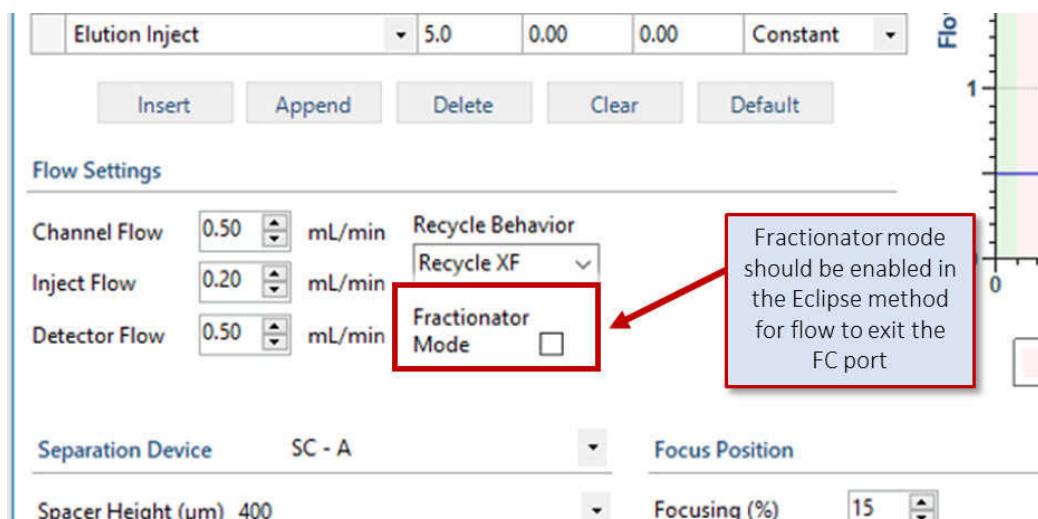


Figure 79. Fluid through the fraction collector port on the Eclipse with DCM option is enabled via the Eclipse method.

Once the Eclipse aspects of setting up the fraction collector are complete, the fraction collector method can be edited. Right-clicking on the fraction collector module when not running will allow access to the current method. Or you can navigate to **Method → Set up Method** to view the overall method, which would include tabs for all HPLC modules and the Wyatt Eclipse. In **Error! Reference source not found.** below, an example window of a fraction collector method is provided. At the basic level, the fraction collector itself can be enabled or disabled through the method for simplicity. While manual collections can be executed directly via the module UI as shown in Figure 77, configuring the system for peak-based trigger collection or timetable-based collections are always done through the method.

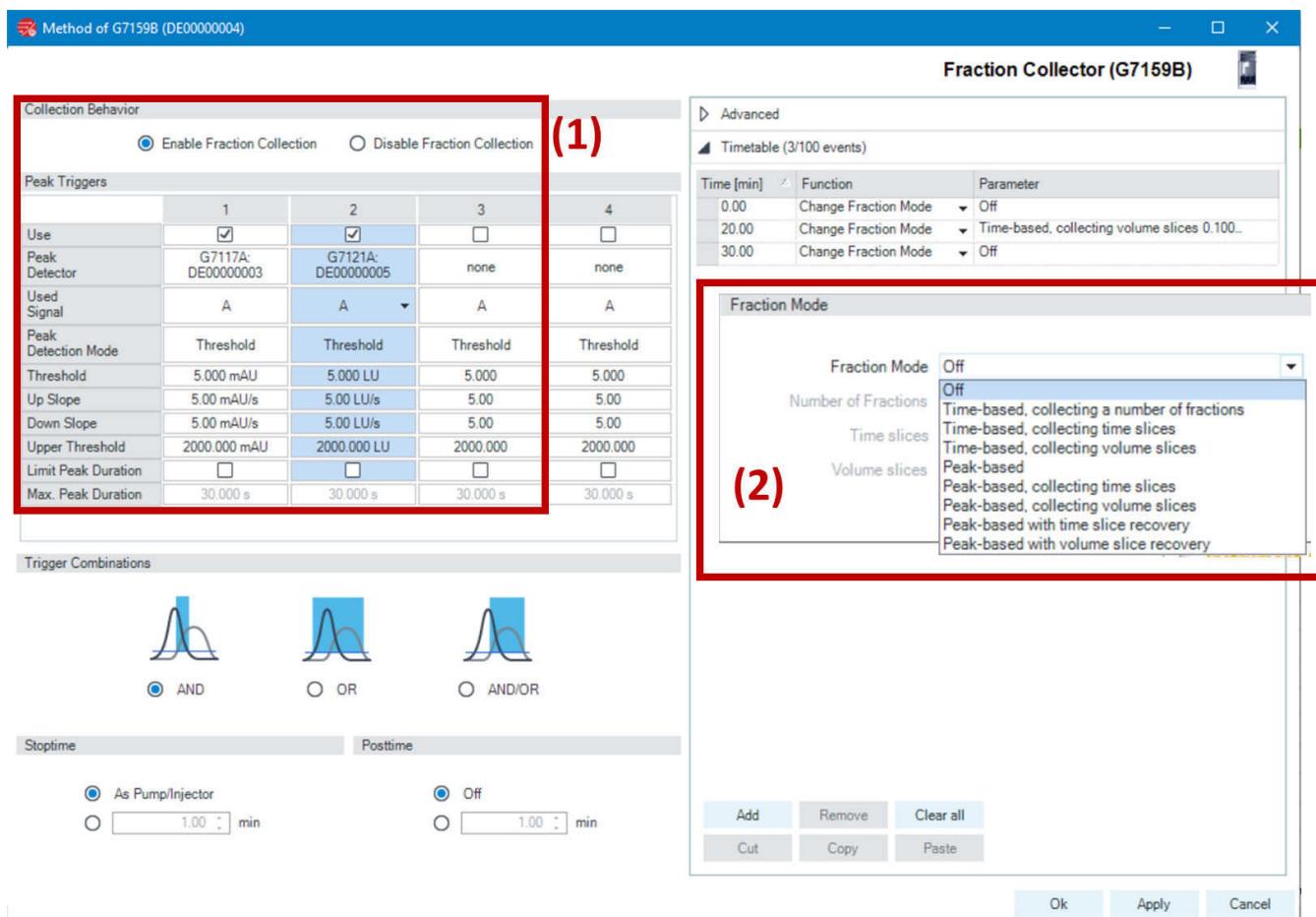


Figure 80. Fraction collector method in VISION RUN. The fraction collector can be enabled or disabled in the method at the upper left. (1) If compatible Agilent UV or FLD detectors are present, they can be configured under ‘Peak Triggers.’ Thresholds and slopes can be set for UV (mAU or mAU/s) and FLD (LU or LU/s). (2) Under the Timetable section of the right side of the window, the fraction collector timetable and mode can be set. Fractions can be collected either time-based or peak-based. For Wyatt Eclipse systems, it is currently recommended to use time-based collections when doing sequences because of limited information regarding collected fractions in VISION DESIGN.

Note: As of May 2021, information about peak trigger-based collections is not collected or shown in VISION DESIGN. When running sequences, it may not be clear what has been collected in what vials. For this reason, using timetable-based collections, where the precise number of vials collected can be more easily obtained from the method, is the recommended method configuration for fraction collection with VISION RUN. If peak-based triggers are required, it is recommended to run experiments individually to track the vials. If the fractogram preview is available, it can also be used to track the vials.

Additional options are provided under ‘Advanced’ which will be described here and expounded in the following sections. An example of the Advanced tab is shown below in [Error! Reference source not found..](#) The option ‘Delay Settings’ allows for the delay volumes calculated in LabAdvisor or stored on the UV/FLD modules to be configured. The fill volume allows for custom fill volumes to be specified—for example, collecting only 0.5 mL fractions in a larger 1.5 mL vial. Overfill behavior can also be defined under the Advanced tab. The last parameter, which is more important for FFF than other techniques, is a ‘3rd party Pump Flow’ which allows a custom flow rate to be set for the fraction collector to account for crossflow in FFF separations. This will be further explained below in [Error! Reference source not found..](#)

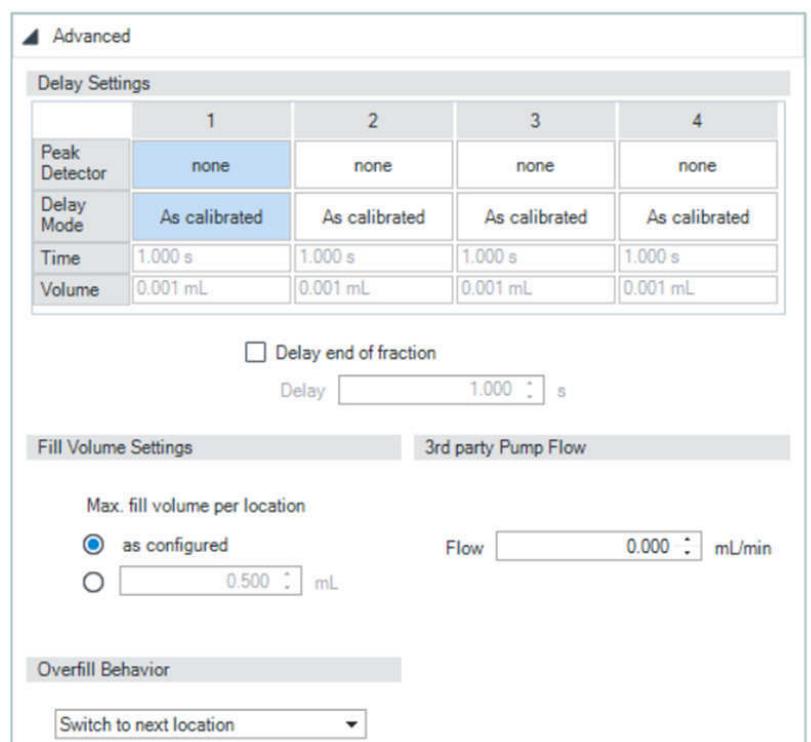


Figure 81. The advanced options under the fraction collector module. Important settings like how to enable custom flow rate to account for detector flow will be covered in the section, Error! Reference source not found..

In addition to the Advanced settings, the delay volume can be added per compatible Agilent detector by right-clicking the fraction collector module and navigating to [Modify](#) → [Detector Delay Volumes](#) as shown in [Error! Reference source not found..](#)

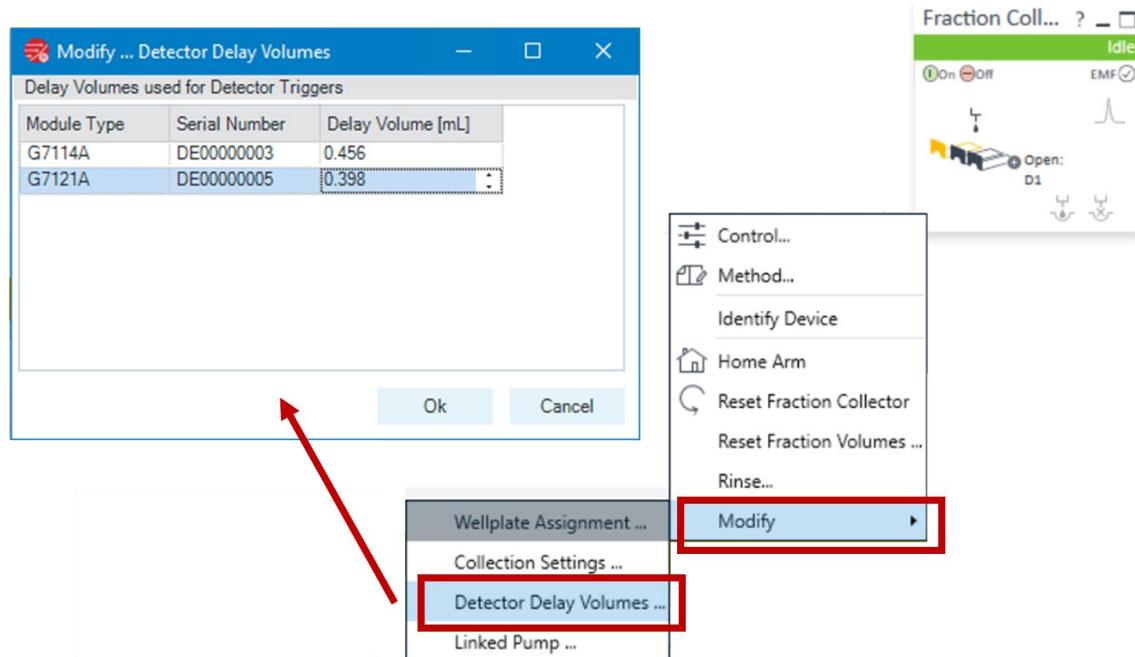


Figure 82. Option for adding delay volumes to the fraction collector module. This is only compatible currently with Agilent-supported UV and FLD hardware.

After the fraction collector settings are set, the method can be saved and executed in a sequence. For information about Eclipse method development please refer to [TN6005 - Eclipse Aqueous Method Development Quick Start Guide](#). The next section will cover important considerations when using a fraction collector with FFF separations before going into setting up sequences.

Considerations for Flow Rate and Vial Volumes

The Wyatt Eclipse is unique in that it utilizes a single pump flow rate and diverts the flow in different ways to achieve detector flow, crossflow, focus flow, and injection flow. Because the fraction collector does not measure flow rate, when a fraction collector is linked to a pump, it may incorrectly expect 3 – 4 mL/min of flow to enter the module; however, due to the nature of FFF separations, it may be that only 1 mL/min goes through the detectors as flow whereas the remainder of the flow goes through the crossflow pathway to waste. With default configurations, this can lead to underfilled vials.

There are two general strategies for accounting for vial volume. The first is de-linking the pump from the fraction collector. The second is adjusting the vial volume to account for the incorrect flow rates the fraction collector is operating with.

De-Linking the Pump and Fraction Collector Modules

This option is not available for all fraction collector and pump module combinations but is the easiest strategy for providing the fraction collector with a correct flow rate. There are a few ways to de-link the pump from the fraction collector. First, it can be de-linked by right-clicking on the fraction collector module in VISION RUN and navigating to [Modify → Linked Pump](#). In the new window, the Linked Pump can be set to “None” as shown in [Error! Reference source not found..](#)

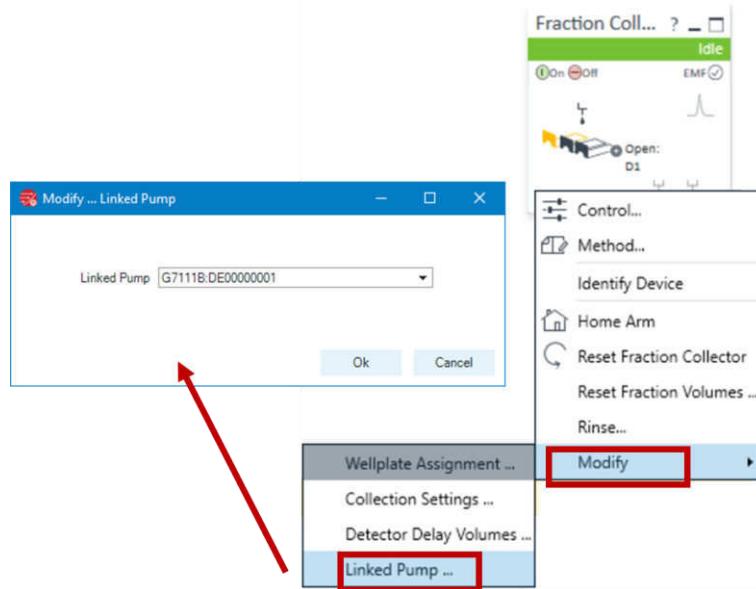


Figure 83. De-linking a pump through the fraction collector module in VISION RUN.

Alternatively, it can be de-linked from the configuration window ahead of launching VISION RUN underneath the fraction collector module as shown below in [Error! Reference source not found..](#)

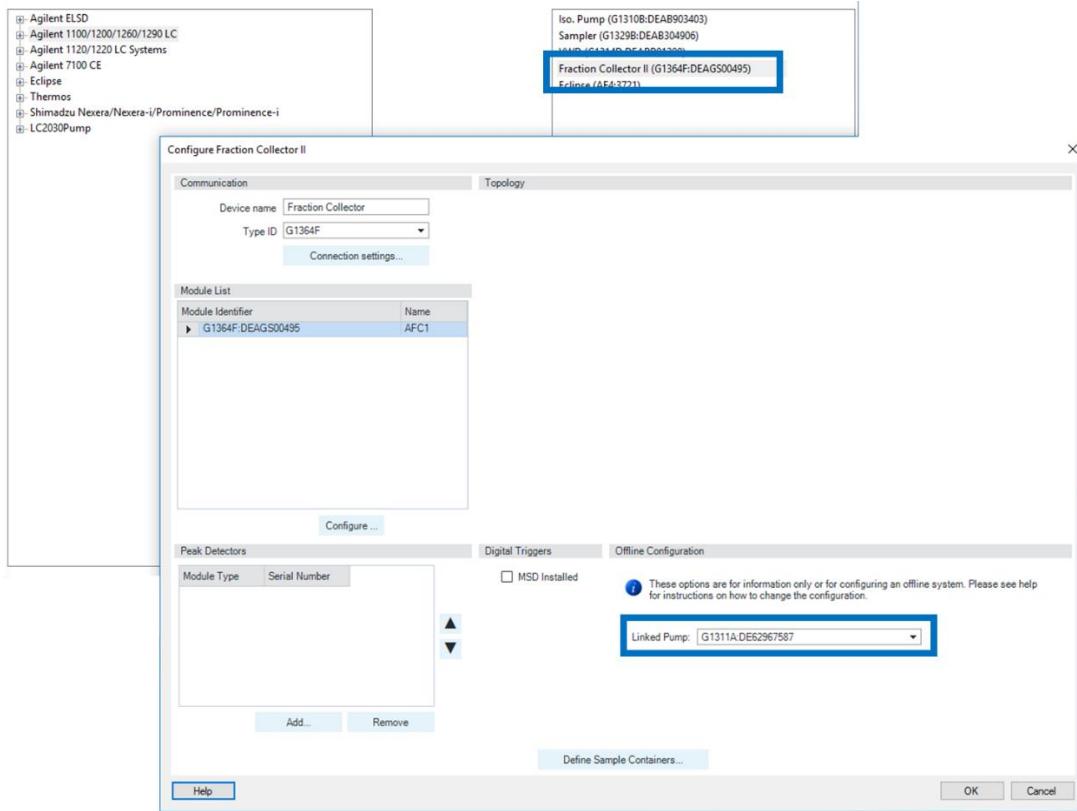


Figure 84. De-linking the pump through the configuration. Select the fraction collector module on the right side of the window and select “Configure” to open a new window. Here, you can enable an offline configuration by setting the Linked Pump to “None.”

In both cases, it may be necessary to also disable the Flow Limit Cluster by right-clicking on the fraction collector module and navigating to “Control.” In the subsequent window, you can select ‘None.’

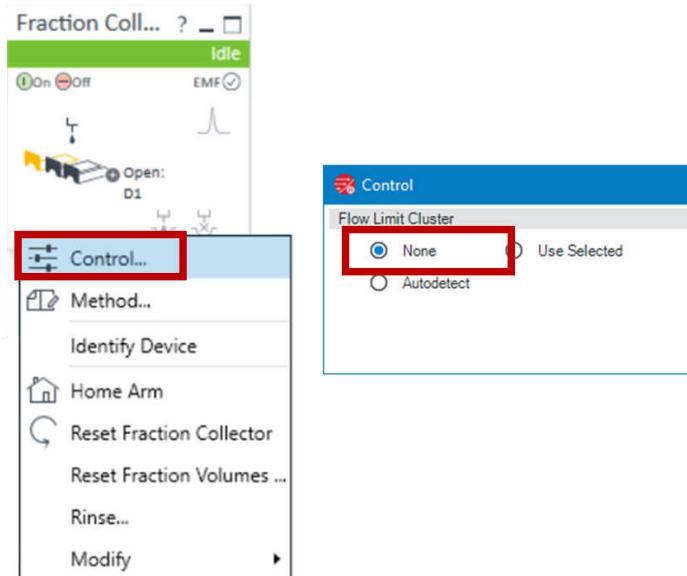


Figure 85. Disabling ‘Flow Limit Cluster’ may enable the ability to set a “3rd party Pump Flow.”

De-linking the pump allows a custom flow rate to be set in the fraction collector method. The flow rate specified here should match the detector flow rate specified in the Eclipse method. To confirm, right-click on the fraction collector and navigate to method and view the information under the Advanced Tab as shown below in **Error! Reference source not found..**

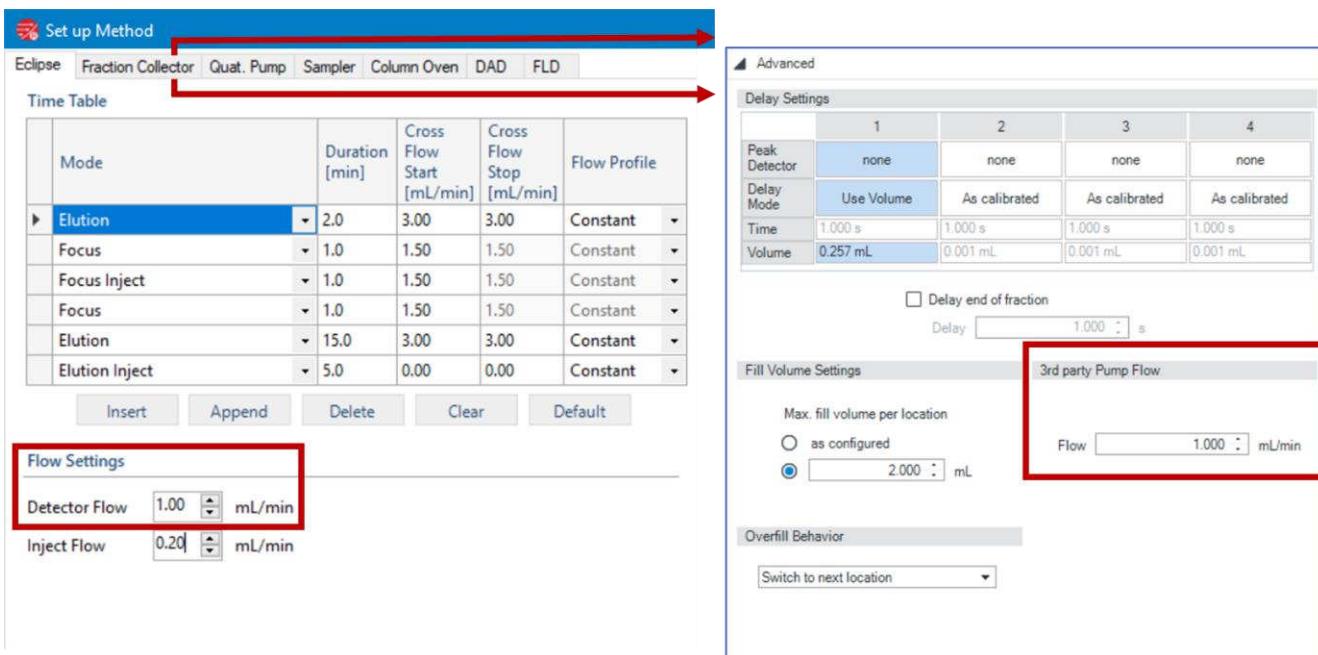
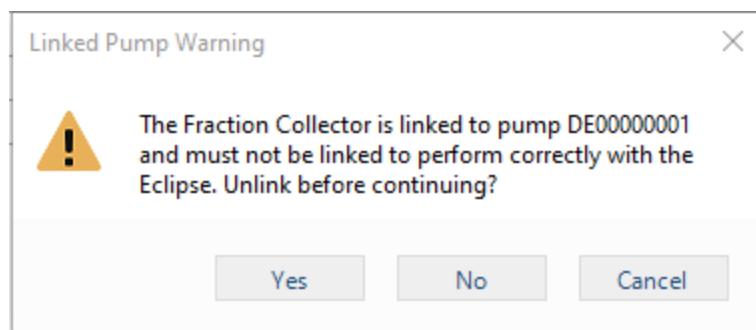


Figure 86. Eclipse method (left) and fraction collector method (right). Once a pump is de-linked the custom flow rate for the fraction collector should be set to match the Eclipse detector flow rate. The excess flow rate from the pump goes through the crossflow pathway.

With the pump de-linked, a custom flow rate can be specified and the fraction collector can be configured to collect volumes consistent with the detector flow.

NOTE: In VISION 3.1.2 and later, the sequence table will automatically check that the pump is de-linked from the fraction collector. If not, an error message will appear that will direct you to the linked pump window for the fraction collector so that it can be switched to “None.”



Adjusting Vial Volume for Detector Flow from Pump Flow

Some fraction collectors may not be de-linked from the pump. In these cases, it is necessary to “trick” the fraction collector by increasing the supposed volume of the vials so that the fraction collector thinks a larger volume is collected. This is because the sum of detector flow and crossflow will be equivalent to the pump flow. This is done by right-clicking the fraction collector module and navigating to [More → Wellplate Assignment](#). To take into account the detector flow (V_d) and crossflow (V_x) during the elution step, the vial volume can be modified per the following equation:

$$V_{mod} = V_{initial} \cdot \frac{V_x + V_d}{V_d}$$

where V_{mod} is the modified vial volume, $V_{initial}$ is the default vial volume, V_x is the crossflow flow rate during elution, and V_d is the detector flow rate.

Setting up a Time Table Based Collection with Fraction Collector

An example time table with a fraction collector is shown in [Error! Reference source not found..](#) In this example, we have initially set the fraction collection mode to “Off.” This will ensure no fractions are collected during the initial focus and focus + injection steps of the Eclipse method, where there is only mobile phase passing through the detectors. For time table-based collections, it is suggested to run an analytical run to assess the elution time of your peaks. Based on the expected time of elution, you can enable the fraction collector to collection fractions at that time—either volume or time-based. Volume based will utilize the 3rd party detector flow rate specified in order to determine how much volume to collect in each vial.

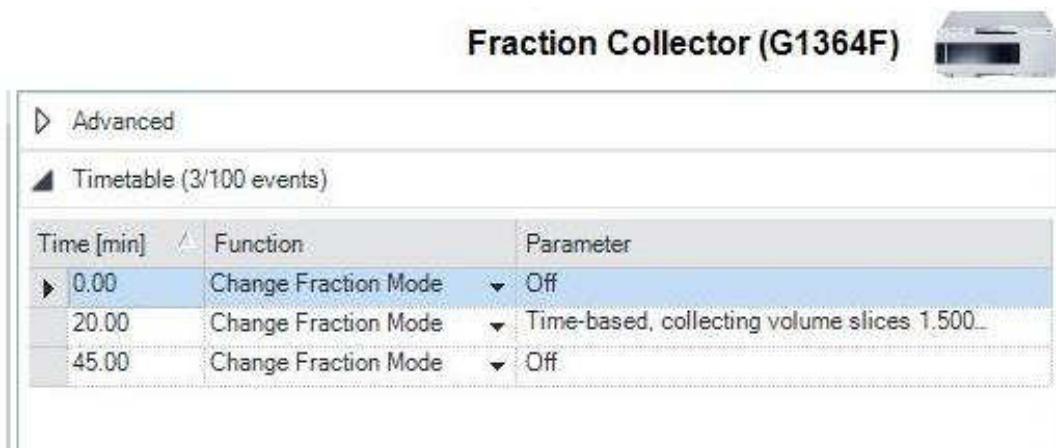


Figure 87. Example time table with fraction mode set to "Off" for the initial focus and focus inject, then switched to "On" with time-based collection by volume slices. At the end of the main elution step, the collection is turned off again.

Running a Sequence with a Fraction Collector

Once these considerations are taken into account and the fraction collector method is created as part of a larger overall experimental method, a sequence is utilized to run this method. Prior to setting up the sequence, the collection orientation can be set by right-clicking the fraction collector and navigating to [More → Collection Settings](#). This will allow you to configure the fill order as shown below in [Error! Reference source not found..](#)

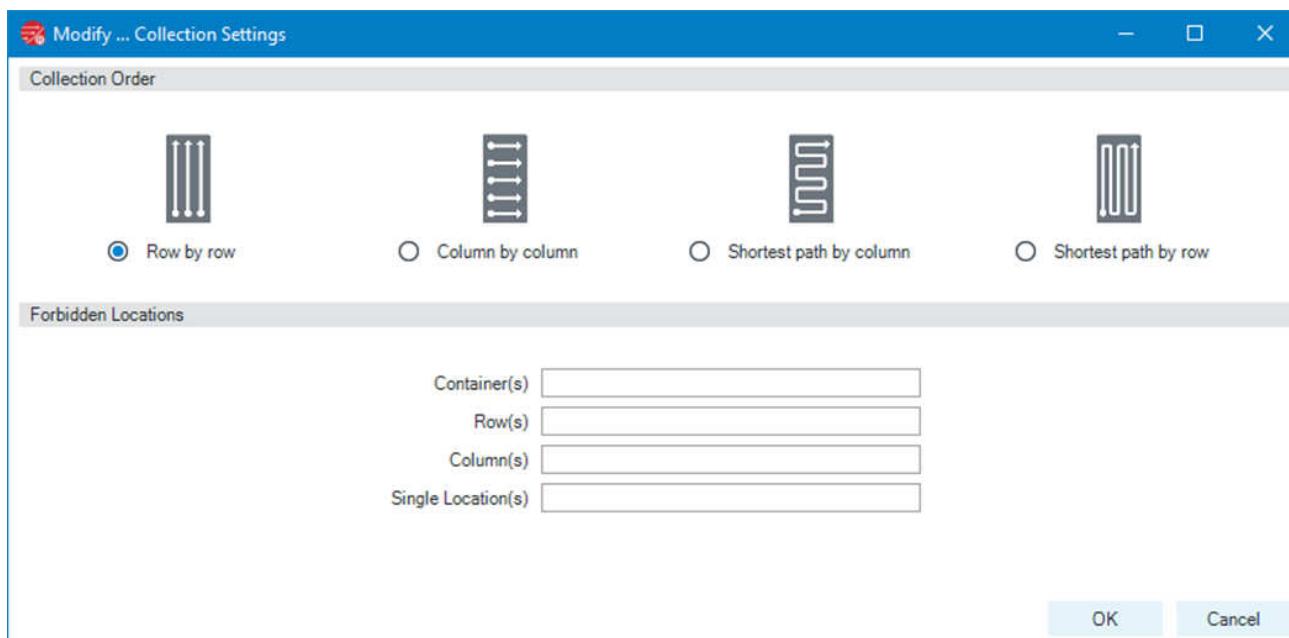


Figure 88. Collection settings for collection order, forbidden locations, and collection mode.

Note: As of January 2021, the Agilent instrument control framework (ICF) has the incorrect image for the description for the collection order. For example, the row-by-row option shows a graphic that is actually describing how the column-by-column fills. Conversely, the column-by-column option's graphic fills vials by column front to back. For example, the column-by-column will actually fill vials per this image below:



Column-by-column collection order

When setting up a sequence, most directions and workflow will be identical to operations without a fraction collector. Some new fields will be present when a fraction collector is configured. Even if the fraction collector is disabled in the method, it may be necessary to set a vial position in the sequence table. Depending on the fraction collector, all possible vial formats may be listed. It is important to select an appropriate format or the sequence may exhibit an error—even if the fraction collector is disabled. The options may include:

- » Next Location
- » Next Container
- » Pooling
- » P#-A-# format
- » Vial # format

It is recommended to use the correct format to prevent sequence errors or select Next Container. The field for entering the vial location is shown below in **Error! Reference source not found.** with a well-plate format. A traditional vial format may be Vial 1, Vial 2, etc. Please check your Agilent fraction collector literature for the correct vial format.

Sequence Table						
Ready						
	Enabled	Vial	Volume [µL]	Inj./Vial	Sample Name	VISION RUN Method
1	<input checked="" type="checkbox"/>	1	5.00	1	Blank	AAV Method.M
2	<input checked="" type="checkbox"/>	2	5.00	3	BSA	BSA Standard Metho...
3	<input checked="" type="checkbox"/>	3	5.00	3	AAV	AAV Method.M

Fraction Collector Start Position
P1-A-1 ▾
P2-A-1 ▾
Next Location ▾

Figure 89. VISION RUN sequence table with field entitled “Fraction Collector Start Position” displayed. The field will display as long as a fraction collector is present in the method, even if the method disables the fraction collection function.

Identifying Collected Vials

As of January 2021, information about peak trigger-based collection tables are not collected or shown in VISION DESIGN. During a run, VISION RUN can display the currently collected vials but across multiple runs (when performing a sequence for example), it becomes unclear what fraction vials correspond to what part of the fractogram. An example of the interface that shows collected vials is shown below in **Error! Reference source not found..**

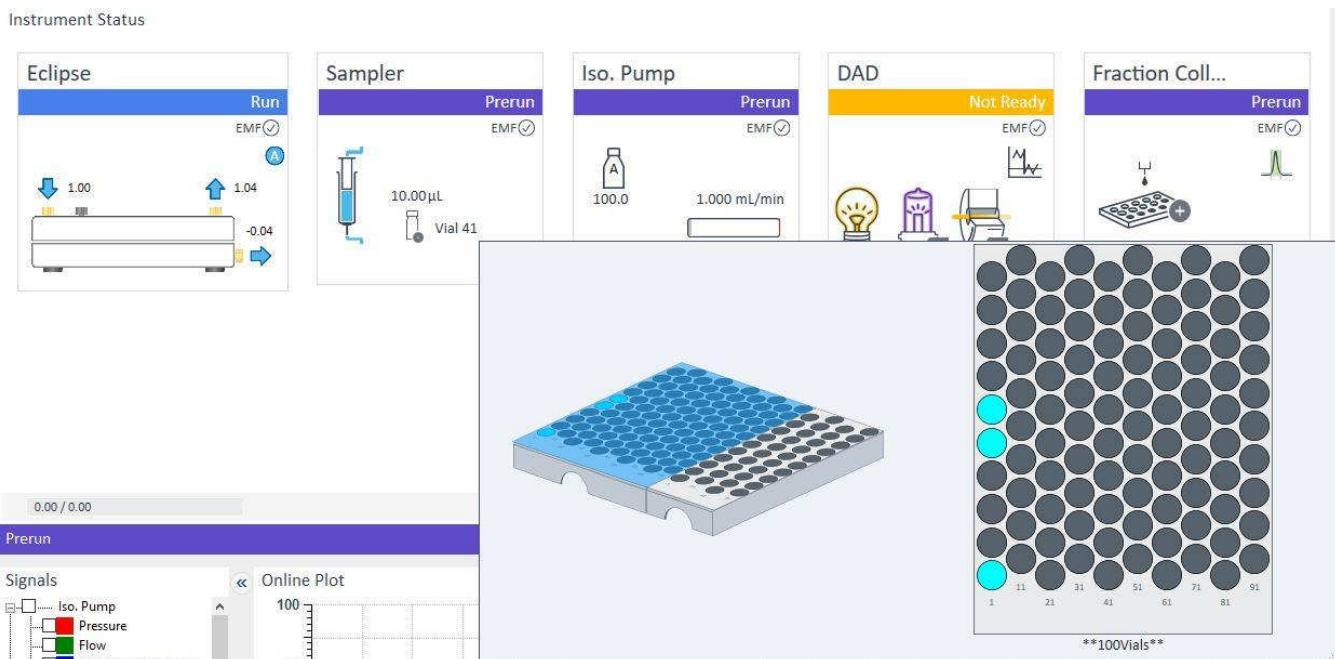


Figure 90. Clicking on the tray in VISION RUN will display the current fraction collector tray and filled vials. Collected vials in VISION are not identified by run or time, and so will need to be determined after the experiment by working backwards.

When running sequences, it may not be clear what has been collected in what vials. For this reason, using timetable-based collections, where the precise number of vials collected can be more easily obtained from the method, is the recommended method configuration for fraction collection with VISION RUN. If peak-based triggers are required, it is recommended to run experiments individually instead of in a sequence initially to track the vials. If the fractogram preview is available, it can also be used to track the vials. The fractogram preview is located in the VISION RUN method in select fraction collector models. This view is shown in **Error! Reference source not found..**

Here, an EMDF (Eclipse Method Data File) from a previous experiment run can be imported in order to preview or estimate the collection time, which can help determine what part of the peak or peaks correspond to which vials. Based on the “starting” location of the collection mode and the vial specified in the sequence, the fractions can be identified. From this point, you can then determine what was collected subsequently based on the vial volume (converting time to volume via the flow rate).

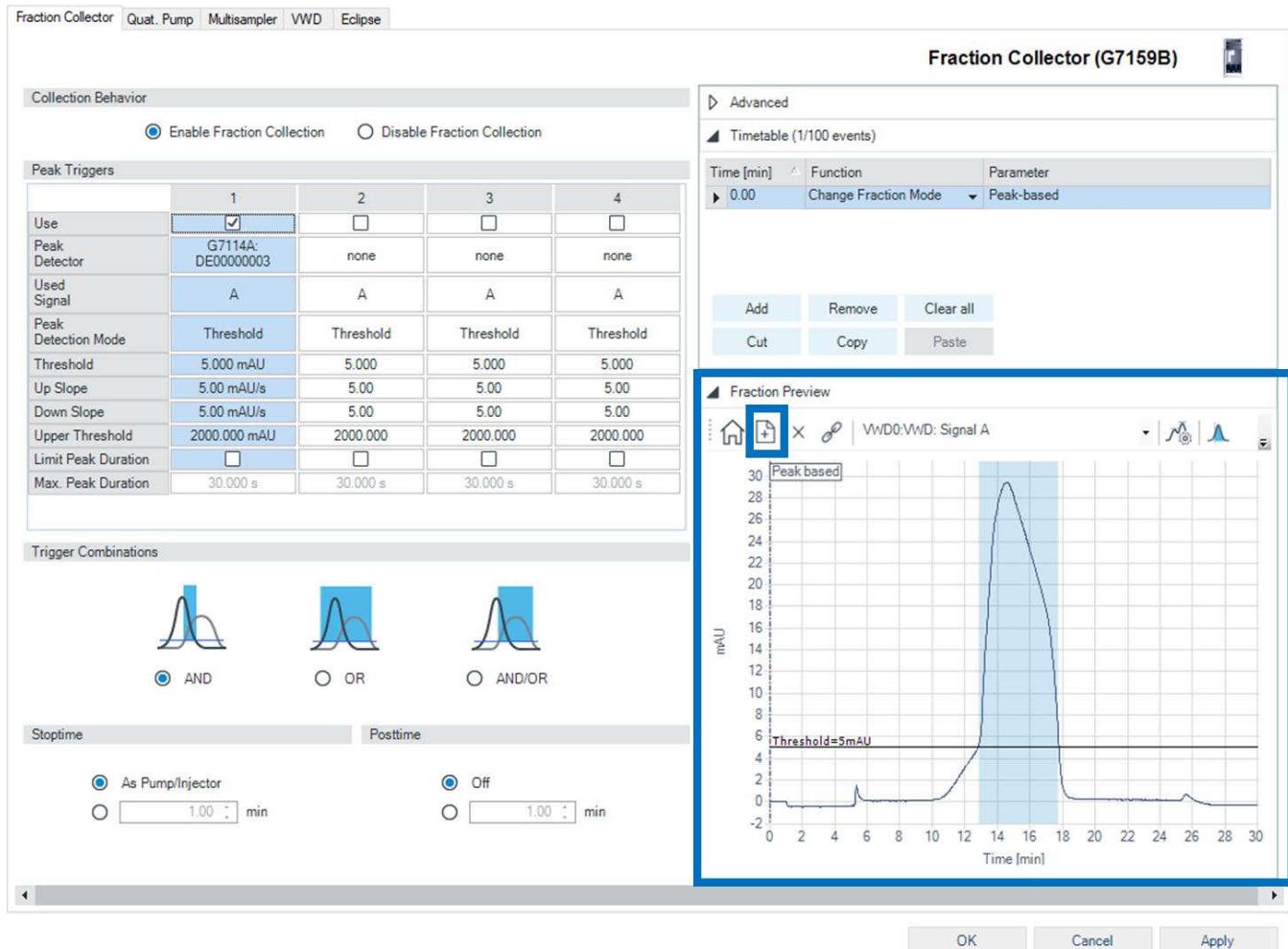


Figure 91. The “Fraction Preview” window is indicated here under the Fraction Collector method. By importing an EMDF (Eclipse Method Data File), the UV or FLD trace can be previewed with the threshold and collected regions highlighted. If the experimental runs are identical and reproducible, the preview can be used to estimate what is collected via peak triggers and help deduce the vials that correspond to relevant peaks.

This view can also be helpful for determining an appropriate time-table based collection. With a time-table based collection, the exact volume can be specified and as a result, determining how the collection volume corresponds to the vial location can be more straightforward. Improving the post-experiment fraction collector data in VISION DESIGN is an ongoing effort but the fraction preview is a useful tool for determining the amount, identity, and vials associated with sample collection.

Digital UV Signals in ASTRA

With the release of VISION 3.1, digital signals for UV traces can be collected in ASTRA. This enables direct digital data collection without the need for implementing hardware connections for analog UV signals. In order to collect digital UV signal data in ASTRA, ASTRA 8.0.1 or higher is required. Other than enabling the UV signals and selecting

the settings in the UV method through VISION RUN, only the ASTRA method needs to be adjusted for collecting digital UV data.

In ASTRA, a method can be created using the Method Builder, which will create a configuration with a generic UV. This generic UV profile needs to be replaced by “VISION UV” in the ASTRA configuration folders, which can be done by right-clicking on the “Generic UV Instrument” configuration and selecting “Replace Generic UV Instrument,” as shown below:

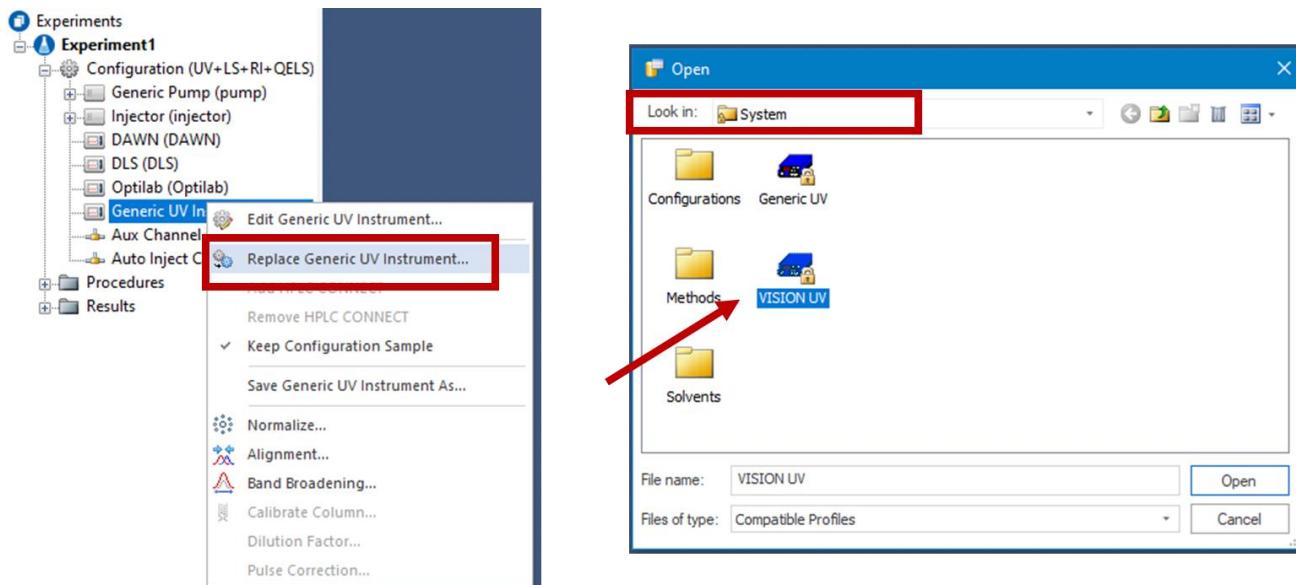


Figure 92. To enable ASTRA to collect digital UV from VISION RUN, the "Generic UV Instrument" configuration must be replaced with the "VISION UV" configuration.

This will convert the ASTRA configuration to one that is compatible with digital UV collection from VISION RUN, as shown below:

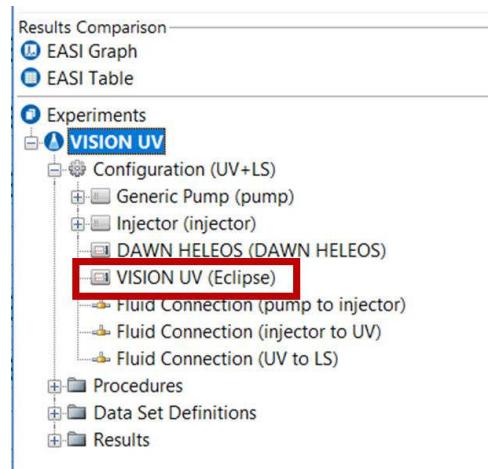


Figure 93. The "generic UV" configuration in ASTRA will need to be replaced with "VISION UV" in order to collect digital signals from VISION RUN.

Creating Fraction Collector Methods

In order to operate the fraction collector, a method needs to be created. The majority of the settings are controlled in the fraction collector method, which will be discussed shortly. If using an Eclipse with DCM option, there is a dedicated fraction collector on the side-panel of the Eclipse as shown in **Error! Reference source not found..**



Eclipse with DCM + SEC (WECDS)

Figure 94. Fraction collector port on side-panel of Eclipse with DCM option.

In order to enable flow to exit that port (instead of going to waste), the Eclipse has to have fractionator mode enabled in the Eclipse method. This can be toggled on in the Eclipse method editor window as shown in **Error! Reference source not found..**

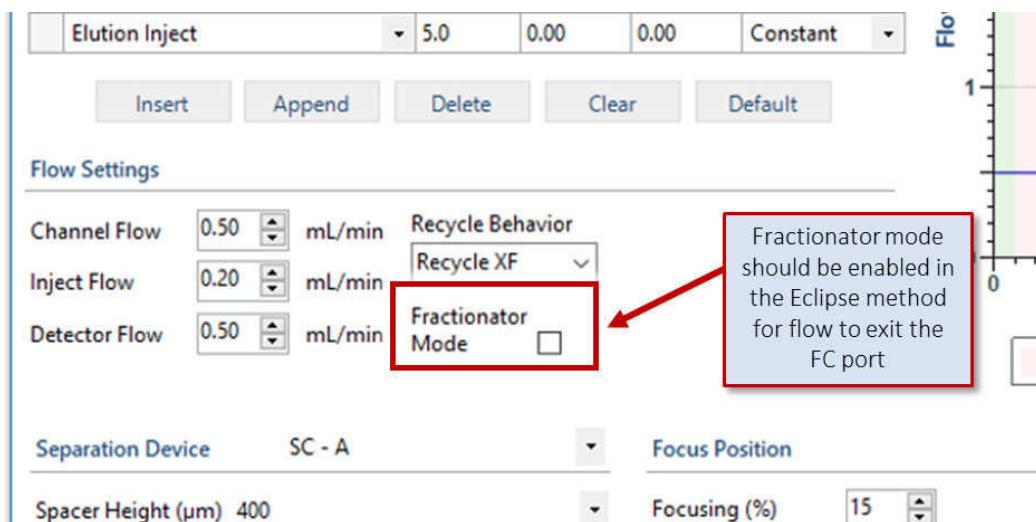


Figure 95. Fluid through the fraction collector port on the Eclipse with DCM option is enabled via the Eclipse method.

Once the Eclipse aspects of setting up the fraction collector are complete, the fraction collector method can be edited. Right-clicking on the fraction collector module when not running will allow access to the current method. Or you can navigate to **Method → Set up Method** to view the overall method, which would include tabs for all HPLC modules and the Wyatt Eclipse. In **Error! Reference source not found.** below, an example window of a fraction collector method is provided. At the basic level, the fraction collector itself can be enabled or disabled through the

method for simplicity. While manual collections can be executed directly via the module UI as shown in [Figure 77](#), configuring the system for peak-based trigger collection or timetable-based collections are always done through the method.

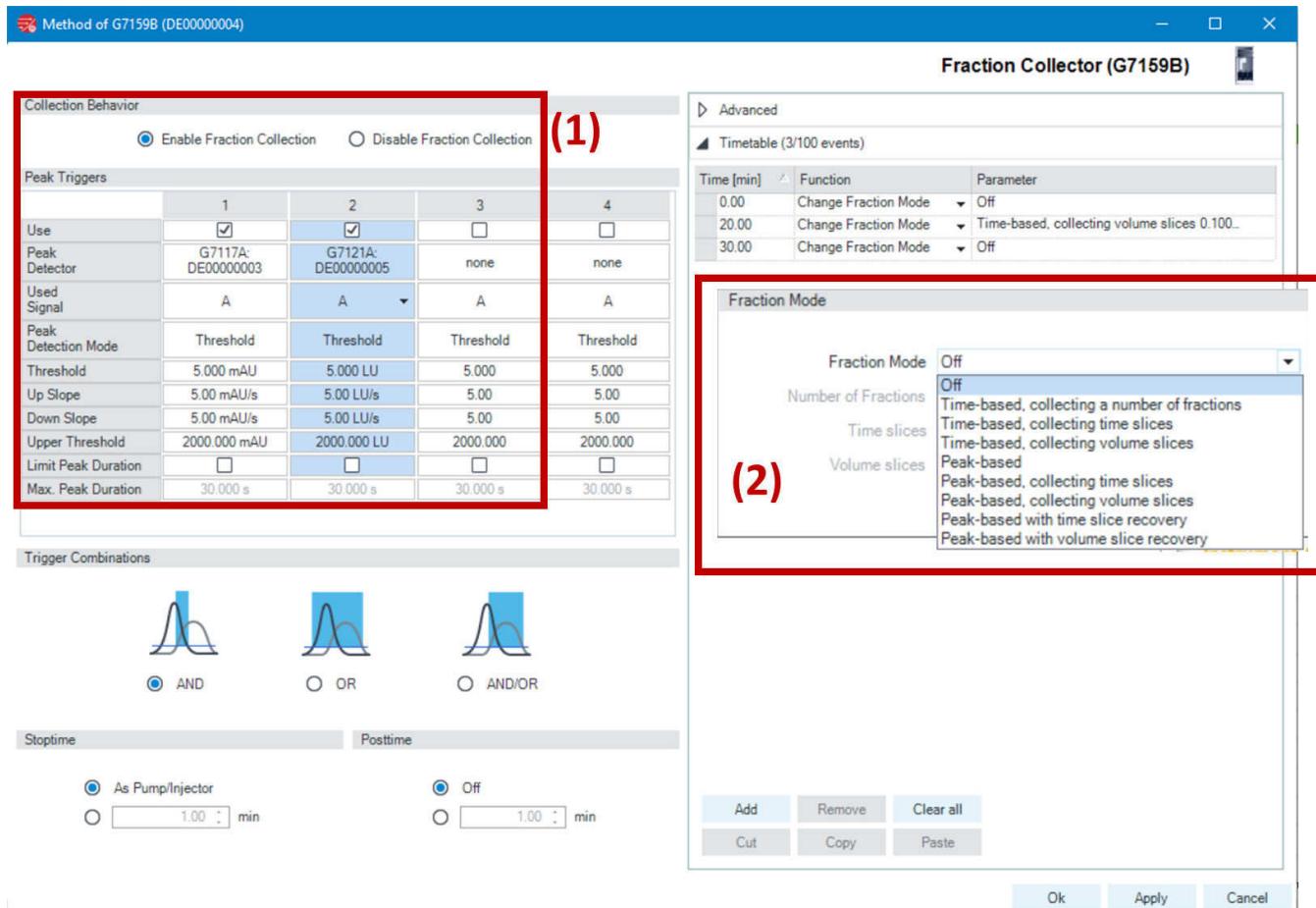


Figure 96. Fraction collector method in VISION RUN. The fraction collector can be enabled or disabled in the method at the upper left. (1) If compatible Agilent UV or FLD detectors are present, they can be configured under ‘Peak Triggers.’ Thresholds and slopes can be set for UV (mAU or mAU/s) and FLD (LU or LU/s). (2) Under the Timetable section of the right side of the window, the fraction collector timetable and mode can be set. Fractions can be collected either time-based or peak-based. For Wyatt Eclipse systems, it is currently recommended to use time-based collections when doing sequences because of limited information regarding collected fractions in VISION DESIGN.

Note: As of May 2021, information about peak trigger-based collections is not collected or shown in VISION DESIGN. When running sequences, it may not be clear what has been collected in what vials. For this reason, using timetable-based collections, where the precise number of vials collected can be more easily obtained from the method, is the recommended method configuration for fraction collection with VISION RUN. If peak-based triggers are required, it is recommended to run experiments individually to track the vials. If the fractogram preview is available, it can also be used to track the vials.

Additional options are provided under ‘Advanced’ which will be described here and expounded in the following sections. An example of the Advanced tab is shown below in [Error! Reference source not found..](#) The option ‘Delay Settings’ allows for the delay volumes calculated in LabAdvisor or stored on the UV/FLD modules to be configured. The fill volume allows for custom fill volumes to be specified—for example, collecting only 0.5 mL fractions in a larger 1.5 mL vial. Overfill behavior can also be defined under the Advanced tab. The last parameter, which is more

important for FFF than other techniques, is a ‘3rd party Pump Flow’ which allows a custom flow rate to be set for the fraction collector to account for crossflow in FFF separations. This will be further explained below in [Error! Reference source not found..](#)

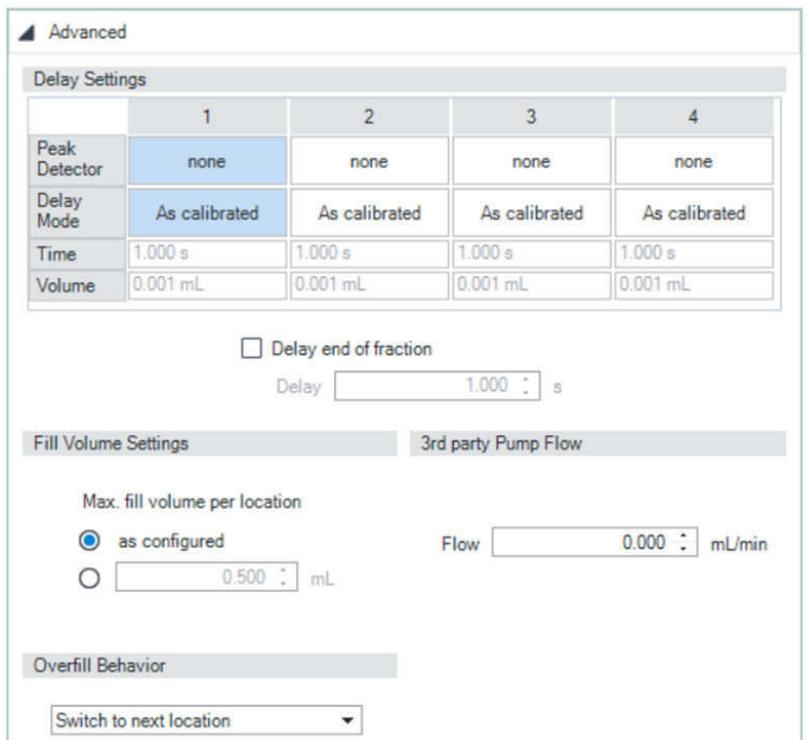


Figure 97. The advanced options under the fraction collector module. Important settings like how to enable custom flow rate to account for detector flow will be covered in the section, [Error! Reference source not found..](#).

In addition to the Advanced settings, the delay volume can be added per compatible Agilent detector by right-clicking the fraction collector module and navigating to [Modify](#) → [Detector Delay Volumes](#) as shown in [Error! Reference source not found..](#)

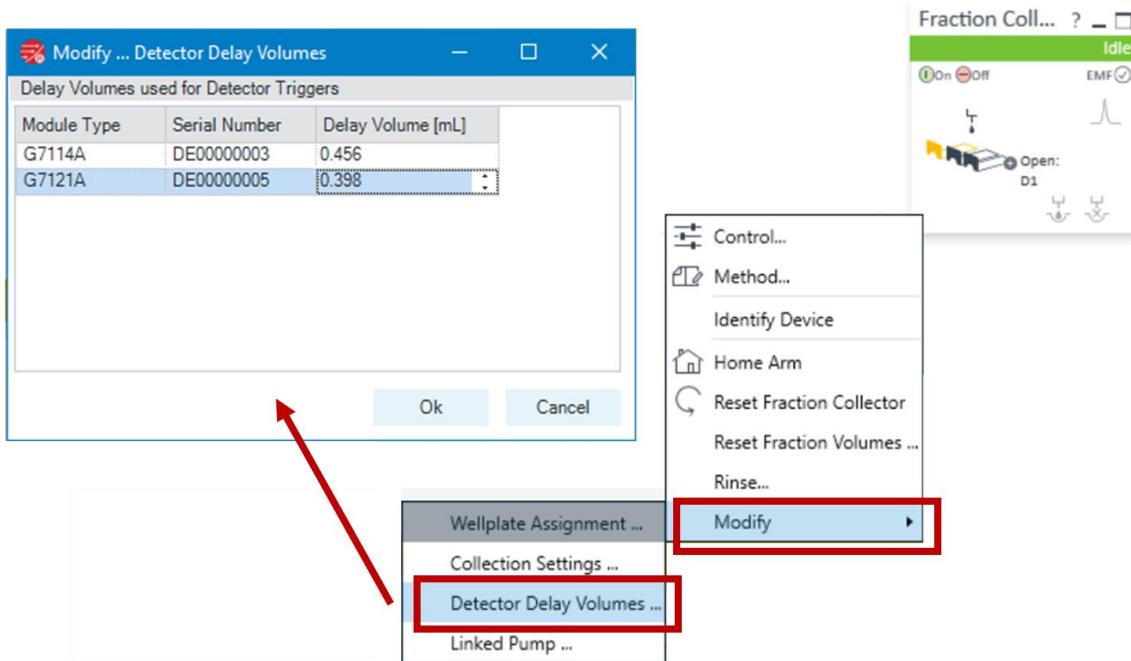


Figure 98. Option for adding delay volumes to the fraction collector module. This is only compatible currently with Agilent-supported UV and FLD hardware.

After the fraction collector settings are set, the method can be saved and executed in a sequence. For information about Eclipse method development please refer to [TN6005 - Eclipse Aqueous Method Development Quick Start Guide](#). The next section will cover important considerations when using a fraction collector with FFF separations before going into setting up sequences.

Considerations for Flow Rate and Vial Volumes

The Wyatt Eclipse is unique in that it utilizes a single pump flow rate and diverts the flow in different ways to achieve detector flow, crossflow, focus flow, and injection flow. Because the fraction collector does not measure flow rate, when a fraction collector is linked to a pump, it may incorrectly expect 3 – 4 mL/min of flow to enter the module; however, due to the nature of FFF separations, it may be that only 1 mL/min goes through the detectors as flow whereas the remainder of the flow goes through the crossflow pathway to waste. With default configurations, this can lead to underfilled vials.

There are two general strategies for accounting for vial volume. The first is de-linking the pump from the fraction collector. The second is adjusting the vial volume to account for the incorrect flow rates the fraction collector is operating with.

De-Linking the Pump and Fraction Collector Modules

This option is not available for all fraction collector and pump module combinations but is the easiest strategy for providing the fraction collector with a correct flow rate. There are a few ways to de-link the pump from the fraction collector. First, it can be de-linked by right-clicking on the fraction collector module in VISION RUN and navigating to [Modify](#) → [Linked Pump](#). In the new window, the Linked Pump can be set to “None” as shown in [Error! Reference source not found..](#)

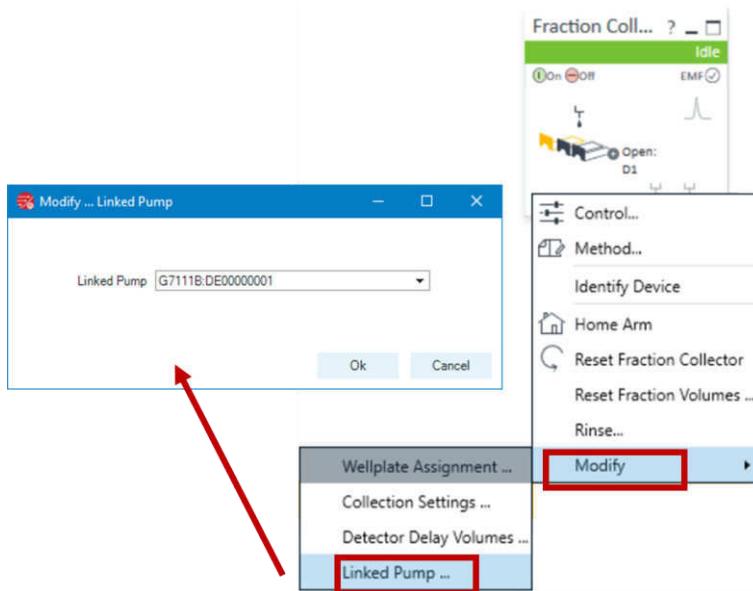


Figure 99. De-linking a pump through the fraction collector module in VISION RUN.

Alternatively, it can be de-linked from the configuration window ahead of launching VISION RUN underneath the fraction collector module as shown below in Error! Reference source not found..

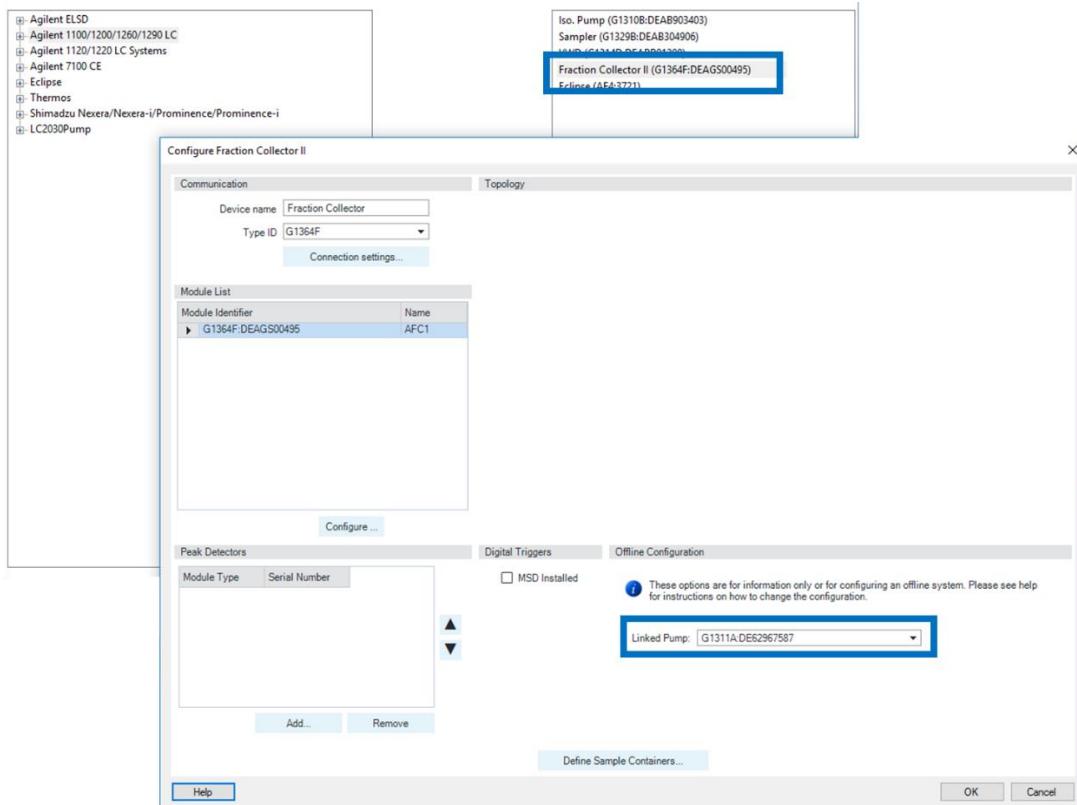


Figure 100. De-linking the pump through the configuration. Select the fraction collector module on the right side of the window and select "Configure" to open a new window. Here, you can enable an offline configuration by setting the Linked Pump to "None."

In both cases, it may be necessary to also disable the Flow Limit Cluster by right-clicking on the fraction collector module and navigating to "Control." In the subsequent window, you can select 'None.'

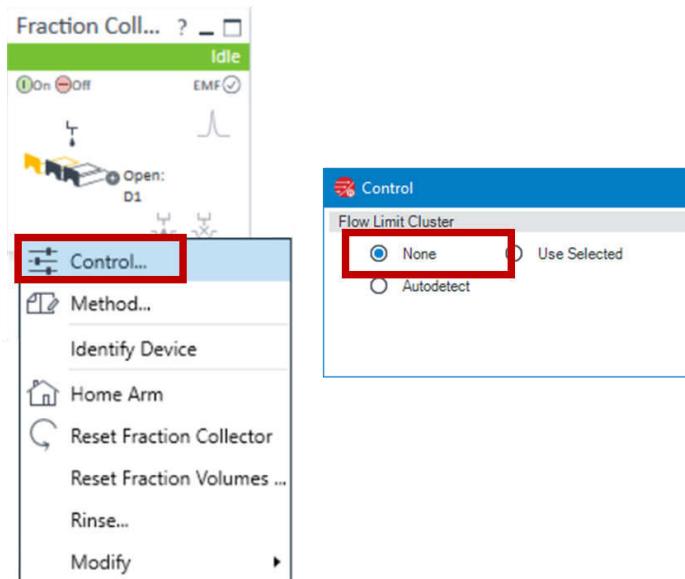


Figure 101. Disabling 'Flow Limit Cluster' may enable the ability to set a "3rd party Pump Flow."

De-linking the pump allows a custom flow rate to be set in the fraction collector method. The flow rate specified here should match the detector flow rate specified in the Eclipse method. To confirm, right-click on the fraction collector and navigate to method and view the information under the Advanced Tab as shown below in **Error! Reference source not found..**

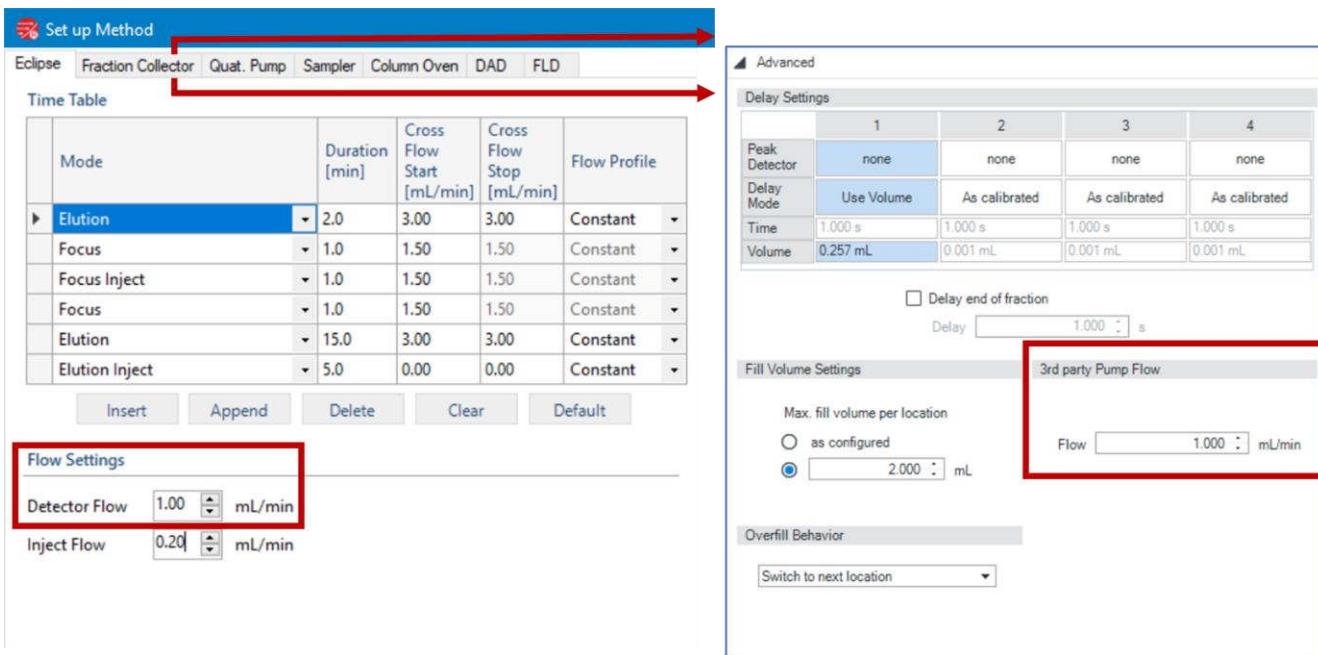
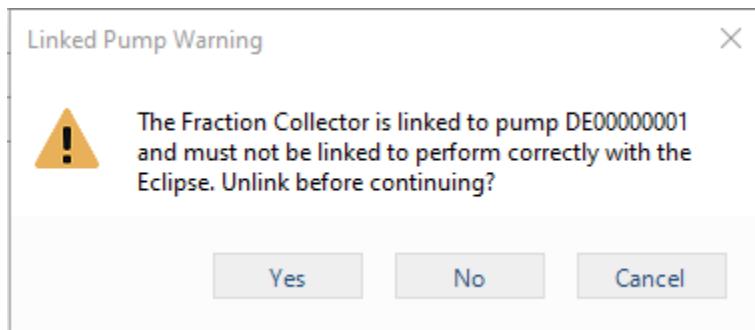


Figure 102. Eclipse method (left) and fraction collector method (right). Once a pump is de-linked the custom flow rate for the fraction collector should be set to match the Eclipse detector flow rate. The excess flow rate from the pump goes through the crossflow pathway.

With the pump de-linked, a custom flow rate can be specified and the fraction collector can be configured to collect volumes consistent with the detector flow.

NOTE: In VISION 3.1.2 and later, the sequence table will automatically check that the pump is de-linked from the fraction collector. If not, an error message will appear that will direct you to the linked pump window for the fraction collector so that it can be switched to “None.”



Adjusting Vial Volume for Detector Flow from Pump Flow

Some fraction collectors may not be de-linked from the pump. In these cases, it is necessary to “trick” the fraction collector by increasing the supposed volume of the vials so that the fraction collector thinks a larger volume is collected. This is because the sum of detector flow and crossflow will be equivalent to the pump flow. This is done by right-clicking the fraction collector module and navigating to [More → Wellplate Assignment](#). To take into account the detector flow (V_d) and crossflow (V_x) during the elution step, the vial volume can be modified per the following equation:

$$V_{mod} = V_{initial} \cdot \frac{V_x + V_d}{V_d}$$

where V_{mod} is the modified vial volume, $V_{initial}$ is the default vial volume, V_x is the crossflow flow rate during elution, and V_d is the detector flow rate.

Setting up a Time Table Based Collection with Fraction Collector

An example time table with a fraction collector is shown in [Error! Reference source not found.](#). In this example, we have initially set the fraction collection mode to “Off.” This will ensure no fractions are collected during the initial focus and focus + injection steps of the Eclipse method, where there is only mobile phase passing through the detectors. For time table-based collections, it is suggested to run an analytical run to assess the elution time of your peaks. Based on the expected time of elution, you can enable the fraction collector to collection fractions at that time—either volume or time-based. Volume based will utilize the 3rd party detector flow rate specified in order to determine how much volume to collect in each vial.

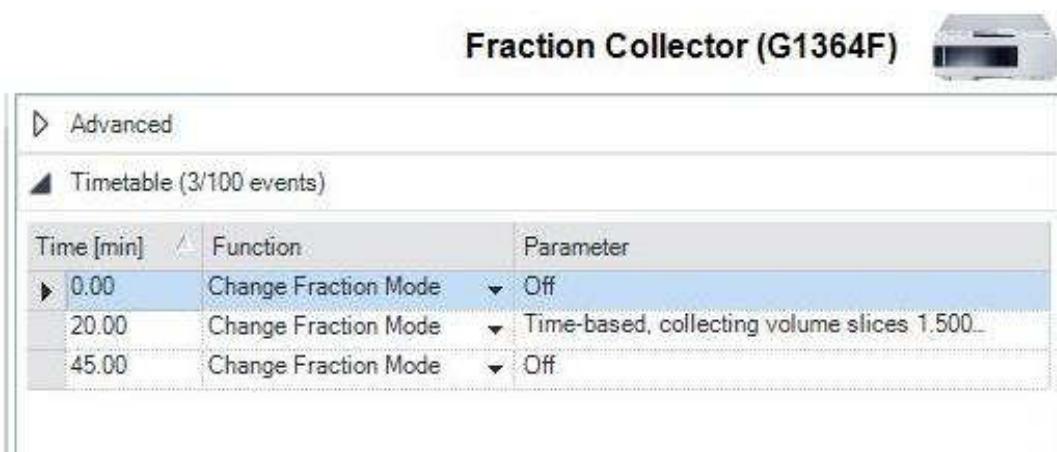


Figure 103. Example time table with fraction mode set to "Off" for the initial focus and focus inject, then switched to "On" with time-based collection by volume slices. At the end of the main elution step, the collection is turned off again.

Running a Sequence with a Fraction Collector

Once these considerations are taken into account and the fraction collector method is created as part of a larger overall experimental method, a sequence is utilized to run this method. Prior to setting up the sequence, the collection orientation can be set by right-clicking the fraction collector and navigating to [More → Collection Settings](#). This will allow you to configure the fill order as shown below in [Error! Reference source not found..](#)

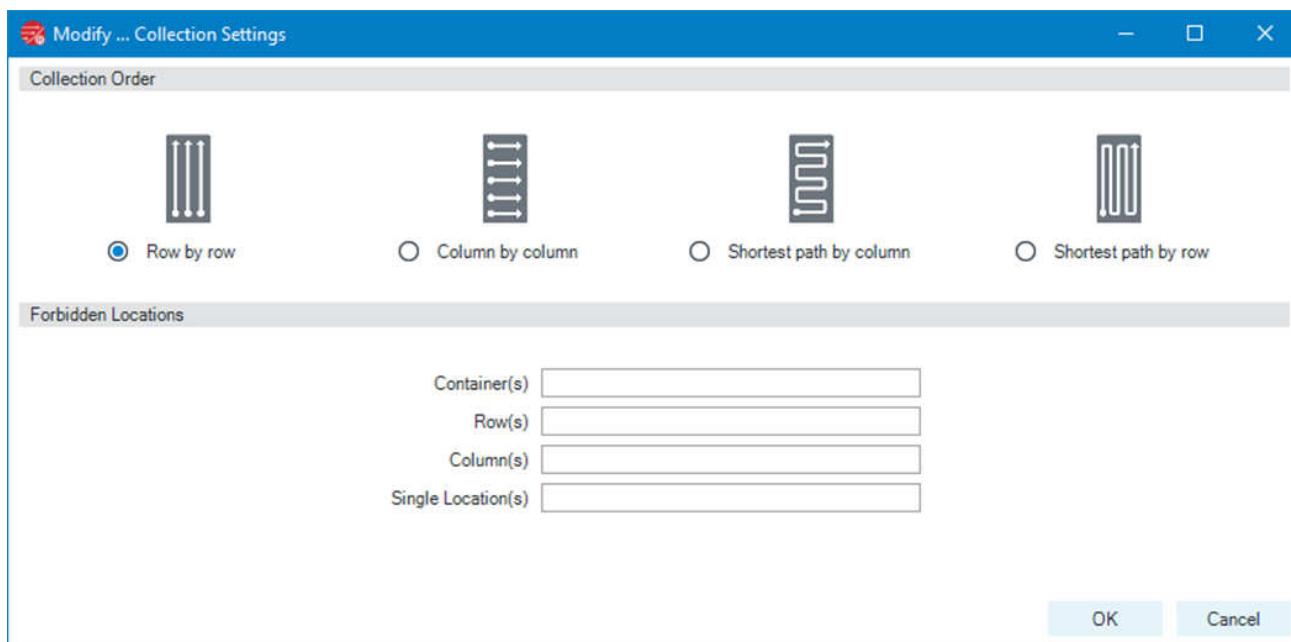


Figure 104. Collection settings for collection order, forbidden locations, and collection mode.

Note: As of January 2021, the Agilent instrument control framework (ICF) has the incorrect image for the description for the collection order. For example, the row-by-row option shows a graphic that is actually describing how the column-by-column fills. Conversely, the column-by-column option's graphic fills vials by column front to back. For example, the column-by-column will actually fill vials per this image below:



Column-by-column collection order

When setting up a sequence, most directions and workflow will be identical to operations without a fraction collector. Some new fields will be present when a fraction collector is configured. Even if the fraction collector is disabled in the method, it may be necessary to set a vial position in the sequence table. Depending on the fraction collector, all possible vial formats may be listed. It is important to select an appropriate format or the sequence may exhibit an error—even if the fraction collector is disabled. The options may include:

- » Next Location
- » Next Container
- » Pooling
- » P#-A-# format
- » Vial # format

It is recommended to use the correct format to prevent sequence errors or select Next Container. The field for entering the vial location is shown below in **Error! Reference source not found.** with a well-plate format. A traditional vial format may be Vial 1, Vial 2, etc. Please check your Agilent fraction collector literature for the correct vial format.

Sequence Table							
Ready							
	Enabled	Vial	Volume [µL]	Inj./Vial	Sample Name	VISION RUN Method	Fraction Collector Start Position
1	<input checked="" type="checkbox"/>	1	5.00	1	Blank	AAV Method.M	P1-A-1 ▾
2	<input checked="" type="checkbox"/>	2	5.00	3	BSA	BSA Standard Metho...	P2-A-1 ▾
3	<input checked="" type="checkbox"/>	3	5.00	3	AAV	AAV Method.M	Next Location ▾

Figure 105. VISION RUN sequence table with field entitled “Fraction Collector Start Position” displayed. The field will display as long as a fraction collector is present in the method, even if the method disables the fraction collection function.

Identifying Collected Vials

As of January 2021, information about peak trigger-based collection tables are not collected or shown in VISION DESIGN. During a run, VISION RUN can display the currently collected vials but across multiple runs (when performing a sequence for example), it becomes unclear what fraction vials correspond to what part of the fractogram. An example of the interface that shows collected vials is shown below in **Error! Reference source not found.**

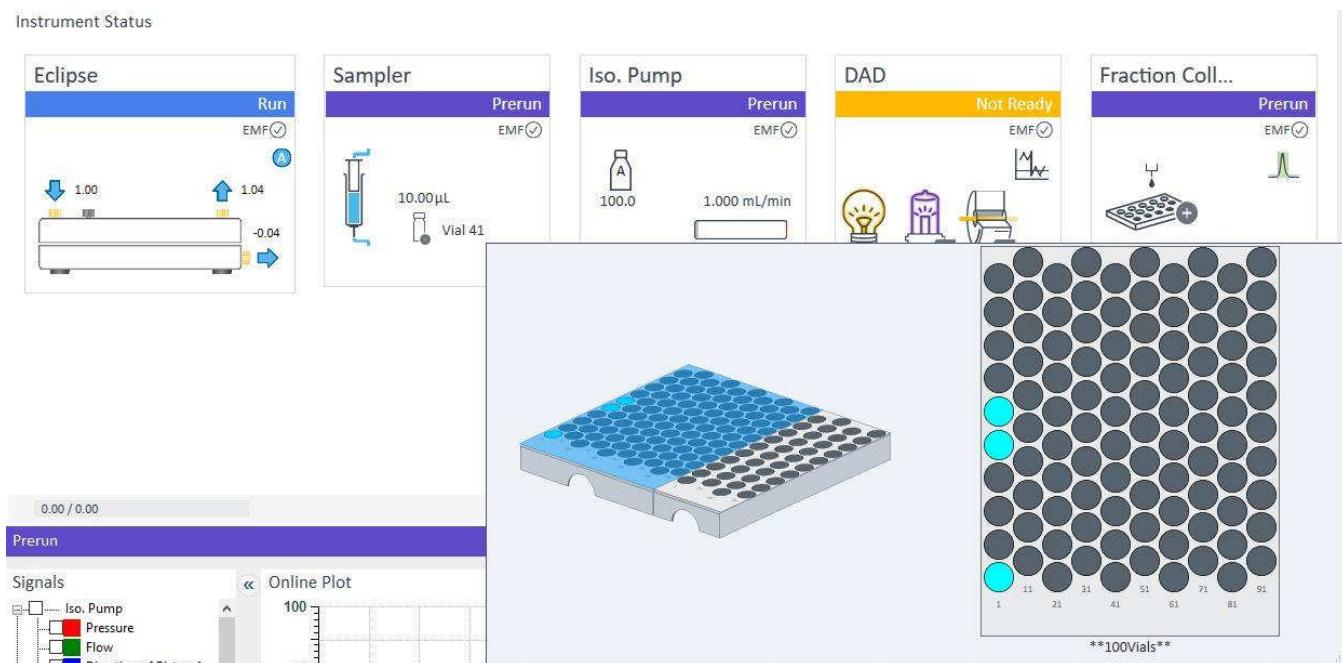


Figure 106. Clicking on the tray in VISION RUN will display the current fraction collector tray and filled vials. Collected vials in VISION are not identified by run or time, and so will need to be determined after the experiment by working backwards.

When running sequences, it may not be clear what has been collected in what vials. For this reason, using timetable-based collections, where the precise number of vials collected can be more easily obtained from the method, is the recommended method configuration for fraction collection with VISION RUN. If peak-based triggers are required, it is recommended to run experiments individually instead of in a sequence initially to track the vials. If the fractogram preview is available, it can also be used to track the vials. The fractogram preview is located in the VISION RUN method in select fraction collector models. This view is shown in **Error! Reference source not found.**. Here, an EMDF (Eclipse Method Data File) from a previous experiment run can be imported in order to preview or estimate the collection time, which can help determine what part of the peak or peaks correspond to which vials. Based on the “starting” location of the collection mode and the vial specified in the sequence, the fractions can be identified. From this point, you can then determine what was collected subsequently based on the vial volume (converting time to volume via the flow rate).

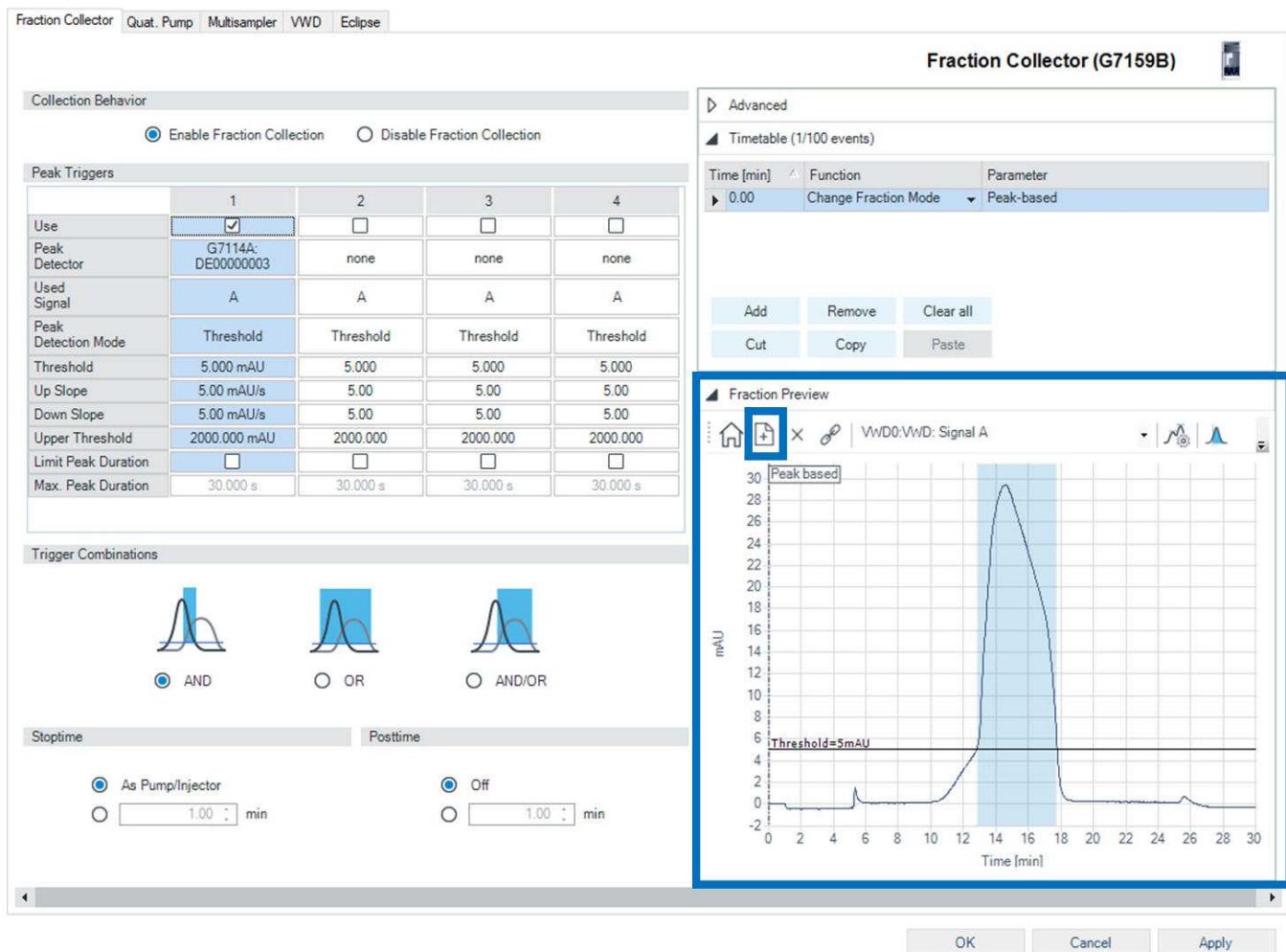


Figure 107. The “Fraction Preview” window is indicated here under the Fraction Collector method. By importing an EMDF (Eclipse Method Data File), the UV or FLD trace can be previewed with the threshold and collected regions highlighted. If the experimental runs are identical and reproducible, the preview can be used to estimate what is collected via peak triggers and help deduce the vials that correspond to relevant peaks.

This view can also be helpful for determining an appropriate time-table based collection. With a time-table based collection, the exact volume can be specified and as a result, determining how the collection volume corresponds to the vial location can be more straightforward. Improving the post-experiment fraction collector data in VISION DESIGN is an ongoing effort but the fraction preview is a useful tool for determining the amount, identity, and vials associated with sample collection.

Digital UV Signals in ASTRA

With the release of VISION 3.1, digital signals for UV traces can be collected in ASTRA. This enables direct digital data collection without the need for implementing hardware connections for analog UV signals. In order to collect digital UV signal data in ASTRA, ASTRA 8.0.1 or higher is required. Other than enabling the UV signals and selecting the settings in the UV method through VISION RUN, only the ASTRA method needs to be adjusted for collecting digital UV data.

In ASTRA, a method can be created using the Method Builder, which will create a configuration with a generic UV. This generic UV profile needs to be replaced by “VISION UV” in the ASTRA configuration folders, which can be done by right-clicking on the “Generic UV Instrument” configuration and selecting “Replace Generic UV Instrument,” as shown below:

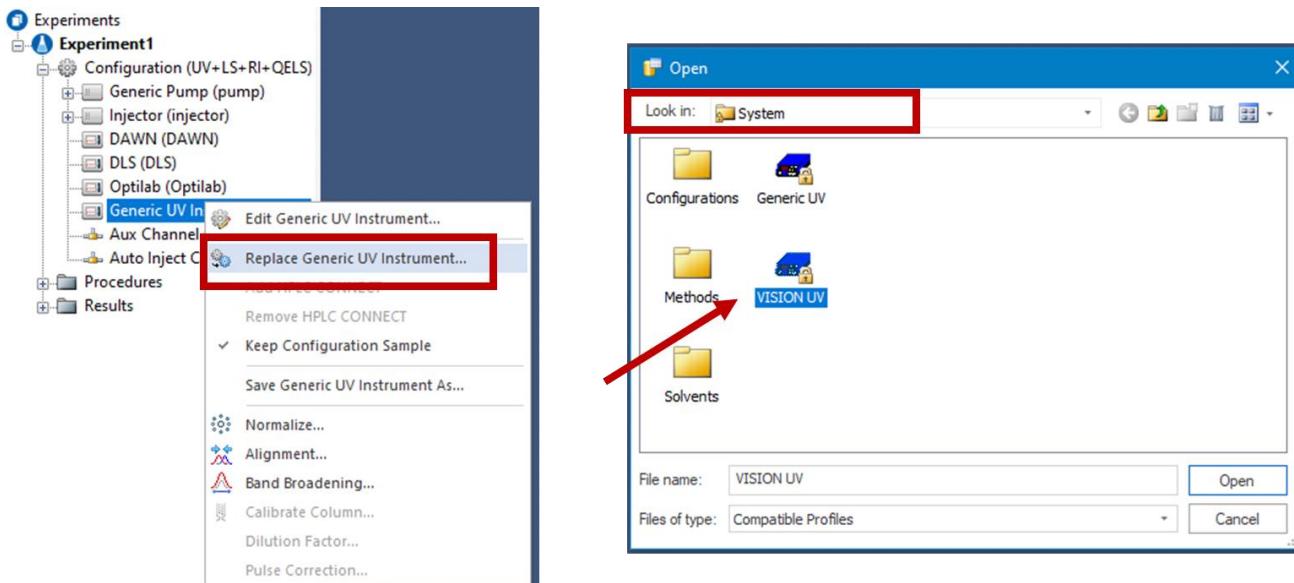


Figure 108. To enable ASTRA to collect digital UV from VISION RUN, the “Generic UV Instrument” configuration must be replaced with the “VISION UV” configuration.

This will convert the ASTRA configuration to one that is compatible with digital UV collection from VISION RUN, as shown below:

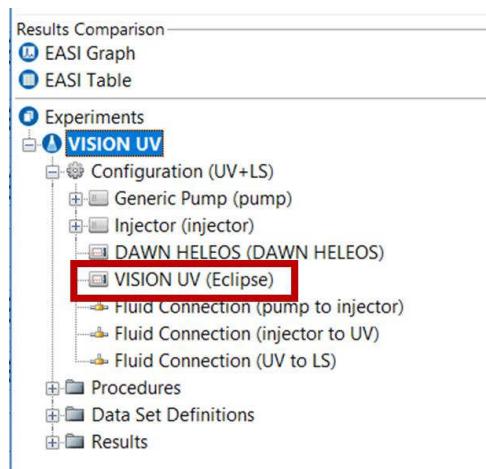


Figure 109. The “generic UV” configuration in ASTRA will need to be replaced with “VISION UV” in order to collect digital signals from VISION RUN.

VISION RUN: Resulting Data Files for VISION DESIGN & ASTRA

Eclipse Measurement Data File (EMDF)

VISION RUN outputs a data file referred to as an EMDF (Eclipse Measurement Data File) that can be opened in VISION DESIGN. The EMDF contains information about the FFF methods, sequence, and recorded digital data for configured detectors. One file is stored per sequence. In the next section, exploring these types of files with VISION DESIGN will be discussed.

ASTRA Data Files & Disabling ASTRA Module

The ASTRA module can be configured on or off in the VISION launch hub. Once correctly configured, VISION RUN will run its sequence table and generate ASTRA data files as part of each sequence row as long as an ASTRA method is configured. You can opt to not collect ASTRA files by leaving the field blank in the VISION RUN sequence table. However, if you do not observe the ASTRA options in VISION RUN, or if you do not want an ASTRA as part of the sequence table, it is possible to enable or disable the ASTRA module in the VISION RUN sequence. ASTRA can be enabled or disabled from the License Manager (shown in Figure 110). The “On” button can be toggled on or off to enable or disable the ASTRA option in the VISION RUN sequence table.

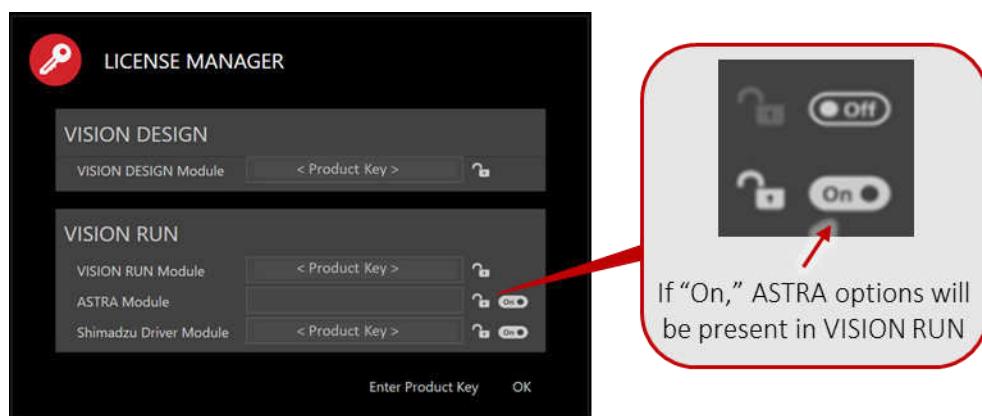
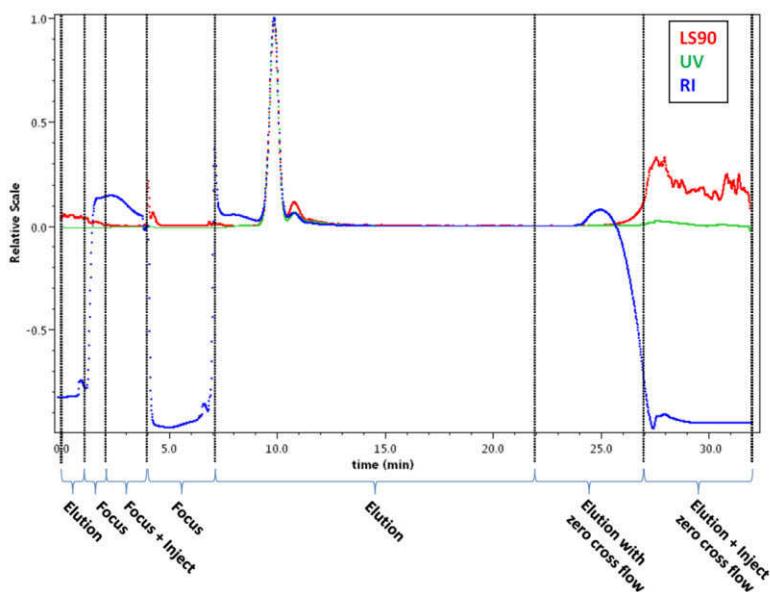


Figure 110. The License Manager window. ASTRA can be toggled on and off on the right side of the ASTRA module line.

ASTRA Data Analysis Tips & Tricks

There are some guidelines for analyzing ASTRA data files of FFF-MALS experiments. More information can be found in the ASTRA Quick Guide or our online resources at the [Wyatt Support Center](#). Because of the fluctuations of flows and pressures, different modes can lead to different detector signals. An example of the fractogram for a BSA run is provided in Figure 111 and shows the RI signal (blue), UV signal (green), and the light scattering signal (red). The RI detector is especially sensitive to the changes in flow rate as a result of the cross flow changes in FFF-MALS separations.



Typical AF4 Method:

- Elution (equilibrate pressures)
- Focus (stabilize focus flow)
- Focus + Inject (transport sample to channel)
- Focus (focus sample further)
- Elution (Method)
- Elution with zero cross flow (clear membrane)
- Elution + Inject (flush autosampler and system)

Figure 111. Changes in RI signal (compared to UV and LS) for different flow modes in FFF-MALS.

As a result, it is often recommended to draw your ASTRA baselines during the main elution step, as shown in Figure 112.

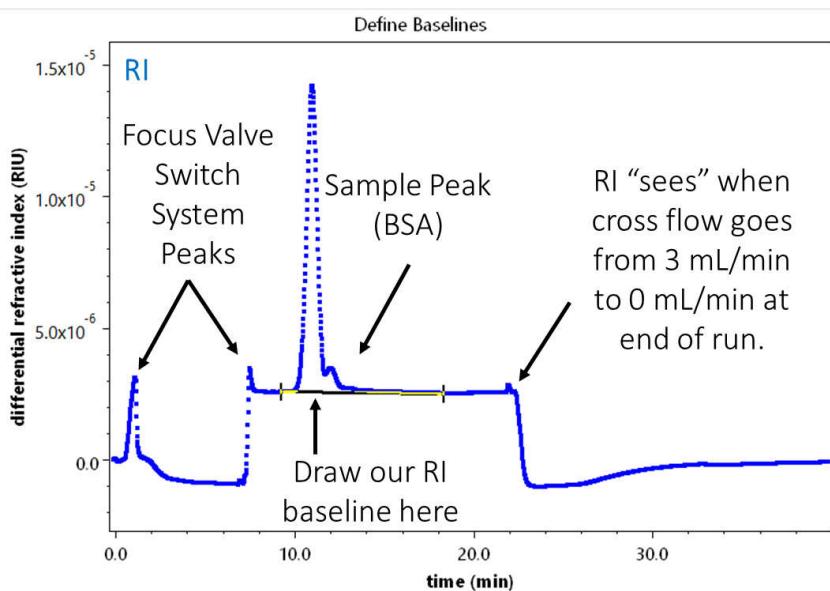


Figure 112. Appropriate RI baseline for a typical FFF-MALS fractogram.

In these cases, there was a constant cross flow across the main elution step. If there is a cross flow gradient, the RI may change with the decreasing flow rate due in part to pressure changes and mobile phase—specifically if containing salts—flux through the membrane (which can affect RI due to concentration changes in mobile phase). This is shown in Figure 113. RI drift from cross flow gradients can obscure signals depending on the amount of drift. As a result, it is often recommended to run a blank injection in order to perform a baseline subtraction in ASTRA. Baselines can be drawn carefully but it is preferable to use the ASTRA Baseline Subtraction Procedure, which can be applied in ASTRA by right-clicking on an experiment to apply a method, then navigating to: System > Methods > Light Scattering > Baseline Subtraction. More information can be found in TN1012 – Using the Baseline Subtraction Procedure in ASTRA.

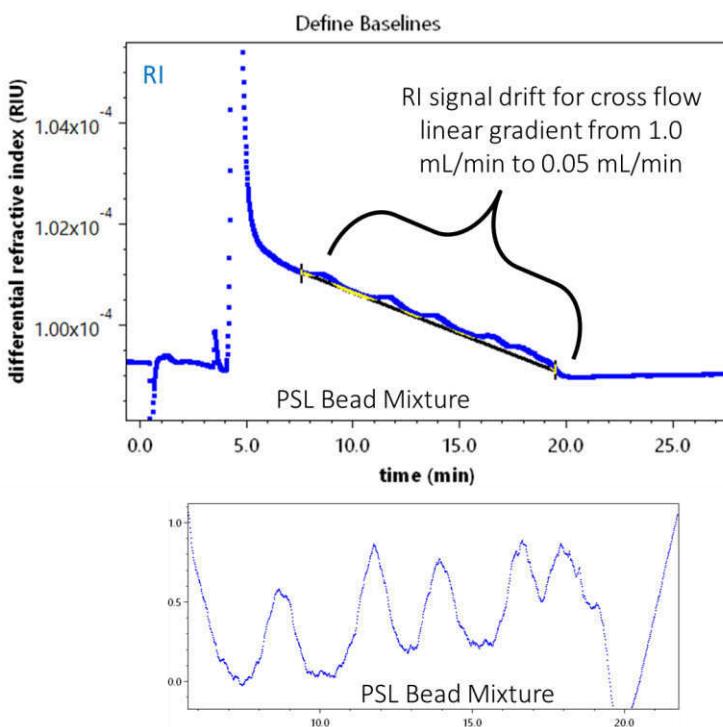


Figure 113. Use baseline subtraction when performing cross flow gradients in FFF-MALS.

Sometimes FFF-MALS conditions can be modified to reduce RI baseline drift when it is caused by poor salt permeability through the membrane. Switching from a 350 µm spacer with a 10 kDa RC membrane to a 490 µm spacer with a 30 kDa RC membrane, can reduce laminar flow near the accumulation wall and with larger MWCO membranes, better salt permeability and fluid flux can improve the RI signal. Higher sample loadings can also assist with better signal-to-noise. When possible, using a UV detector which is sensitive just to UV-active chromophores, can lead to data that is easier to process than when using RI signals alone.

In the next section, we will explore VISION DESIGN and simulating, analyzing, or refining methods. For more information about analyzing mobility data with the Eclipse Mobility Module, please refer to the [Eclipse Mobility Module](#).

VISION DESIGN Operation Workflow

VISION DESIGN is a method development and analysis software for the Eclipse hardware. VISION DESIGN can guide you through all stages of an experiment – from the planning phase via simulation and method development to analyzing your samples.

NOTE: Analyzing mobility data from EAF4 experiments is discussed in the [Eclipse Mobility Module](#).

You are able to set up virtual experiments based on FFF theory without any existing experimental data. Based on real experiments, you are also able to do further method refinement. A comprehensive design strategy helps you optimize your method. By applying your optimized method to your real sample, VISION DESIGN can even determine the size distribution for your particles. For the calculations, you can choose between FFF theory using patented algorithms and external standard calibration procedures based on hydrodynamic radius standards.

NOTE: It is important to enable appropriate signal selections in VISION RUN before analyzing data in VISION DESIGN. This is covered in the section, [Signal Selection Window](#).

Structure of VISION DESIGN

This section will give a short overview of the layout and organization of VISION DESIGN, highlighting specific information that may be relevant for different procedures.

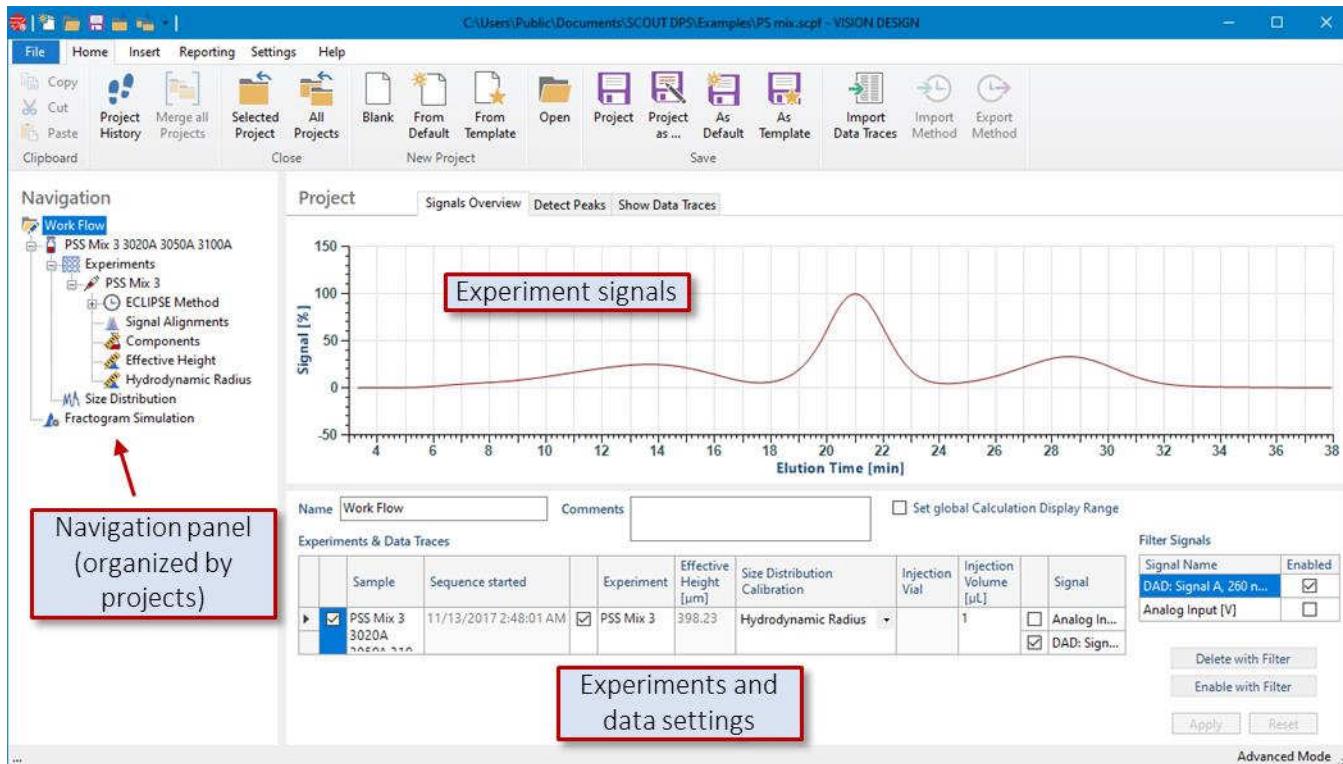
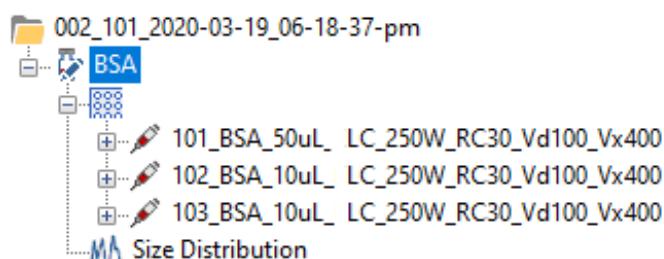


Figure 114. Overview of VISION DESIGN user interface

VISION DESIGN has a navigation panel on the left side used for viewing currently opened projects and EMDF files. In a Project, different measurements can be analyzed. Below the top menu bar under the Home tab, Projects can be created or opened. Key features will be explained in the next sections.

All the measurements from a sequence are stored in the navigation tree. Additional EMDF files from other sequences can be added to the current project by either drag and dropping from the file explorer or via the menu bar (Open). Doing this will merge the two projects together. The measurements will be sorted according to the sample names. If multiple measurements have the same sample name, these measurements will be combined under one sample tree. Every measurement will be displayed in the sample tree as an injection as shown below.



For every measurement, the method that was employed can be found under ECLIPSE Method. You can also find the effective height which is important for the method development and the calculation of the hydrodynamic radius from the FFF theory.

In the main window section called **Project**, all signals are visible. There are three different options you can use to look at the data. Under **signal overview** all signals are displayed which are marked with “detector” in the signal selection profile. Under **detect peaks** the set baseline and peaks will be displayed for every measurement. Under **Show data traces** all signals which are selected in the profile will be displayed. For every measurement it is possible to check if the set values for flow and pressure of the pump and the Eclipse matched the read values. Further information of these functions will be provided in the subsequent sections.

Typical Workflow with VISION DESIGN

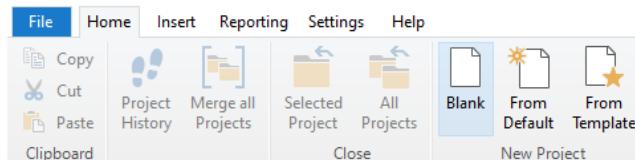
The following examples will show the typical process when using VISION DESIGN. This section will guide users through all the stages of an experiment – from the planning phase via method development to analyzing your results. For analyzing MALS data with ASTRA for molar mass, RMS radius and particle concentration calculations, please refer to the ASTRA User’s Guide or ASTRA Quick Guide.

Simulation

The first step is the planning phase. In this phase, it is important to know which samples will be measured and what is the goal of the measurement. Follow the step below to simulate an FFF method:

NOTE: If you want to simulate an FFF method for an unknown sample it is very helpful to have DLS data from your sample. These data will give you useful information about the hydrodynamic radius which you can use in VISION DESIGN. Before you start measuring unknown sample you should always measure a standard sample to check that the Eclipse system, the separation device, and the Wyatt detectors are working properly.

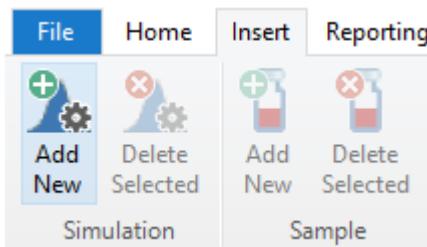
1. First launch VISION and then VISION DESIGN. Then navigate to Home → Blank (under new project)



2. In this new empty project, sample information can be inserted. Navigate to **Sample (new)** and add the sample information. One of the key parameters to know is the hydrodynamic radius of the sample. If the sample has multiple components an estimated value of the relative quantity can be noted in the mass fraction column. The amount of each component will only affect the appearance of the peaks (useful for quickly differentiating peaks visually) and will not affect the calculations based on FFF theory.

ID	Description	Mass Fraction [%]	Hydrodynamic Radius [nm]	Molar Mass [kDa]	Electrophoretic Mobility [10⁻⁸ m²/(V·s)]
1	Duke 20	59.00	10		
2	Duke 50	35.00	25		
3	Duke 100	6.00	50		
*					

3. The next step is to add the simulation. Navigate to **Insert → Add new** (under simulation)



4. In the fraction simulation level, sample and setup information can be inserted. There are three different sections on the right side of the simulation which should be edited in the following order: **Sample & Experiment**, **Separation Device** and **Time Table & Flows**. In the middle panel, the simulated result of the input separation method—calculated with FFF theory—will be shown.

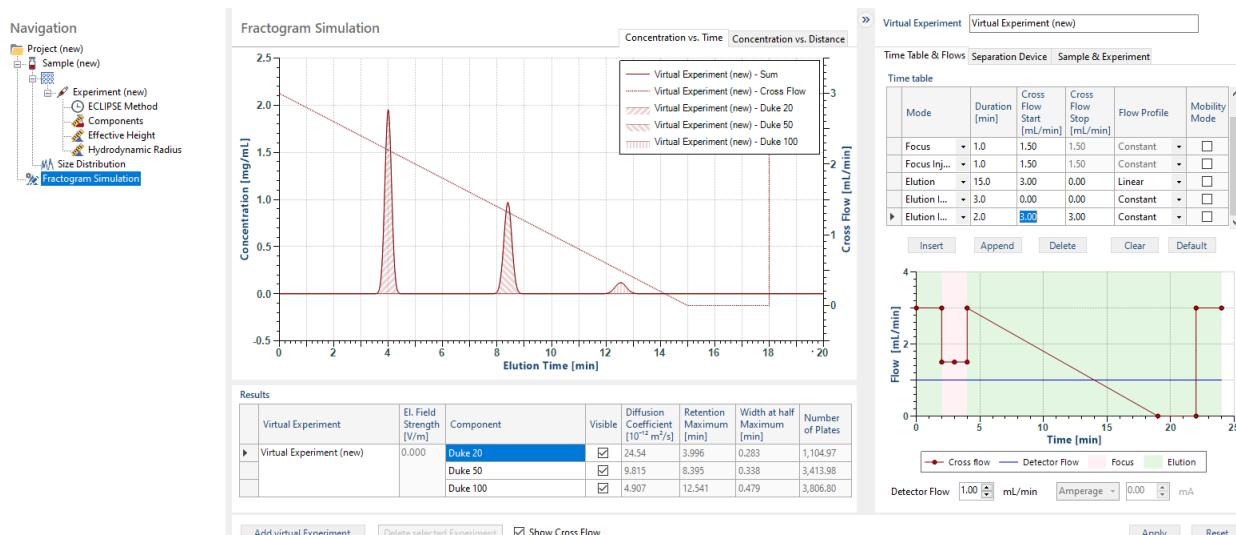


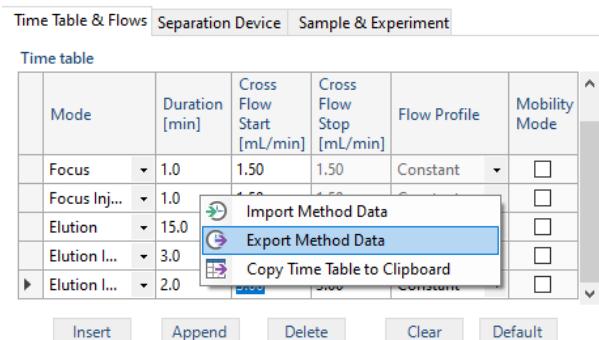
Figure 115. Example of Fraction Simulation in VISION DESIGN

Under **Sample & Experiment** the values which were input previously for the sample will be transferred; however, new components may also be added here. Additionally, some information about the solvent and the injection volume can be added. Under **Separation Device** the channel, the spacer, and membrane can be selected. You can observe how a different channel height (different spacer) can change the separation and peak shape. Under **Time Table & Flows** the method for the Eclipse separation will be shown. You can adjust the cross flow profile and duration of different elution steps to simulate the peak resolution and determine the experiment run time for the desired fractionation. A good starting point is a screening method with a linear gradient as you see above.

NOTE: The fractogram which is displayed in the middle panel shows only the signal after the last focus step when a short or a long channel will be used. Before this step the sample will not separate and therefore this time is not shown. Please keep in mind when comparing the x-axis time between the method (which includes focus time) and the simulated peaks (which shows just elution time post-focus).

5. Next, you can transfer the method which was created in the simulation in the previous step via the export function. By right-clicking on the Eclipse method table, **Export Method Data** can be selected and the method can be saved as an ECMF (Eclipse Method File). This method can be imported in VISION RUN by

navigating to the Eclipse method, right-clicking on the method table and selecting **Import Method Data**. The table will be updated and include the flow values and durations.

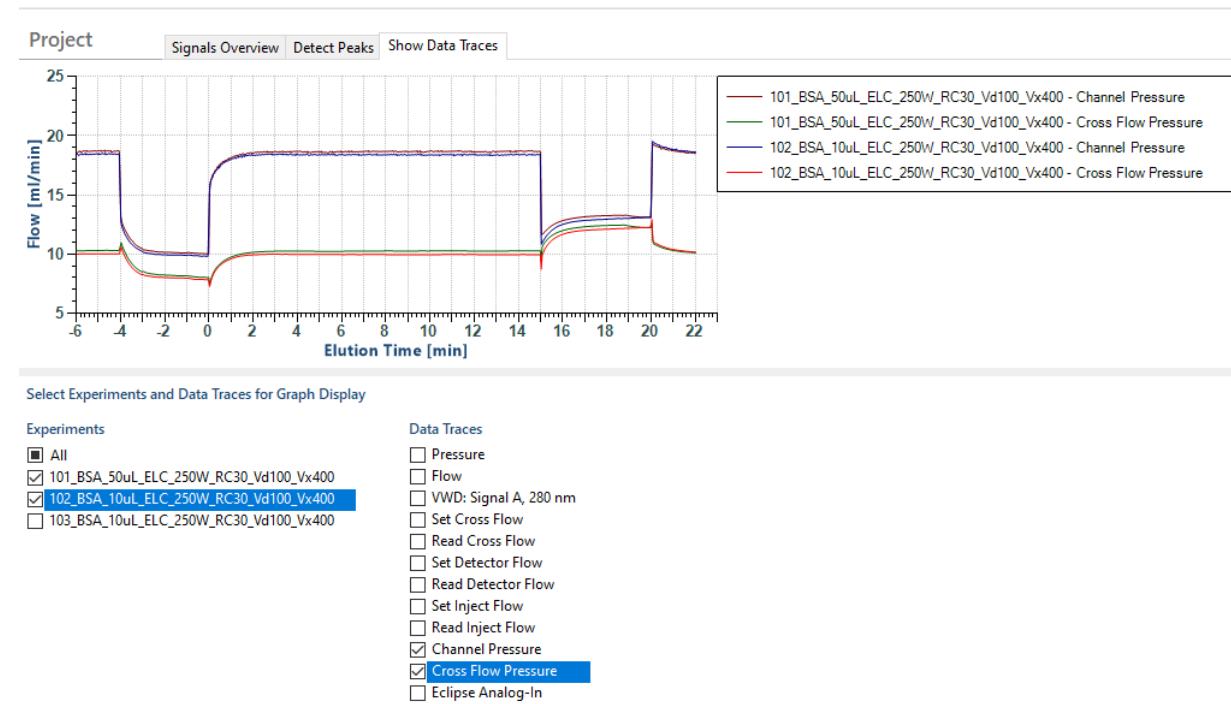


6. You can then setup up and run a sequence in VISION RUN with the simulated method. Please check other module settings, such as solvent reservoir, UV wavelength, and other HPLC settings when saving your new method.

Reviewing Data

After using the simulated method to run an experiment, you can usually use the experimental data to further optimize the method. The first step for improving the method further is to review the data.

1. Launch VISION DESIGN and open the EMDF from the sequence.
2. When opening the file, the signal from the detector (typically UV) will be shown in the **Signals Overview**. As a first step, the different system values should be checked in the tab **Show Data Traces**. Select the measurements and the system parameters via Graph Display beneath the graph. In this view, a comparison of the data or confirming that the flow rates and pressures were as expected can be done.



3. When comparing the actual data signals to the simulated data, it is expected to observe some differences. The example below shows that the result from the first measurement differs from the simulation. If the

results are different, this only means that the sample did not elute under perfectly ideal FFF theory. The combination of membrane, solvent, and sample may all contribute to a deviation from ideal FFF theory.

Fractogram simulation

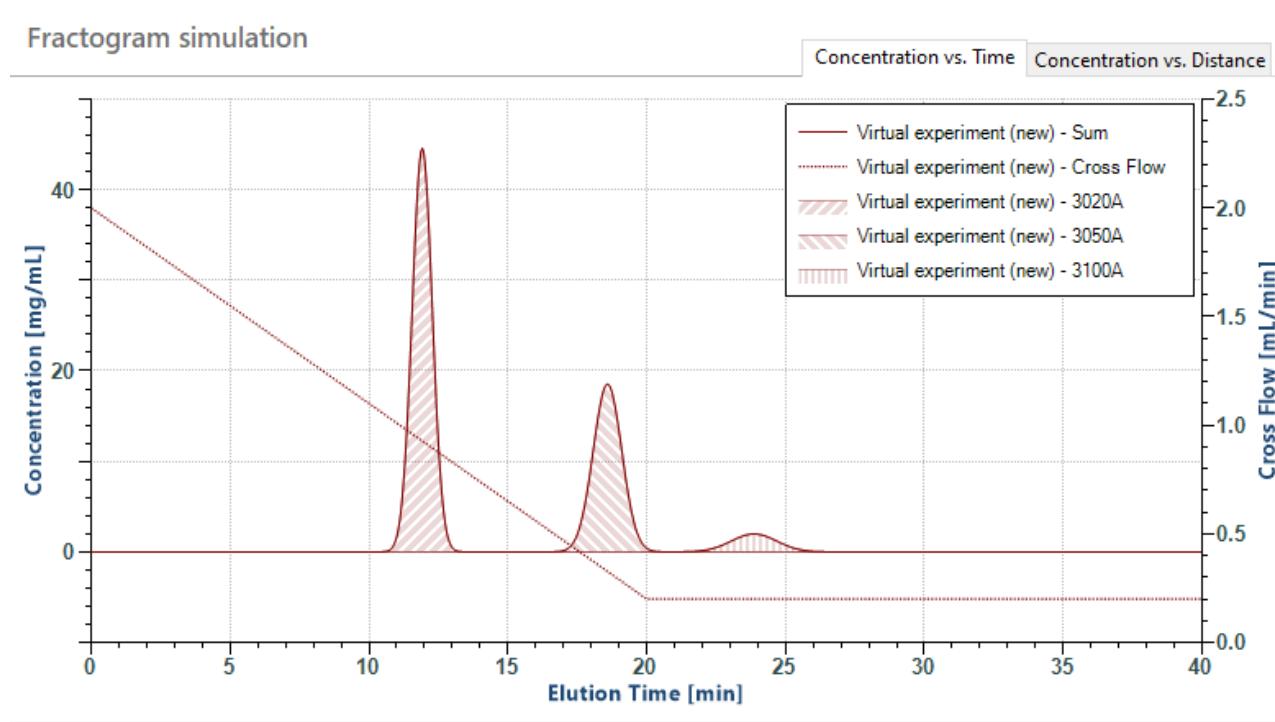


Figure 116. Example of the “Fractogram Simulation” used to simulate the Eclipse method within the VISION DESIGN software. In this case, the goal is to separate three different spherical polystyrene standard latex particles.

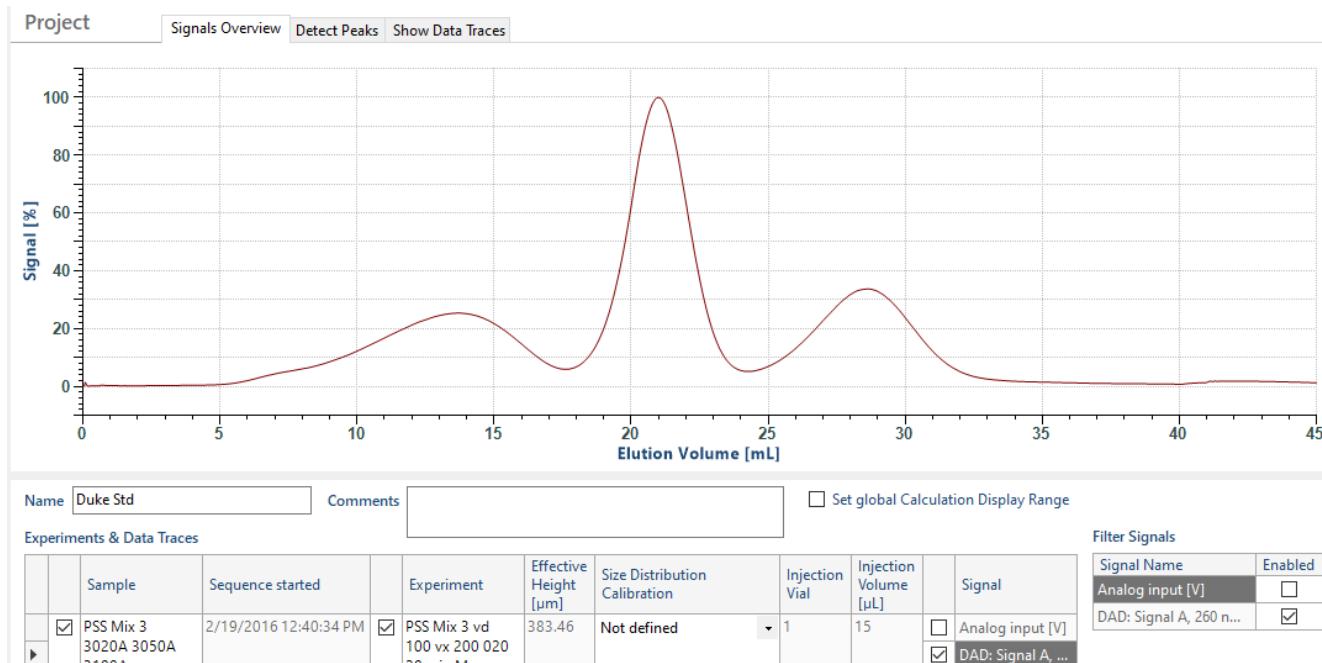
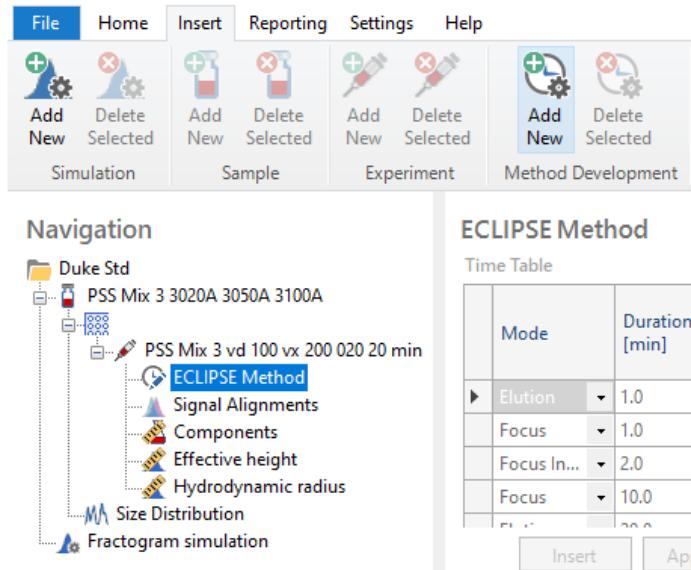


Figure 117. Screen shot of the “Signals Overview” within the Wyatt VISION DESIGN software of real data. Three different spherical polystyrene standard latex particles separated with the simulated method from Figure 116 above.

Method Refinement

In this section, method refinement from an existing data file to further improve the separation will be detailed. After reviewing the data, method development is recommended for optimizing the method.

- For adding the method development option in VISION DESIGN, first click on ECLIPSE Method. Then Navigate to Insert → Add new (under method development)



- The method development option will open as an additional submenu. In the middle panel, two different signals will be displayed. The red signal is the source: this is the signal of the measurement which was measured. The green signal is the target; this is signal which will be calculated if the Eclipse method settings are changed. On the right side, the Eclipse method table displayed. The method can now be changed to improve the separation from the measurement which was made before. If you are interested in more details regarding method development, please refer to [TN6005 Eclipse Aqueous Method Development Quick Start Guide](#).

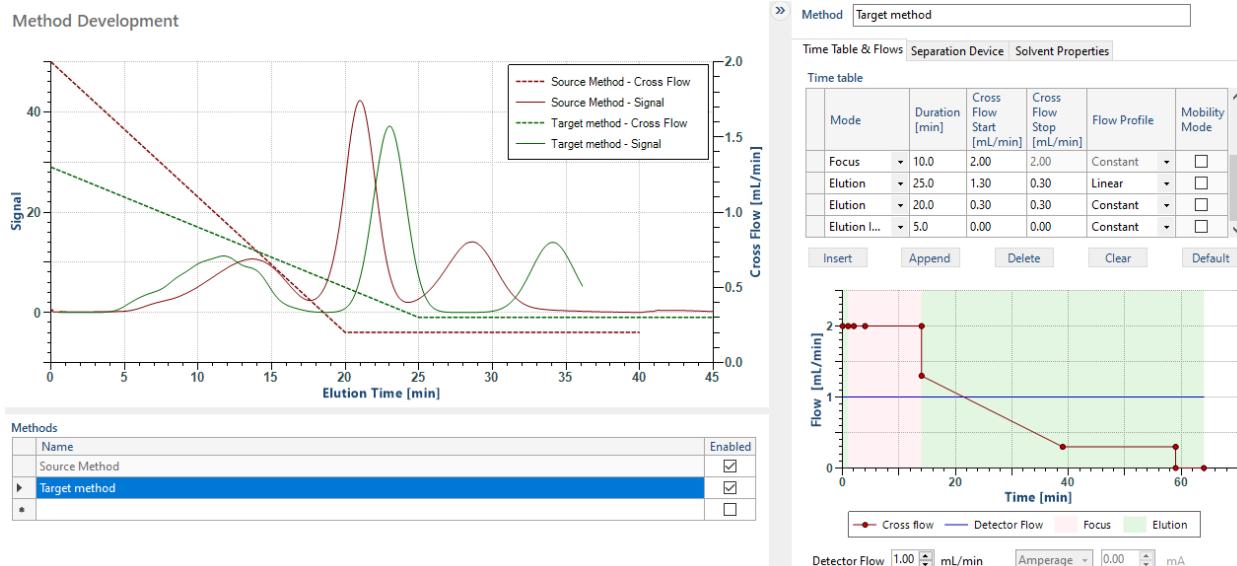


Figure 118. Screen shot of the “Method Development” within the VISION DESIGN software. The flow gradient and the height of the cross flow were changed in order to get a better separation.

3. The improved method can be transferred via the export function as shown earlier by right-clicking on the Eclipse method table. And subsequently imported into VISION RUN by right-clicking on the Eclipse method table.

Analyzing VISION DESIGN Data

Once a method is optimized and high-quality data is acquired, the data can also be analyzed further.

NOTE: Analyzing mobility data from EAF4 experiments is discussed in the Eclipse Mobility Module .

Peak Analysis

1. To compare different measurements from different optimizations, the EMDFs from different sequences can be opened. They will be merged into one project and the data can be saved under that project.

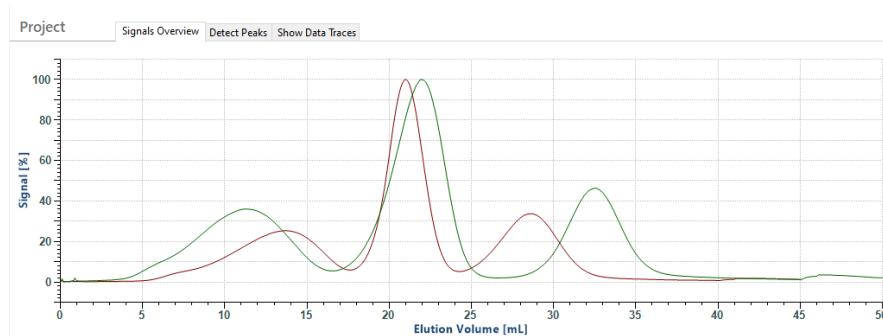


Figure 119. Screen shot of the “Signals Overview” within the Wyatt VISION DESIGN software. The red signal is the first measurement and the green signal is the optimized measurement.

2. The first important step before analyzing the data is to determine the calculation display range. In an FFF-MALS measurement, there are often some system peak spikes which occur because of the switching of the valves (for example when the valve is switching from elution to focus mode) and are visible in the signal. These peaks typically manifest in the early stages of the experiment or at the end of the experiment when cross flow reaches 0 mL/min. These signals are not important for the analysis and therefore should be excluded with the function **Set global Calculation Display Range**. Navigate to **Signals Overview** and check the checkbox **Set global Calculation Display Range** below the fractogram (Figure 8). Then the two yellow lines can be moved to exclude the area which should not be analyzed. To accept the changes, uncheck the checkbox and the fractogram will only contain the desired peaks. This step is very important because VISION DESIGN will function more reliably if these system spikes are excluded from the analysis.

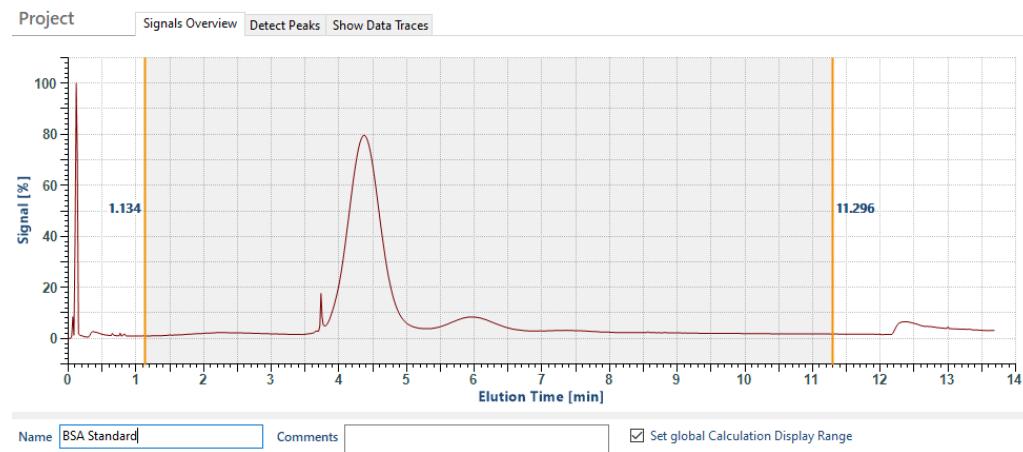
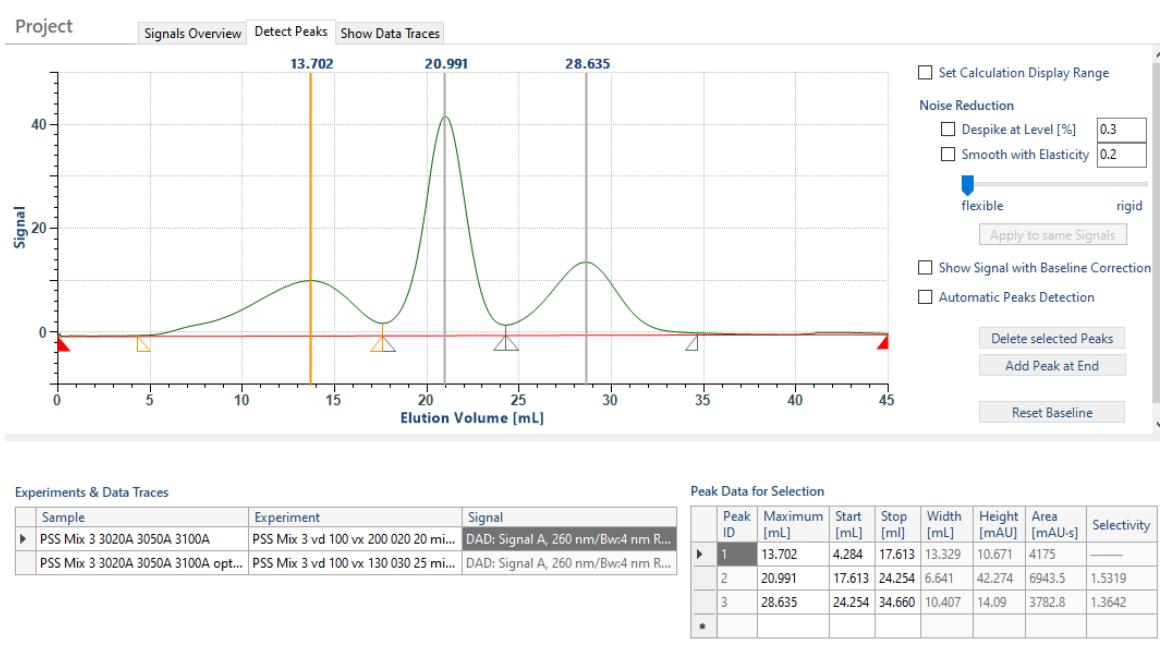


Figure 120. Example for the function Set global Calculation Display Range

- Under **detect peaks**, the baseline and peaks can be set or changed. VISION DESIGN will automatically set a baseline and peaks. The easiest way to set the peaks is to use the function **Automatic Peak Detection**. You can toggle the checkbox, set the area where VISION DESIGN should look for peaks, and then click on find. Peaks can be set also manually. Therefore, it is the easiest way to change the start and stop for each peak in the table **Peak Data for Selection** in the bottom right-hand corner. To add a new peak, simply click in the last empty row and add a start and stop time. To delete a peak just mark this line and use the DELETE key on your keyboard to remove the line.



- The **Peak Data Selection** table also contains some other useful data. To add these to the table just right click on the table and a menu will open. Select the checkboxes from the values you want to add.

Peak Data for Selection								
	Peak ID	Maximum [mAU]	Start [mL]	Stop [mL]	Width [mL]	Height [mAU]	Area [mAU·s]	Selectivity
▶	1	13.699	3.476	17.617	14.142	10.627	4141	—
	2	20.997	17.617	24.260	6.643	12.241	6030.4	1.5325
	3							

[Copy Table to Clipboard](#)

Select items to display

Maximum Area Content [µg]
 Start Selectivity Content [µmol]
 Stop Plates Concentration [mg/mL]
 Width Resolution Concentration [µmol/mL]
 Height Symmetry Recovery
 FWHM Component Signal to Noise

Close

5. One important point is the **Channel Height Correction**. After the correction, the method development simulation will also be more precise. The channel height is necessary because the membrane can swell in the channel or slightly different torque settings can lead to different effective heights in the channel. In FFF theory, the height of the channel is an important factor for the calculation of the retention time of the sample. With a known standard this can be calculated and used for all measurements made with this channel assembly. Navigate to **Effective height** under the experiment, where the correction should be done. At the bottom, **Reference from Experiment** (or from another file) can be chosen and a signal will be loaded into this window with the **play button** ▶. Then fill in the correct hydrodynamic radii for every peak in the table and the correction value will be automatically calculated. The **Apply to All** button will apply this channel height correction value to all experiments which were measured with the same channel.

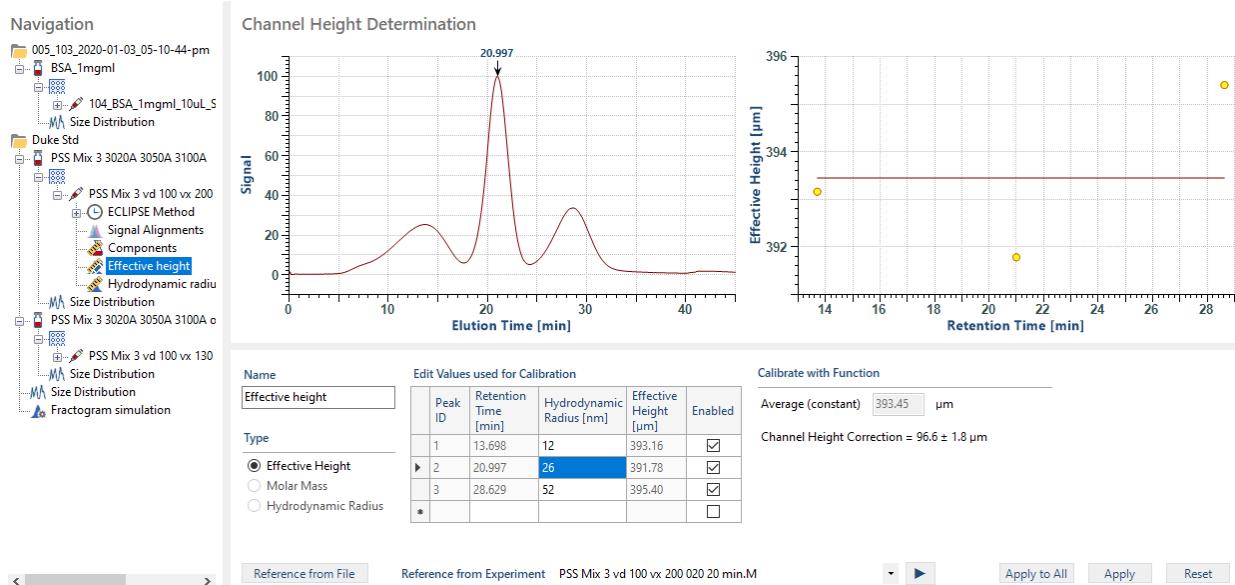
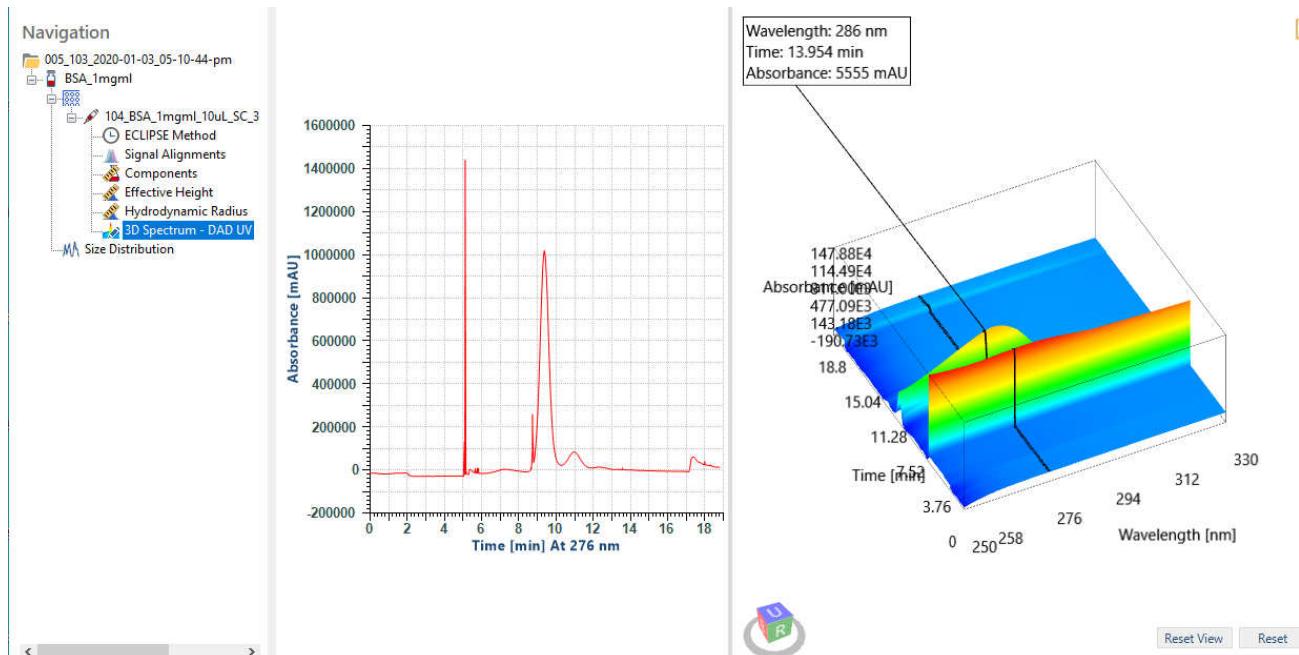


Figure 121. Example for the Channel Height Determination in VISION DESIGN

3D Spectral View

In VISION DESIGN, it is also possible to view a 3D spectrum of the DAD or FLD data. This allows for data across multiple wavelengths to be viewed simultaneously. DADs allow for continuous acquisition of different UV wavelengths at the same time. On the other hand, VWDs and FLDs can scan a wavelength range in order to acquire spectral data. VISION can digitally read data from both collection strategies to generate 3D plots. This allows for a quick inspection of several data streams and the ability to gauge the appropriate UV or FLD wavelengths for analysis. Please refer to the section, [Enabling 3D Spectral View in VISION DESIGN](#), for the correct steps to enable spectral data collection. The data can then be analyzed in VISION DESIGN per the instructions below.

- VISION RUN will generate a corresponding VISION DESIGN data file with each experiment run, that will include a Spectrum View node for those data. The spectral data for a collection can be found under the experiment header, as shown below. Once selected, the 3D spectral view will be provided in the right-side window and the 2D graph showing the detector signal versus time will be displayed on the left. This layout is shown below.



- To control and change the view of your spectral data:
 - Right-click and drag to rotate the spectral view (alternatively, the arrow keys can be used).
 - Middle-click and hold to pan (alternatively, the Shift + arrow keys can be used).
 - Click on the box in the lower left of the spectral view to adjust the view to a particular direction. (For example, the 'U' face of the cube will show the 3D graph from directly above.)
 - Left-click on the graph itself and drag to highlight and select an area of interest and focus on only that section. Clicking 'Reset' in the lower right of the view will undo this.
 - Select 'Reset View' in the lower right to reset the camera angle and zoom.
- If you would like to use the spectral data in a report, the clipboard icon at the upper right can be left- or right-clicked to copy the image of the current graph showing absorbance versus wavelength and time. Pressing CTRL + C will copy both the graph and the callout that displays the wavelength, time, and absorbance. The table data can also be copied, and saved as a .csv file.

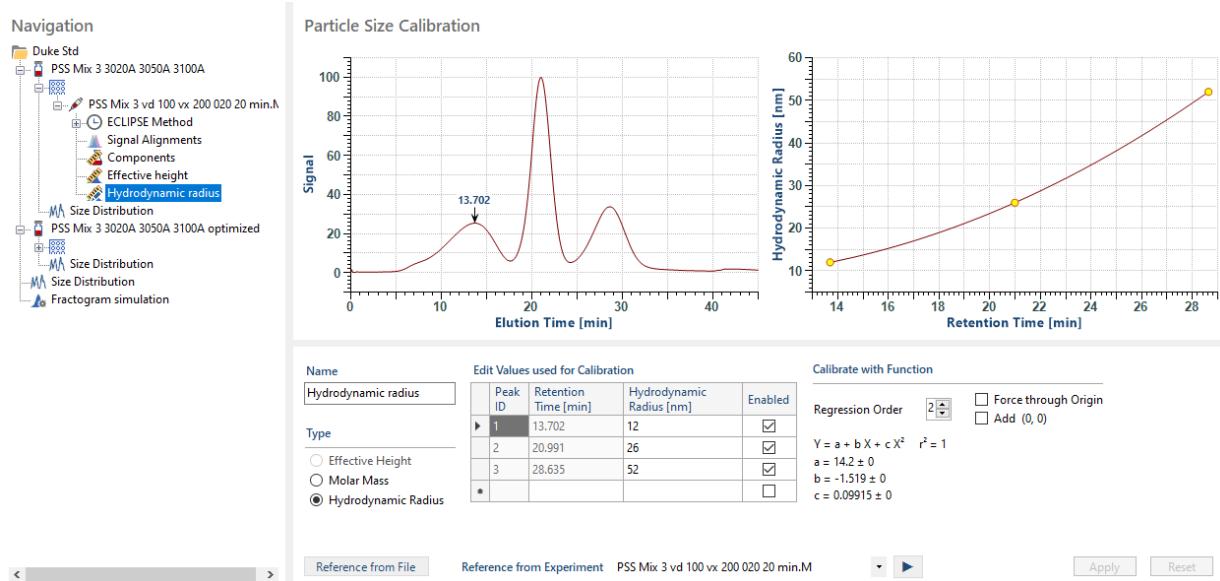
Particle Size Calibration

If you are interested in external standard calibration procedures based on hydrodynamic radius standards, you can calibrate the channel. This is particularly advantageous when measuring very large particles—as FFF can effectively separate particles up to 10 microns in radius whereas MALS has an upper characterization limit of 1 micron in radius with a shape model. Large size standards can be used to estimate the size of a larger, unknown sample that may not be easily characterized by MALS.

- After performing the channel height correction this measurement of the standard can be also used for the calibration. There is only one requirement: the unknown sample and the hydrodynamic radius standards

have to be measured with the same Eclipse method conditions (not only the same channel, but also the same flow rates and cross flow profile).

2. Navigate to **Hydrodynamic Radius** and load the channel height correction experiment with the standards with the **play button** . After adding the hydrodynamic radii for the standards, a calibration curve will be calculated.

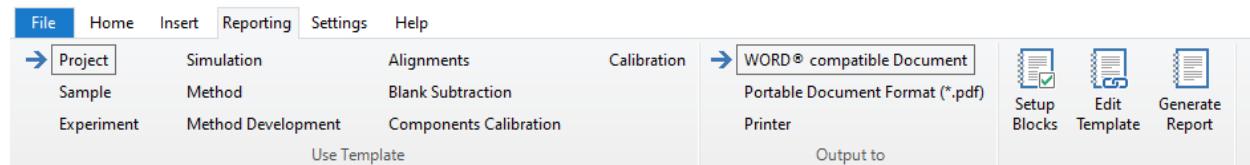


3. Under **Size distribution** the results will be displayed. The results of the calibration with the hydrodynamic radius standards can be compared with the results from the FFF theory.

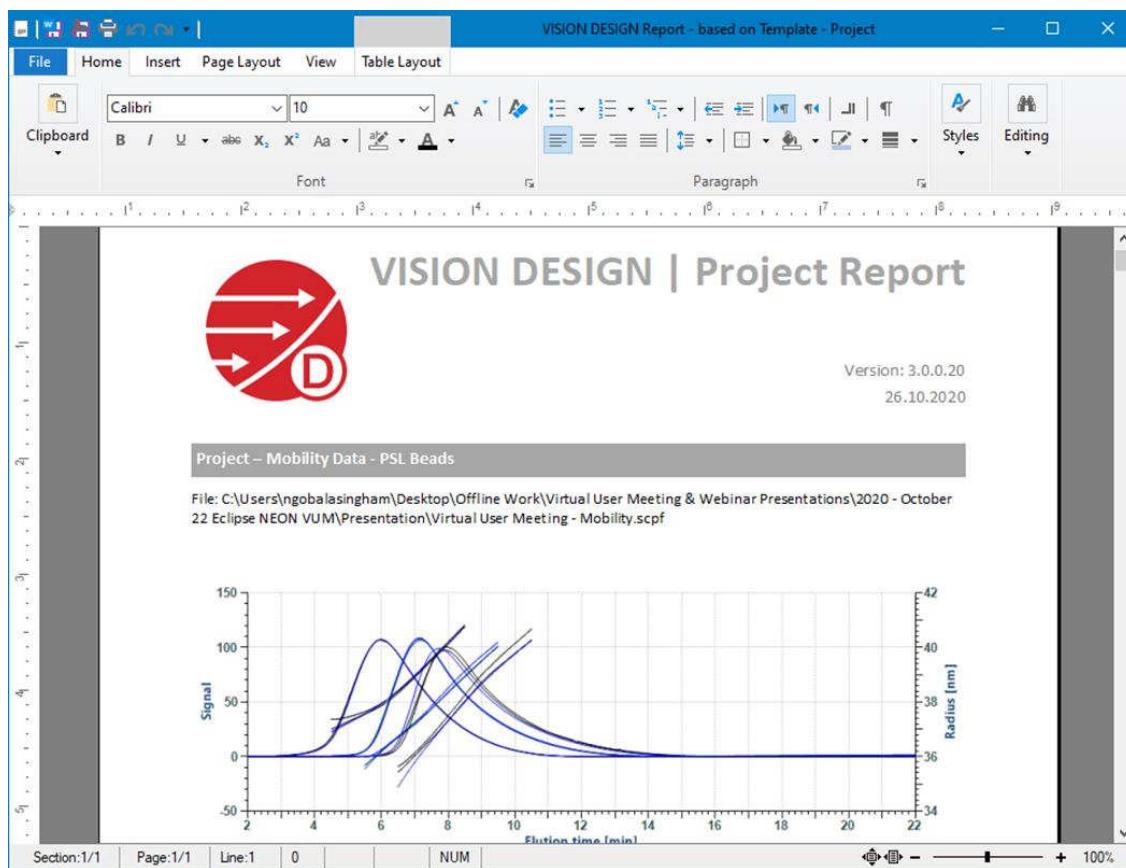
Reports in VISION DESIGN

In VISION DESIGN, you can also generate report documents for your projects. There are different options.

1. To find the options for the report navigate to **Reporting**.



2. Different templates for the report are available. Select a template and choose **edit template** to have a look at the report or to customize the report.

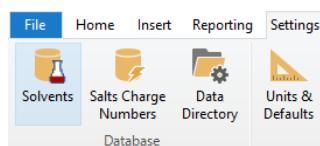


3. The output can also be chosen. The report can be printed or saved as a Word or .pdf file. Just by clicking on **generate report** the report will be saved and/or printed.

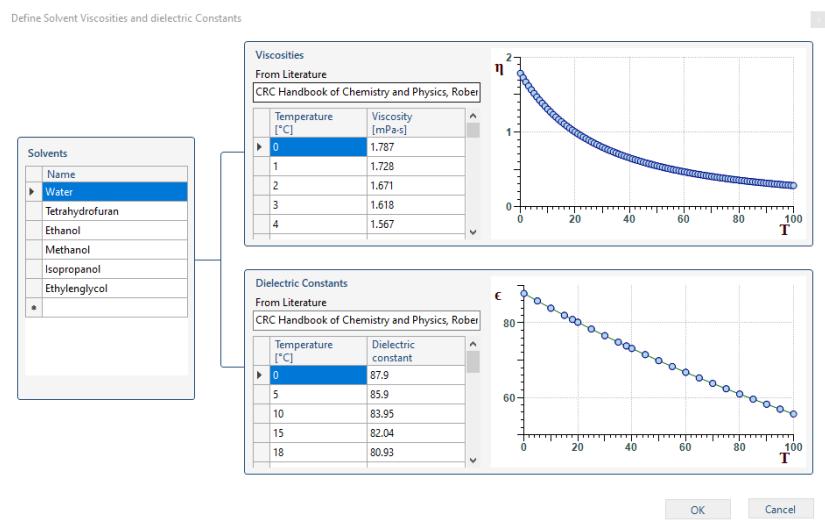
Solvent Settings & Adding New Solvents

As FFF theory uses the viscosity of the solvent to calculate the retention times, the different solvents can be added and edit using a database.

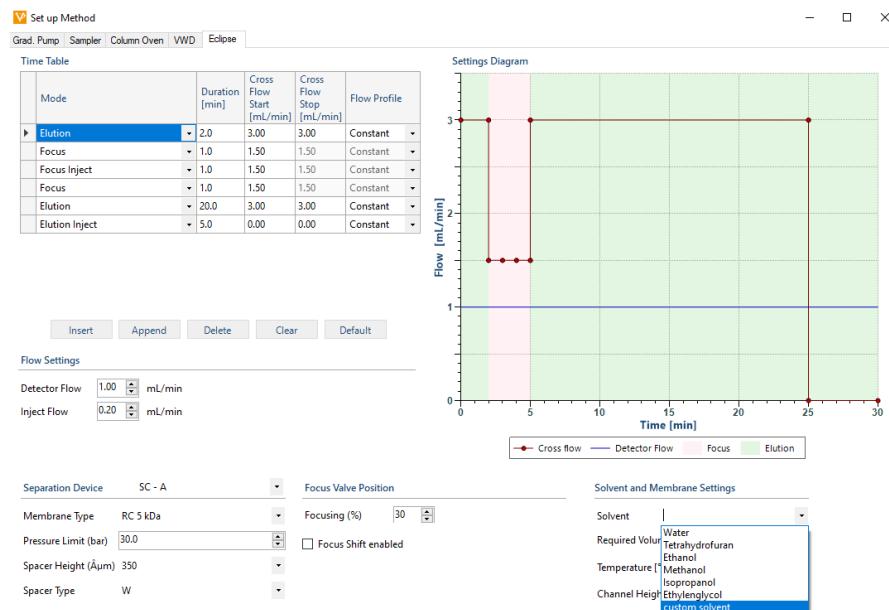
1. Navigate to **Settings → Solvents** (under database).



2. Some solvents come pre-configured and are stored in the database with their viscosity and temperature values. A new solvent can be added by just clicking in the last line of the table. A name for the solvent can be added and values for the viscosity and temperature dependence can be copied from the literature or from outside experiments.



3. The added solvent will now also be added in VISION RUN in the method in the Eclipse Method editor.

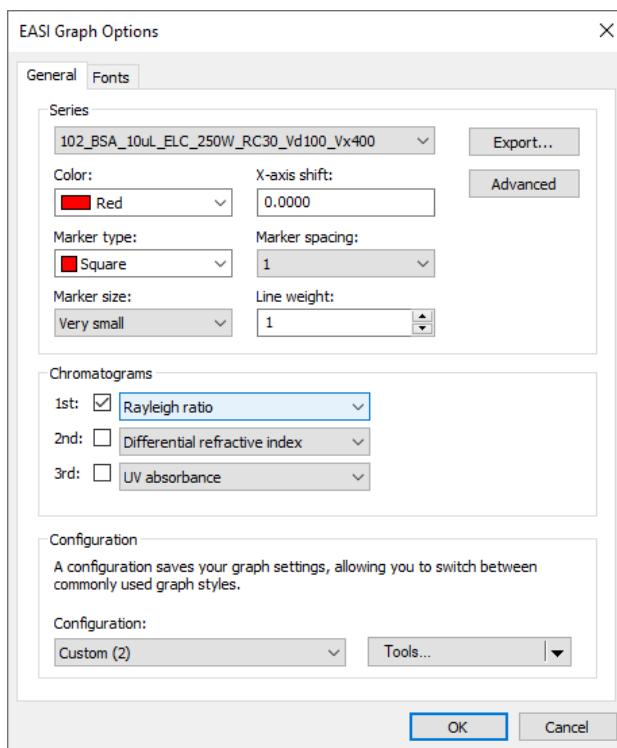


Import Data Traces

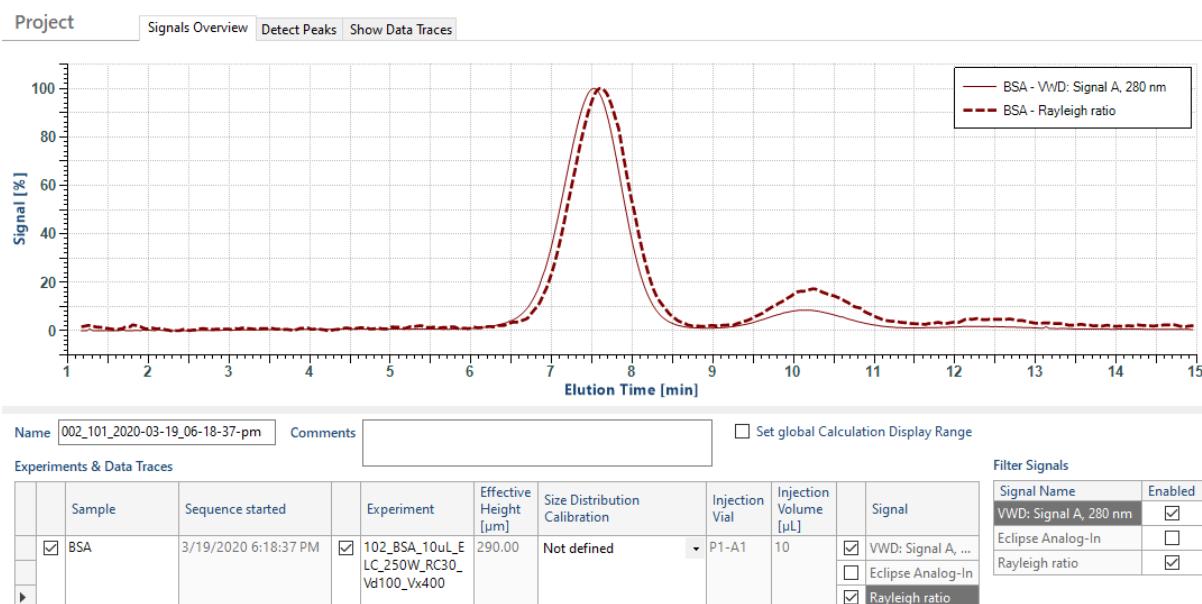
The EMDF contains detector signals that were configured in VISION RUN; however, other detector signals can be imported into VISION DESIGN and be used as well. These include data traces from ASTRA data files. VISION DESIGN can import .txt or .csv files. In this example workflow below, the MALS data trace from the 90-degree detector will be imported into VISION DESIGN in order to have both a UV and a MALS signal to perform method development with.

1. Launch ASTRA and open the experiment that has the desired signals for import into VISION DESIGN. The data should be analyzed, including an appropriate baseline and peaks should be selected. It is important that the ASTRA file has exactly the same name as the measurement in the EMDF. Otherwise, the MALS data traces will not be assigned to the correct measurement in VISION DESIGN.
2. Open the **EASI Graph** and choose the **Display: Chromatograms only**. Only the MALS signal should be displayed, which can be done by right-clicking on the EASI Graph and selecting **edit**. Under Chromatograms

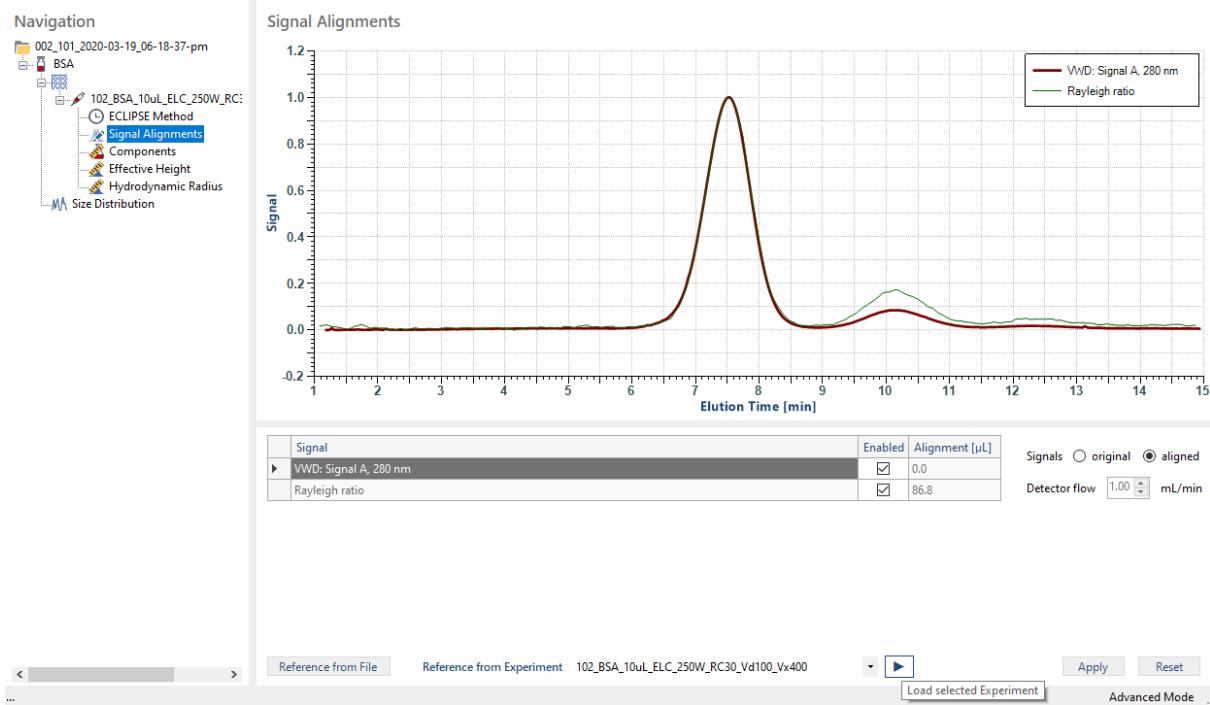
there are three different options are shown. For displaying the MALS data, the **Rayleigh Ratio** should be selected. You can press **OK** to save the modifications.



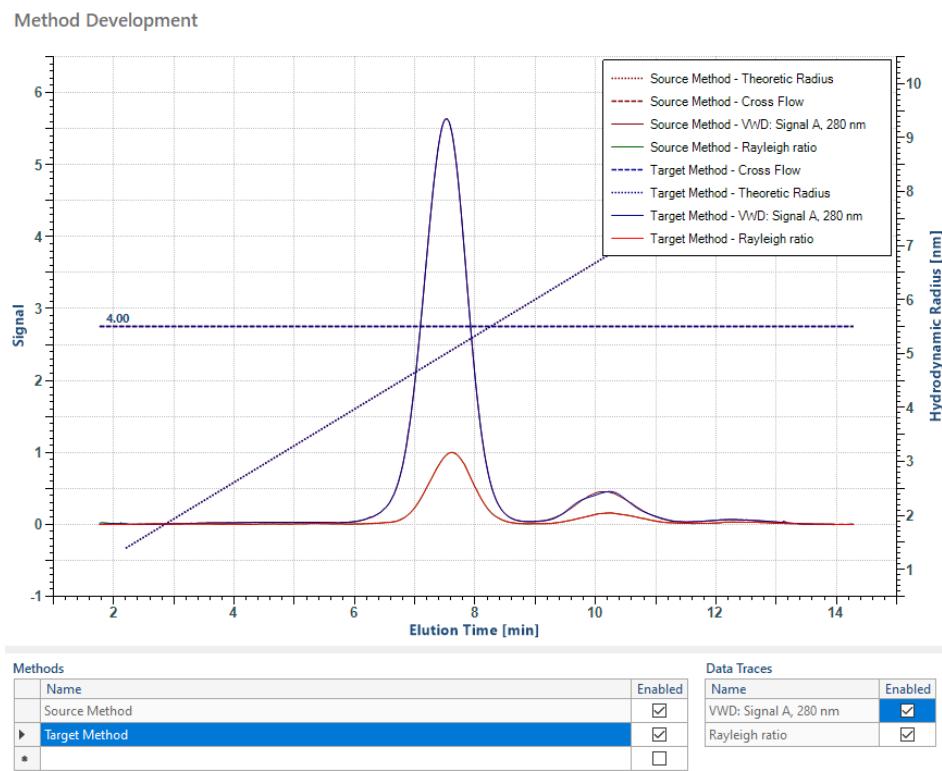
3. To export the file right click on the graph and select **edit**. The same window in the step above will open. Click on the button **Export...** the data trace can be saved as a .csv file.
4. Launch VISION DESIGN and open the file where the MALS data should be imported. To import the data traces just drag and drop the .csv file onto the graph of the **Signals Overview**. The signal of the MALS detector will be added to the graph and as a signal in the table.



5. The two signals can be aligned by selecting the **Signal Alignment** node under the experiment name on the left navigation panel. Load the measurement and apply this. The alignment will be calculated automatically.



- The method development procedures described above can now be done with either the signal from the UV detector or the signal from the MALS detector.



Eclipse Mobility Module Guide

Eclipse Mobility consists of the **Mobility Module** (the control box) and the **Mobility Channel**. This section will cover key information regarding Eclipse Mobility, especially where it may differ from the Eclipse operations without Mobility. Mobility enables the addition of an electrical field to the fluid flow fields of AF4 (thus EAF4). In addition to the cross flow that is applied, the electrical field can also be applied (and in different field directions) as shown in Figure 122.

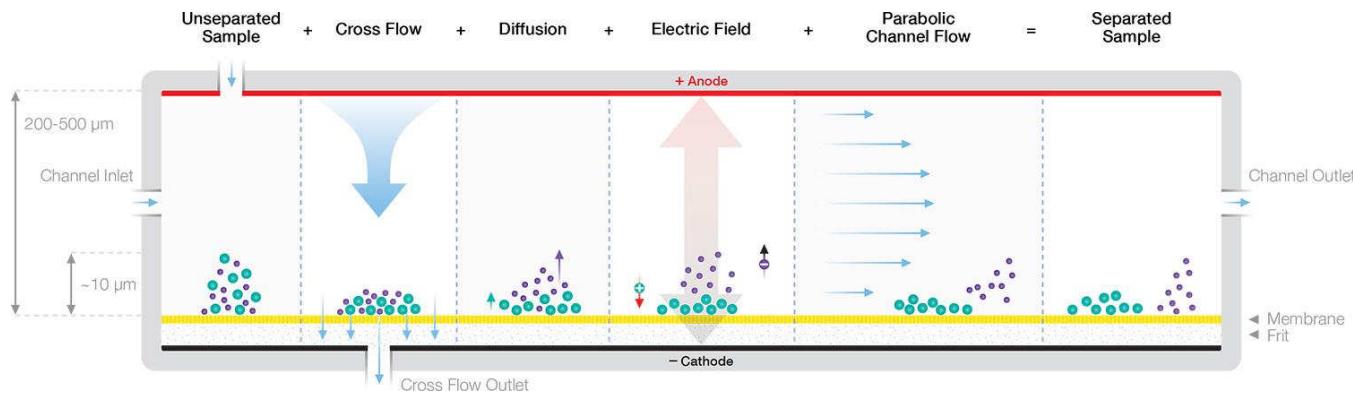


Figure 122. Forces in play during EAF4 separations.

As a result—and depending on the electric field—samples with some surface charge may be drawn to or from the electrodes. This causes them to enter either faster or slower parabolic flow regimes. An example is illustrated below for two samples with identical size (would behave identically under cross flow) in Figure 123.

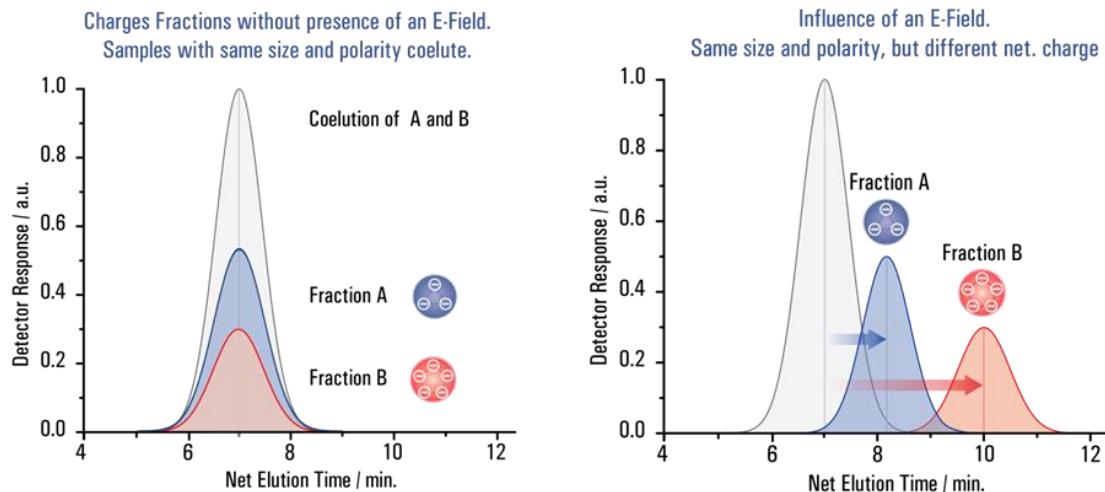


Figure 123. Without an electrical field, two species with the same size but different surface charge may elute at the same time. With an electrical field—and depending on the field direction—different net charges can be exploited with EAF4.

With identical cross flow gradients with increasing electrical field, a sample is expected to shift respectively. It is this shift in retention time that enables determination of electrophoretic mobility. More theory is provided in Appendix B – Basic Theory.

Eclipse Mobility Module Specifications

With an applied electric field, it is important to understand the chemical and salt limitations of the system.

With regards to pH compatibility, the pH limitation will mostly be defined by the membrane and flow cell, so typically pH 2 - 10. Strongly basic solutions (pH > 11) may etch the flow cell over long periods of time.

With regards to maximum salt concentration, the system is chemically compatible with higher salts (50 – 75 mM NaCl); however, the practical limit is usually lower. Mobility is best performed with lower salt concentrations (0 - 15 mM NaCl). There are two challenges:

- When we apply a current, the field is inversely proportional to the solvent conductivity. So a high conductive solvent will generate low electrical fields, and thus low peak shifts. If you attempt to use higher currents to overcome the low fields, you will generate air bubbles in the fluidics.
- If you will generate air, you may need to increase the pressure on the system to keep bubbles from forming. If you need to increase the pressure, the limitation is the Optilab which can only handle 2 bar of back pressure. If you bypass the Optilab, the pressure limit for the Mobility box is 6.7 bar.

Eclipse Mobility Module Installation

If you have an Eclipse Mobility module, the installation and the connections require a few additional steps to those described earlier in [Hardware Installation Quick Guide](#). On the back of the Mobility module, there will be a power cable (powered by the Eclipse rear panel), LAN connection, and connection to the mobility channel. These are summarized in Figure 124.



Figure 124. Rear panel of the Eclipse Mobility module and the keyed connection to the mobility channel and power connection to the Eclipse rear panel.

The mobility module is powered by the Eclipse and so does not require a dedicated power cable. Power to the channel is provided via a keyed connector.

Eclipse Mobility Module Configuration

The Eclipse can be configured in VISION RUN to have a Mobility addon. The default static IP address for the Mobility Module is 192.168.254.50 with subnet mask 255.255.255.0. Please refer to Step 5 in the section, [VISION RUN Instrument Configuration](#), for more information on configuring the Eclipse with Mobility Module.

Eclipse Mobility Fluid Connections

The Eclipse Mobility module is typically installed after the last analytical detector, often the RI detector. Your Mobility ship kit will come with pre-swaged tubing to connect to the Mobility module. A flow diagram of the entire system is provided in Figure 125. Because the Optilab cannot handle much backpressure due to the sensitive nature of the flow cell, it is recommended to install 0.030" I.D. tubing (often color-coded as green PEEK tubing) from the Optilab outlet to the Mobility module's conductivity cell inlet. Then, the conductivity cell is connected to the pH cell via a short piece of green 0.030" I.D. PEEK tubing. The conductivity and pH cell are connected via Super Flangeless™ fittings. A set of tubing for flushing the cells for either checking or calibration is included with the Mobility hardware kit.

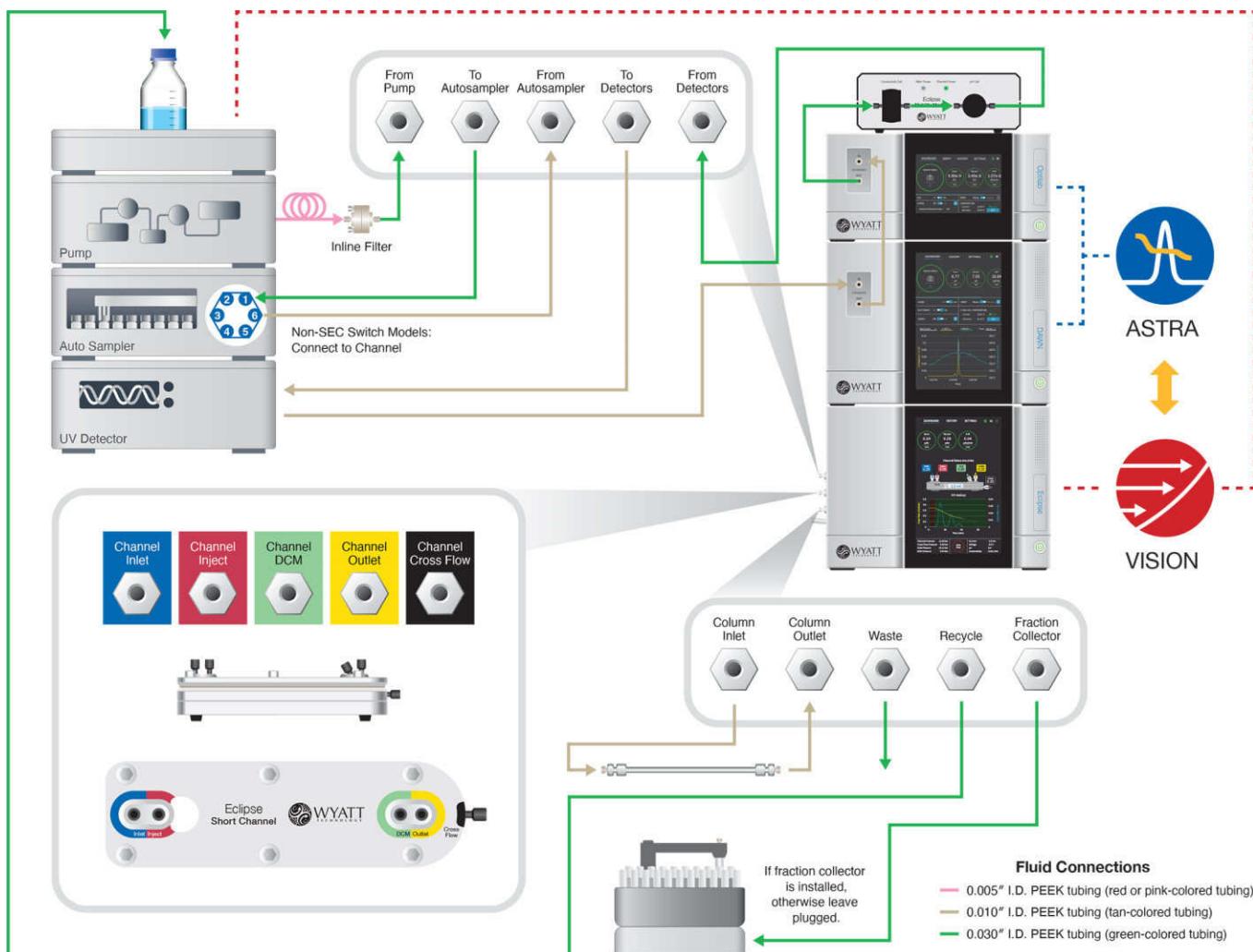


Figure 125. Flow diagram for Eclipse system with Mobility module

Eclipse Mobility Channel Installation

Installation of the membrane and general cleaning strategies are similar to that of standard channels, as described in the section, [Eclipse Channel Overview and Membrane Installation](#). There are several key differences that will be expanded on in this section, which include:

- Insulating washers: The mobility channel bolts are secured with two washers—one of which is an insulating fiberglass washer that prevents the channel from shorting.

- Ten bolts: The two blocks of the mobility channel are secured by ten bolts, though the recommended torque settings are the same as other channels.
- Platinum electrodes: The mobility channel has two platinum electrodes that are powered by an electrical cable.

Mobility Channel Power Cable

On the exterior of the channel, the design and temperature regulator are similar to other channels. The Mobility channel contains two platinum electrodes that are powered by a cable from the Mobility module on the top and bottom block. This power cable is equipped with a 4-pin connector that can only be installed in one orientation, as shown below in Figure 126. This prevents accidental reversal of the polarity and safe installation.

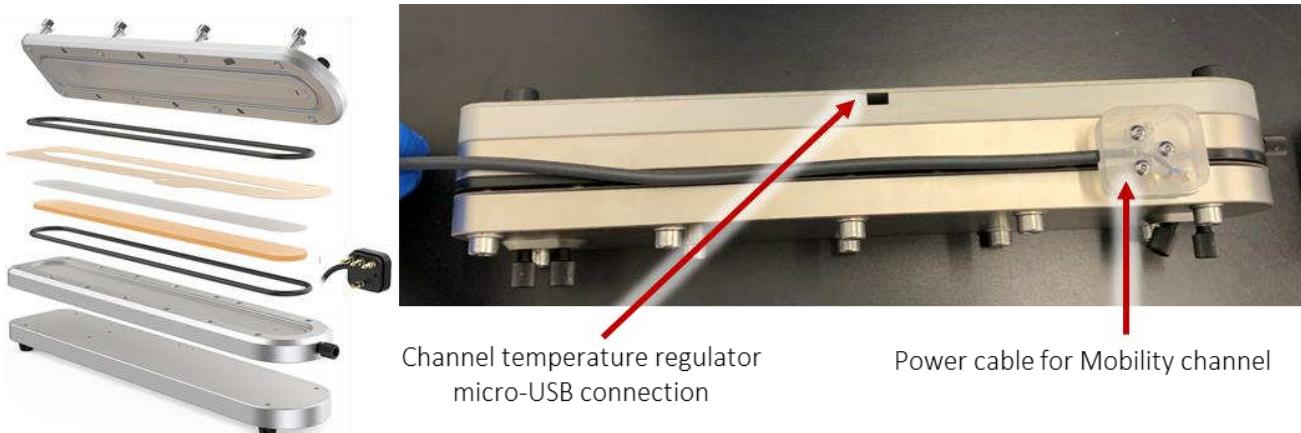


Figure 126. Mobility channel and power cable connecting to channel. The channel is also equipped with a channel heater.

Mobility Channel Assembly

The mobility channel has some differences from the other channels. In order to avoid shorting the channel and keep the platinum electrodes from contacting each other, the bolts that secure the two blocks of the channel together are equipped with an insulating washer, as shown in Figure 127. The insulating fiberglass washer should be placed on the bottom, with the stainless-steel washer on top, and the bolt is secured through the two washers.

CAUTION: If the mobility channel will be used as a standard long channel (i.e., no connection to the mobility module), it is important to bridge the top and bottom electrodes with a shortening plug that will prevent unwanted electric fields. Please contact Wyatt Technology if a shorting plug (p/n 164745) is needed.



Figure 127. Mobility channel bolts have an insulating fiberglass washer.

Like other channels, the mobility channel does have two O-rings for the top and bottom blocks and the ceramic frit is matches the channel geometry. The top and bottom blocks have alignment pins and pinholes at the bottom of the channel, and sealed with a silicone O-ring. This assembly is shown in Figure 128.

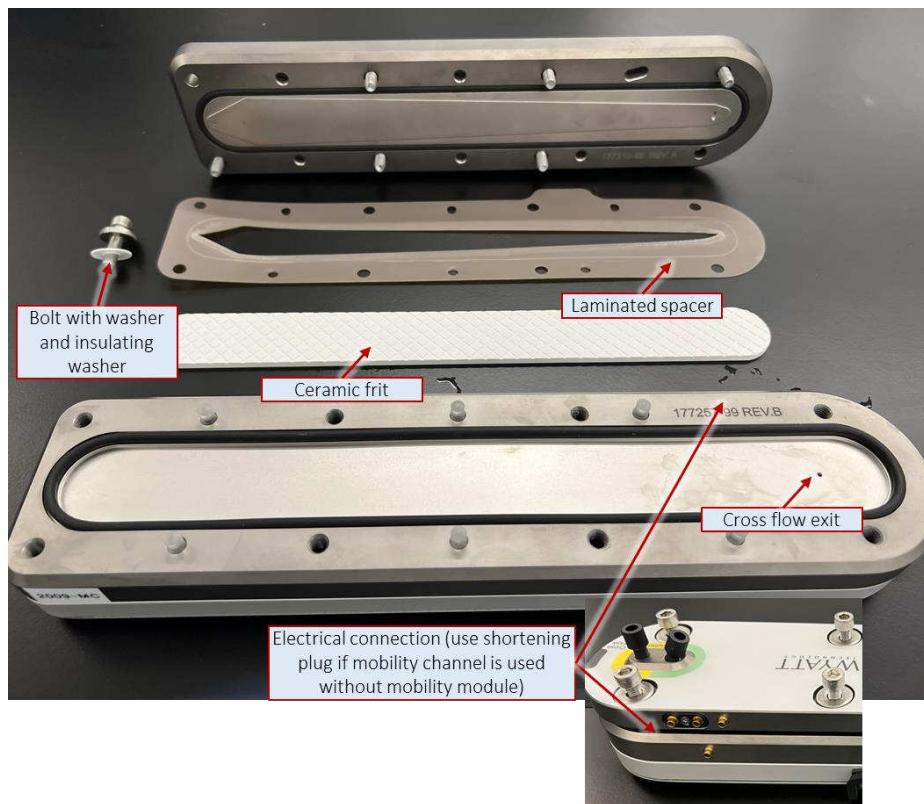


Figure 128. Mobility channel assembly. (Top) Ceramic frit alignment pins on the bottom block of the channel. (Middle) Ceramic frit installed. (Bottom) Membrane and silicone O-ring installed. **Not shown:** Spacer installed on top of O-ring and membrane.

When assembling the channel, the torque settings and procedure are similar to long channels. and is illustrated in Figure 129.



Figure 129. Order for tightening the bolts in the mobility channel. The same procedure of starting with 1 Nm, 2 Nm, then 3 Nm, then 4 Nm is applied for variable height versions. The fixed height channels are tightened in the same crisscross pattern but no torque wrench is required.

Please refer to the section, [Eclipse Channel Overview and Membrane Installation](#), for other relevant information for channel assembly. All mobility settings and parameters are defined in the software.

Mobility Experiments with VISION RUN

Methods are created in VISION RUN for Mobility experiments in a similar nature to other FFF-MALS experiments as described in [VISION RUN Operation Workflow](#).

The key difference with the Eclipse Mobility configured is that you can enable mobility mode for desired rows in the Eclipse timetable as shown in Figure 130.

Time Table						
	Mode	Duration [min]	Cross Flow Start [mL/min]	Cross Flow Stop [mL/min]	Flow Profile	Mobility Mode
▶	Elution	1.0	1.00	1.00	Constant	<input type="checkbox"/>
	Focus	1.0	1.00	1.00	Constant	<input checked="" type="checkbox"/>
	Focus Inject	5.0	1.00	1.00	Constant	<input checked="" type="checkbox"/>
	Focus	2.0	1.00	1.00	Constant	<input checked="" type="checkbox"/>
	Elution	20.0	0.50	0.50	Constant	<input checked="" type="checkbox"/>
	Elution	1.0	0.50	0.00	Linear	<input checked="" type="checkbox"/>
	Elution Inject	10.0	0.00	0.00	Constant	<input type="checkbox"/>

Enable mobility mode for desired steps

For equilibration, it is recommended to apply mobility during Focus, though mobility will affect separations during the main elution steps.

Figure 130. Mobility adds an extra column to the Eclipse time table.

The mode and applied current or voltage is defined under Flow Settings in the Eclipse method editor window, as shown in Figure 131. The mode—either current or voltage—and the value for either mA or V can be input. The current mode is most commonly utilized. These modes and values can also be defined in the sequence table.

Flow Settings					
Channel Flow	1.00	mA/min	Recycle Behavior	Current Mode	<input checked="" type="radio"/>
Inject Flow	0.20	mA/min	Recycle XF	Voltage Mode	<input type="radio"/>
Detector Flow	0.50	mA/min	Fractionator Mode	<input type="checkbox"/>	Current 0.00 mA

Select desired mode and current/voltage. This can be edited in the sequence table as well.

Figure 131. In the Eclipse method editor window, the mobility mode and value can be set for the method.

A minimum of three different current or voltage values are required for determining electrophoretic mobility, so a minimum of three runs are needed. Although a unique method can be generated for each current or voltage value, the mobility settings can also be conveniently adjusted in the sequence table so that only one method needs to be created. The steps for which mobility mode are enabled are always defined by the method, and it is recommended

to apply mobility settings during focus and main elution separation steps. The mobility mode and values can be adjusted in the sequence table as shown in Figure 132.

NOTE: For the Eclipse Mobility module, the maximum current is +/- 50 mA. The maximum operating voltage is +/- 30 V. The modes are either constant current or constant voltage.

Sequence Table												
	Enabled	Vial	Volume [µL]	Inj./Vial	Sample Name	In/dc [mL/g]	UV Ext. [mL/(mg·cm)]	A2 [mol·mL/g ²]	Mobility Value	Mobility Type	Comment	
1	<input checked="" type="checkbox"/>	1	20.00	3	50 nm PSL	0.1850	6.670E-001	0.0000E+000	0	Current		
2	<input checked="" type="checkbox"/>	2	20.00	1	blank	0.1850	6.670E-001	0.0000E+000	0	Current		
3	<input checked="" type="checkbox"/>	1	20.00	3	50 nm PSL	0.1850	6.670E-001	0.0000E+000	-1	Current		
4	<input checked="" type="checkbox"/>	2	20.00	1	blank	0.1850	6.670E-001	0.0000E+000	0	Current		
5	<input checked="" type="checkbox"/>	1	20.00	3	50 nm PSL	0.1850	6.670E-001	0.0000E+000	-2	Current		
6	<input checked="" type="checkbox"/>	2	20.00	1	blank	0.1850	6.670E-001	0.0000E+000	0	Current		
7	<input checked="" type="checkbox"/>	1	20.00	3	50 nm PSL	0.1850	6.670E-001	0.0000E+000	-3	Current		

The mobility mode and values can be defined in the sequence table.

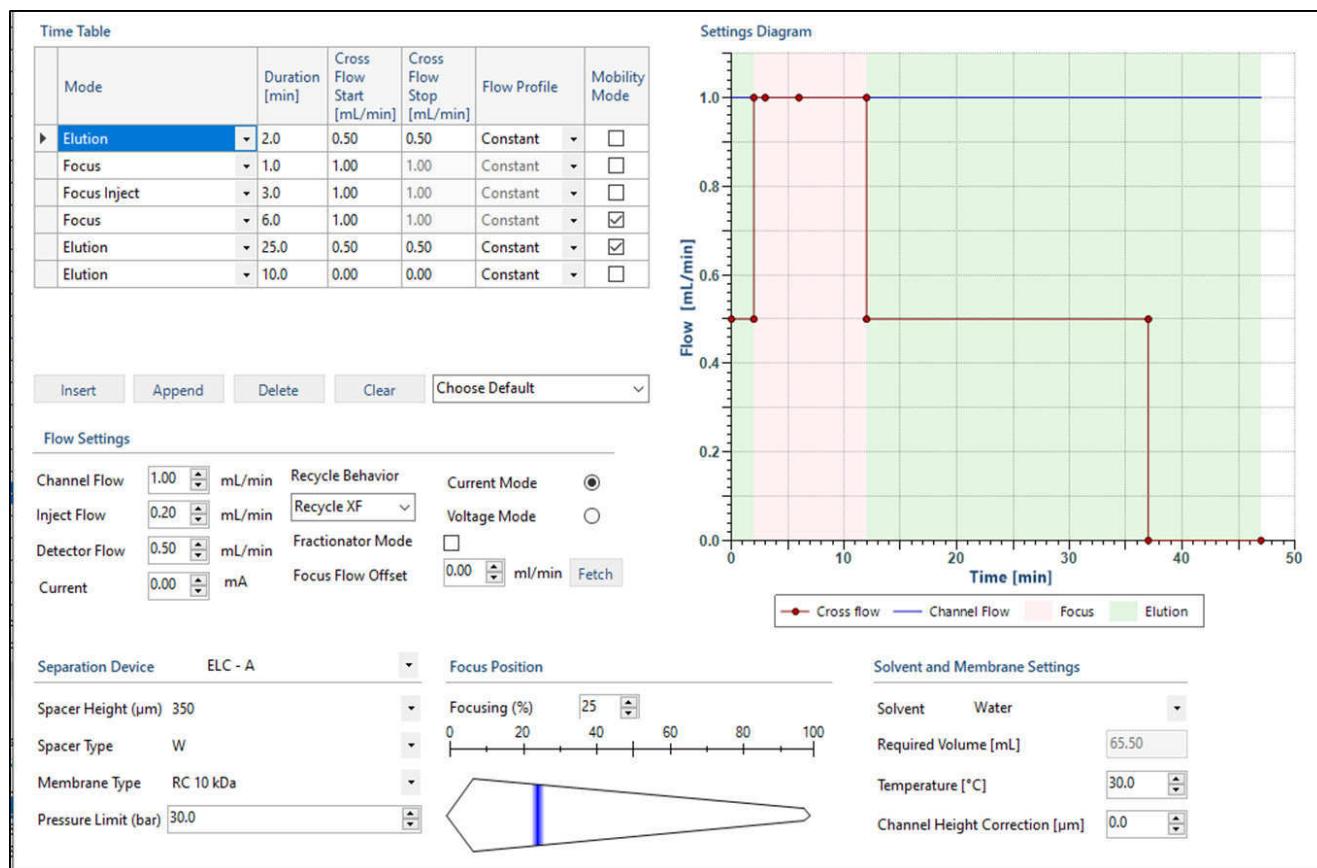
Figure 132. A typical sequence table for mobility experiments. Each application of current or voltage is separated by a blank injection to equilibrate the system and discharge the platinum electrodes. Values are applied from lowest (i.e., 0 mA) to highest (i.e., -3 mA). It is recommended to run a 0 mA run in between measurements with a blank injection to re-equilibrate the system.

It is recommended to run a blank injection between mobility experiments to ensure the platinum electrodes are fully discharged, the system is equilibrated, and there is no carry over in the channel. In general, application of current or voltage starting with 0 followed subsequently by incrementally stronger absolute current or voltage is recommended. Start with lower currents to observe shifts before progressing to stronger currents. Samples with low surface charge, such as liposomes, can begin with higher currents, such as -10 mA and -20 mA.

Carboxylate-Modified PSL Mobility Channel Method

For mobility channel validation, we recommend Thermo Scientific™ C37233 (CML-60 nm bead) standards. These tend to perform much better under electrical fields than the Thermo Scientific™ Nanospheres™ PSL beads. The stock 4% w/v solution is diluted to 0.24% w/v with water to make at least 300 µL of solution. For example, a 600 µL volume of stock 4% solution can be diluted with water to make 10 mL of 0.24% solution. The solution should be used fresh or stored at 2 – 8 °C.

For mobile phase, we recommend 5 mM phosphate buffer from mono- and di-basic sodium phosphate. Importantly, the pH is adjusted to 7.4 as mobility is pH-dependent.



For mobility verification, multiple replicate runs are performed at currents of 0, -3 mA, and +3 mA with 20 µL injections of the above described standard diluted to 0.24% w/v. An example sequence table is shown below.

Sequence Table									
Ready									
	Enabled	Vial	Volume [µL]	Inj./Vial	Sample Name	VISION RUN Method	Detecto Flow	Mobility Value	Mobility Type
► 1	<input checked="" type="checkbox"/>	1	20.00	5	CML 60nm	ECDS - QC Mobility Test - ELC.M	▼ 0.50	0	Current ▾
2	<input checked="" type="checkbox"/>	1	20.00	3	CML 60nm	ECDS - QC Mobility Test - ELC.M	▼ 0.50	-3	Current ▾
3	<input checked="" type="checkbox"/>	1	20.00	3	CML 60nm	ECDS - QC Mobility Test - ELC.M	▼ 0.50	3	Current ▾

Analyzing Mobility Experiments with VISION DESIGN

When mobility data is collected, the EMDF that is generated by VISION RUN will automatically populate with “Electrophoretic Mobility” analysis level at the experiment level under the Navigation panel on the left side of VISION DESIGN. This is shown in Figure 133. Combining all relevant experiments into a single project will generate an additional analysis called “Electrophoretic Mobility Comparison.”

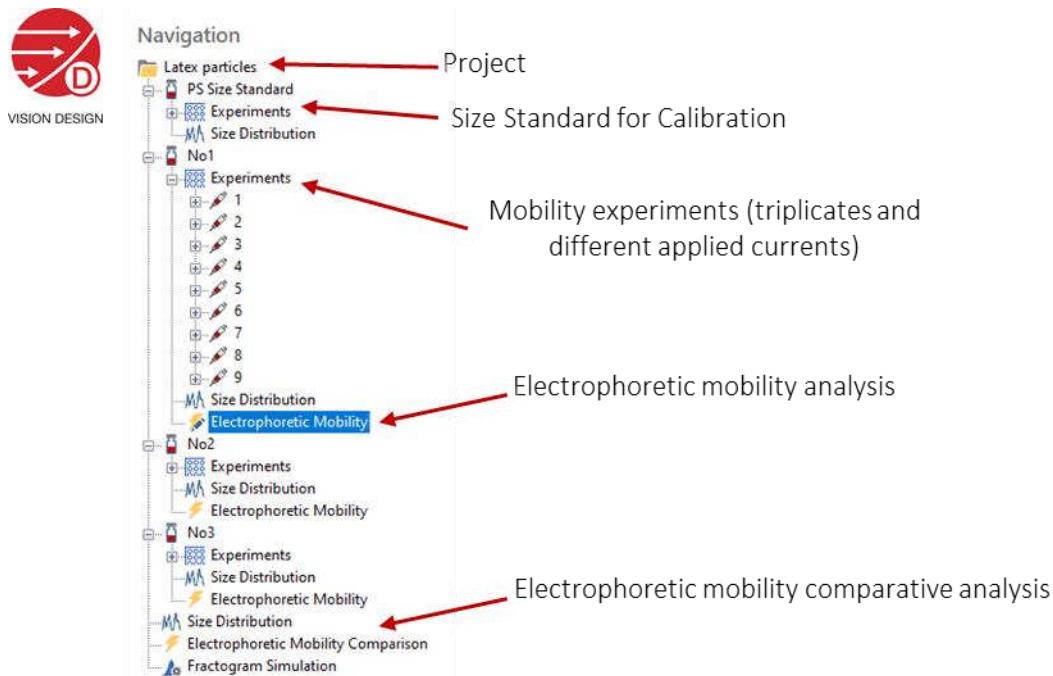
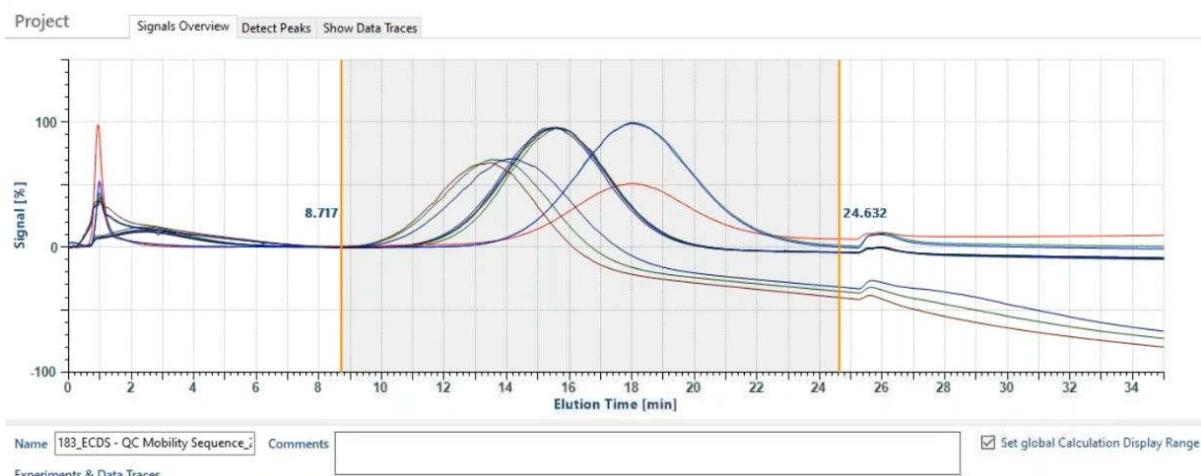


Figure 133. The Navigation pane of VISION DESIGN when EMDFs are loaded with mobility data.

A general process for analyzing mobility data can be done as follows:

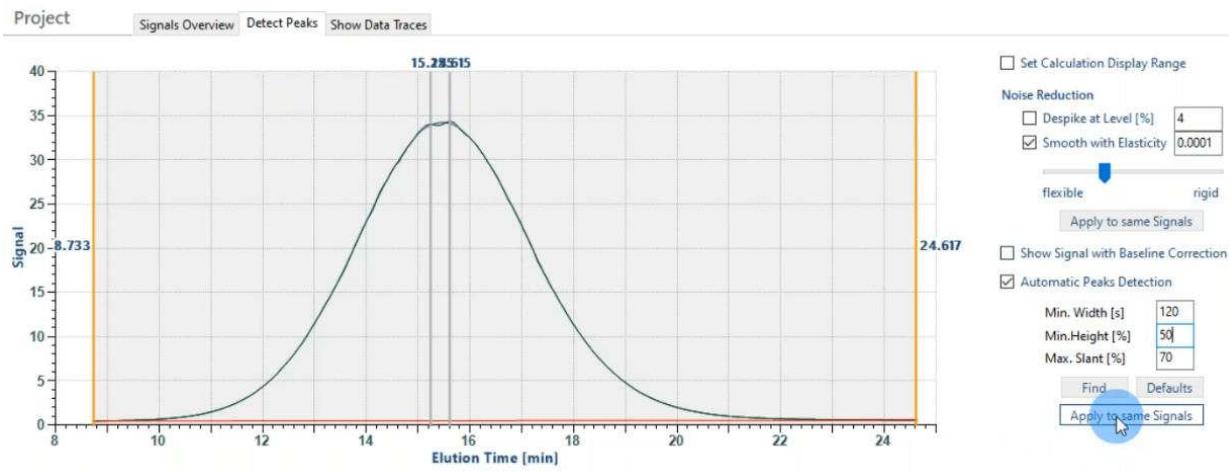
1. Open the EMDF(s) containing your sample runs. Opening an EMDF automatically generates a project file, so the raw data is untouched in the subsequent steps.
2. At the project-level (signals overview), you can use the “delete with filter” option to remove all signals except the one you will use for determining the elution times.
3. Set “global calculation display range” to include just the peak region.



4. Manually de-select any outlier runs.

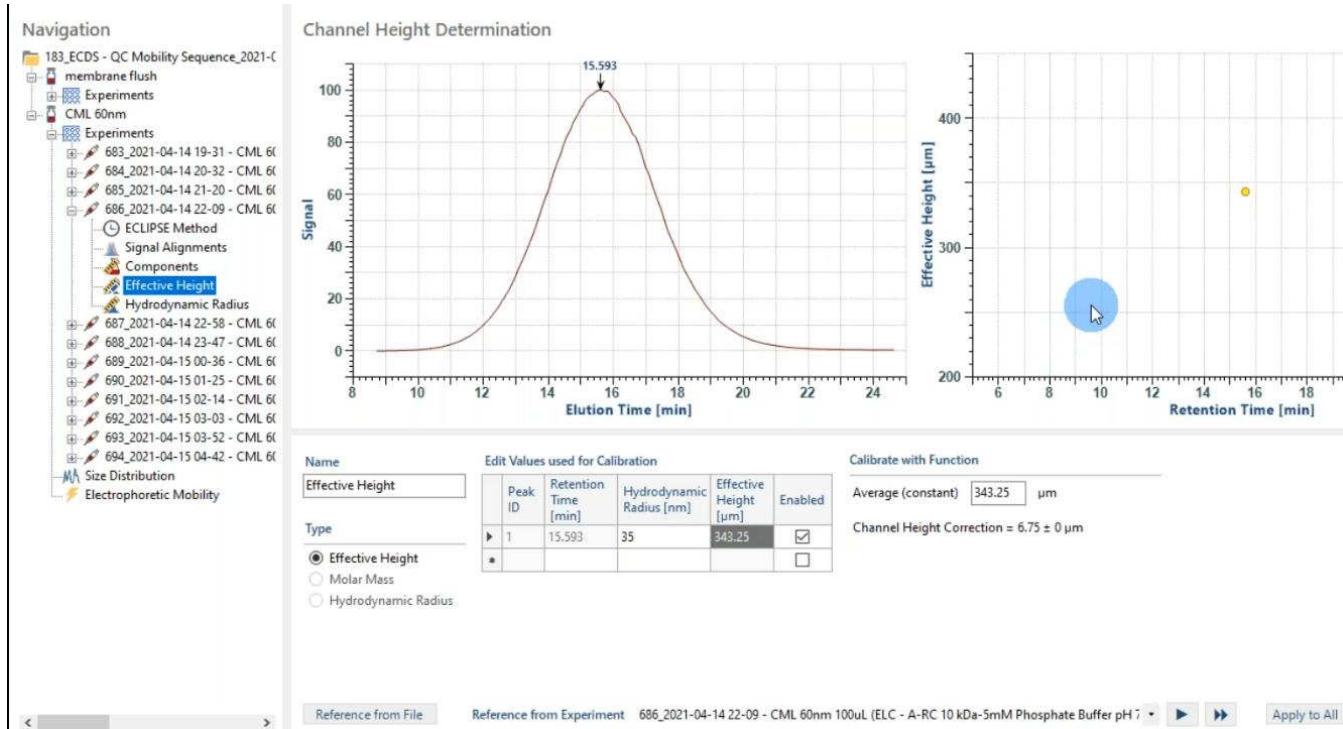


5. Use the “automatic peaks detection” and “Smooth with Elasticity” options as needed to ensure only one peak per run and true apex is detected:
 - a. Smooth with Elasticity should generally be set to 0.0001, can add extra zeroes after the decimal if needed.
 - b. Recommended settings for peaks detection: Min Width 120s, Min Height 50%, Max Slant 70%
 - c. Click “apply to same signals” after making any changes



6. Determine effective height
 - a. Find the best zero-current run, expand the experiment, and select “effective height”
 - b. Click the button at the bottom to load the chromatogram from the selected experiment, and confirm that only one peak is detected.

- c. Enter in known hydrodynamic radius for the peak. Effective height should appear when you press Enter.
- d. Click “apply to all” at bottom.



7. [Optional] To get charge & zeta potential, fill in ionic strength.

- a. Click on an individual experiment.
- b. Enter the buffer recipe in the “Ionic Strength Calculation” table
- c. Click “apply to all” at bottom

Navigation

- 183_ECDS - QC Mobility Sequence_2021-C
 - membrane flush
 - Experiments
 - CML 60nm
 - Experiments
 - 683_2021-04-14 19-31 - CML 60
 - 684_2021-04-14 20-32 - CML 60
 - 685_2021-04-14 21-20 - CML 60
 - 686_2021-04-14 22-09 - CML 60
 - ECLIPSE Method
 - Signal Alignments
 - Components
 - Effective Height
 - Hydrodynamic Radius
 - Size Distribution
 - Electrophoretic Mobility

Experiment

Name: 686_2021-04-14 22-09 - CML 60nm

Inject: from Vial: 1

Volume: 100 [µL]

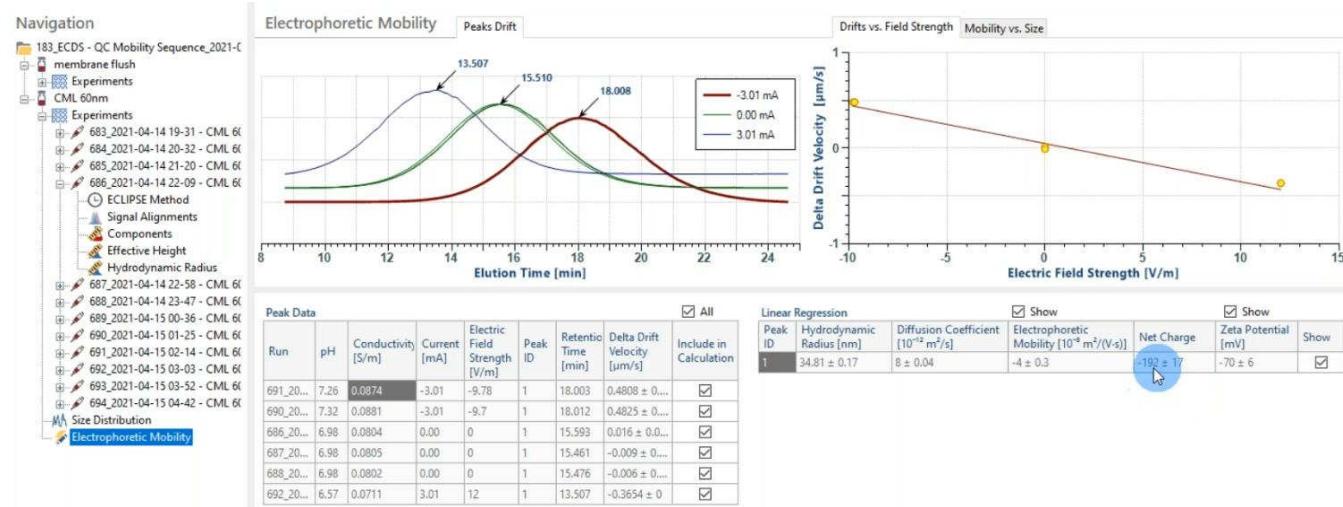
Concentration: 0 [mg/mL]

Ionic Strength Calculation

Salt	Formula	Concentration [mg/L]	Concentration [mmol/L]	Ionic Strength [mmol/L]
Sodium Phosphate dibasic	Na ₂ HPO ₄	550	3.874	11.623
Sodium Phosphate monobasic Dihydrate	NaH ₂ PO ₄ · 2H ₂ O	155	0.994	0.994
*				

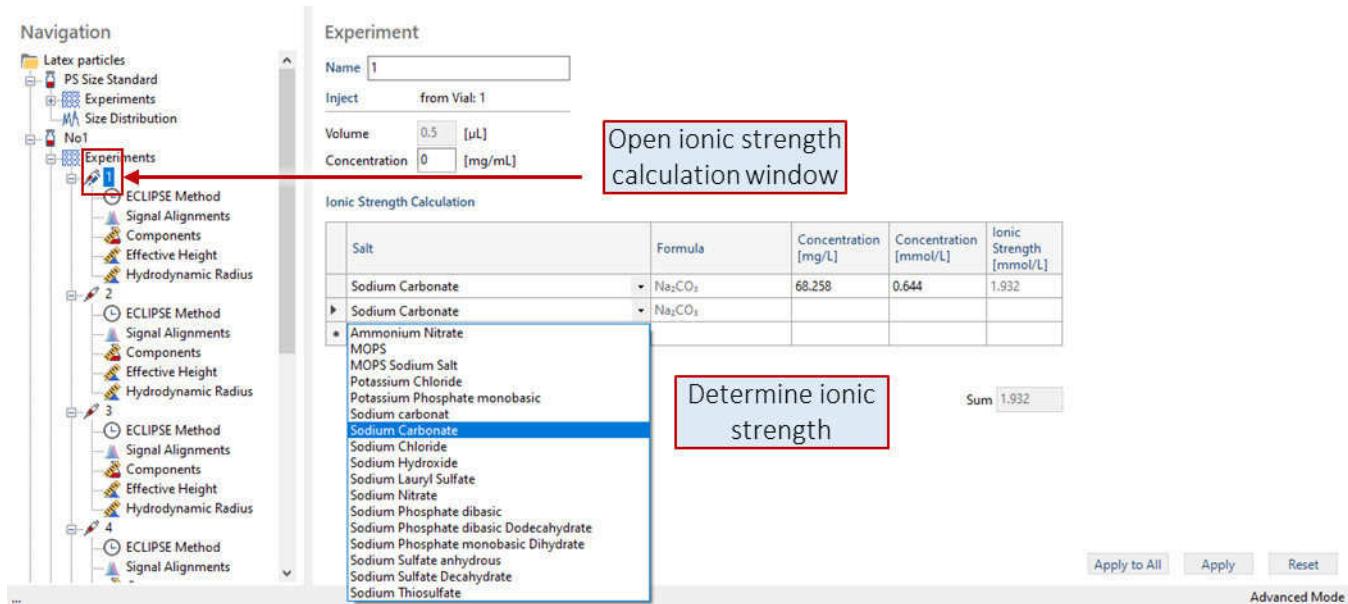
Sum: 12.617

8. Select “Electrophoretic Mobility” at the bottom of the left-hand window pane. There should be no outliers in the plot of “Drifts vs. Field Strength”. Electrophoretic mobility results appear in the table below.



Analysis via Shift of Peak Maxima or Radius Curves

Electrophoretic mobility is derived from retention time drifts as a function of electrical field change. The retention times can be derived with two different methods. In the first strategy, the peak maxima positions can be used to correspond a time with field charge. In the second strategy, particle size information that correlates time with radius or molar mass can be imported and plotted vs. elution time. If using peaks, they should be defined as described in the section, [Peak Analysis](#), under [Analyzing VISION DESIGN Data](#). Once the peaks are detected, they can be analyzed by navigating to the Electrophoretic Mobility level. Before navigating to that level, which is described in the next section, the ionic strength conditions of the experiment should be selected at the injected sample level. Several common salts are included in VISION DESIGN, and their corresponding concentration can be specified.



Additionally, the effective height should be determined and referenced in the experiments that will be analyzed. This is also described in the section, [Peak Analysis](#), but specifically in the step discussing channel height correction in Step 5 on page [162](#). The effective channel height correction can be applied to all experiments in the project that used this channel.

From Shift of Peak Maxima

With the peaks defined as described in the preceding section, the peak drift can be analyzed. In Figure 134 below, this experiment consists of 9 runs, with 3 triplicates each at currents of 0 mA, -0.5 mA, and -1 mA. The peak data, including the pH, current (mA), electric field strength (V/m), retention time, and delta drift velocity are provided in the table below and can be included or omitted from the calculations via the radio button in the table. On the right, the drifts vs. field strength can be plotted, and the linear regression data provided below.

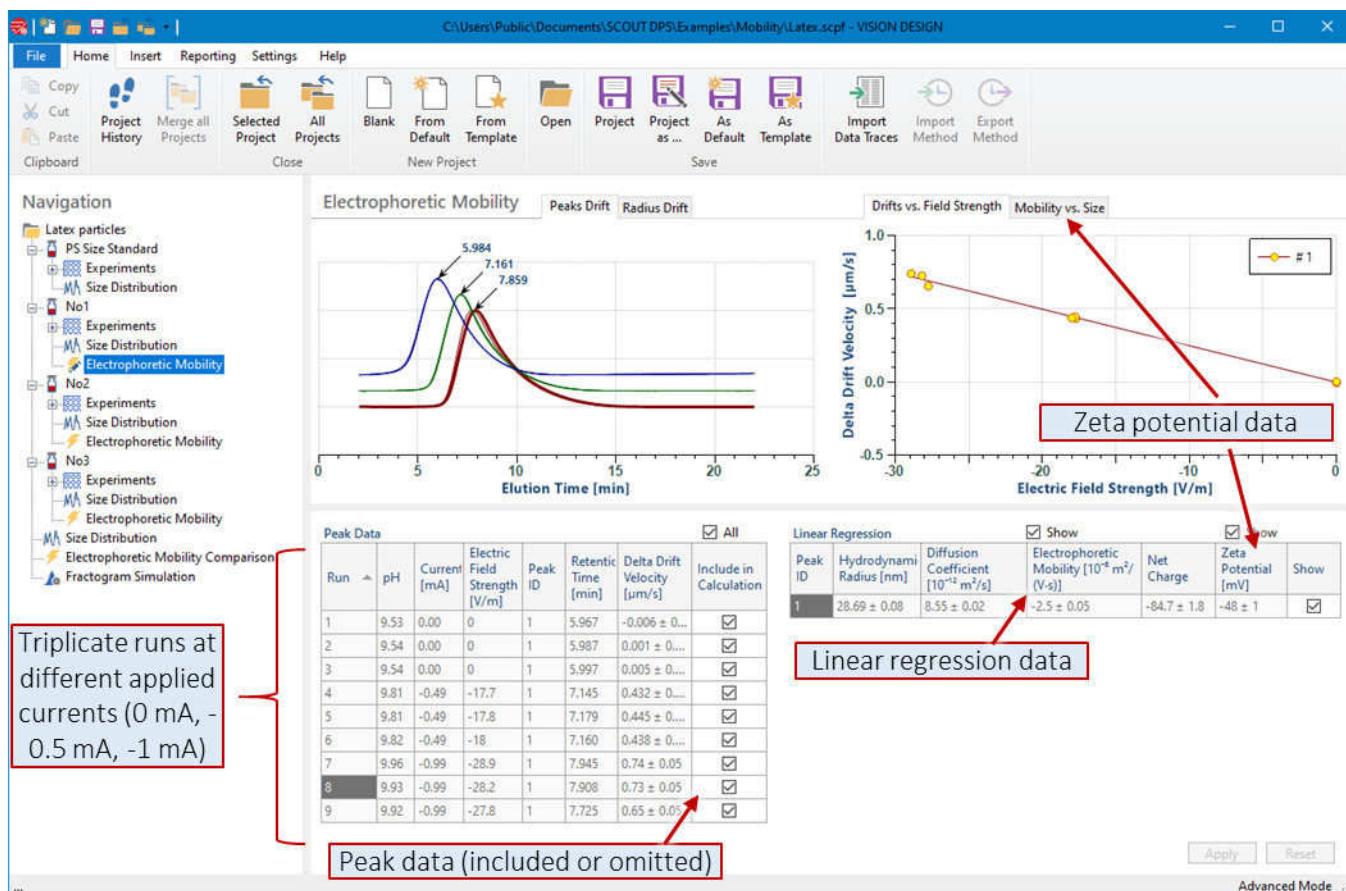


Figure 134. Electrophoretic Mobility level window in VISION DESIGN. Peaks Drift is selected for analysis.

Selecting the Mobility vs. Size graph on the right-side of the window will allow you to see the electrophoretic mobility for each peak vs. hydrodynamic radius, as well as Zeta potential.

From Shift of Radius Curves

In addition to analyzing via peak retention time shift, the data from ASTRA can be imported to do a radius shift analysis for electrophoretic mobility calculations. Please refer to [Import Data Traces](#) for instructions on importing ASTRA data into VISION DESIGN. This view can be accessed by selecting Radius Drift in the Electrophoretic Mobility window. On the left, the cubic polynomials for each radius data trace are displayed. The color of the line corresponds to the electrical field strength group. The data trace selected in the table (“Traces Data”) below graph will result in a slightly thicker line in the graph to visually distinguish it. Clicking the line on the graph will change the selection to the appropriate row in the table.

The original imported ASTRA data can be viewed by selecting the Show Points radio button under the graph, with or without log scaling. The analysis via shift in radius curves is shown in Figure 135. The Show Radius Lines and Show Radius Data Range are used to determine the radii used for calculations. In the Lines selection, the gray highlighted region will indicate which data points are used to give the retention times for calculating drift velocities. The Data Range selection allows for the maximum range for possible radius to be determined. Drag the lines to select an optimum range. Finally, the Set button will allow new radii classes to be created and populate the Drift Data table.

As with the peaks shift analysis, the shift in radius curves can be used to determine electrophoretic mobility and zeta potential data.

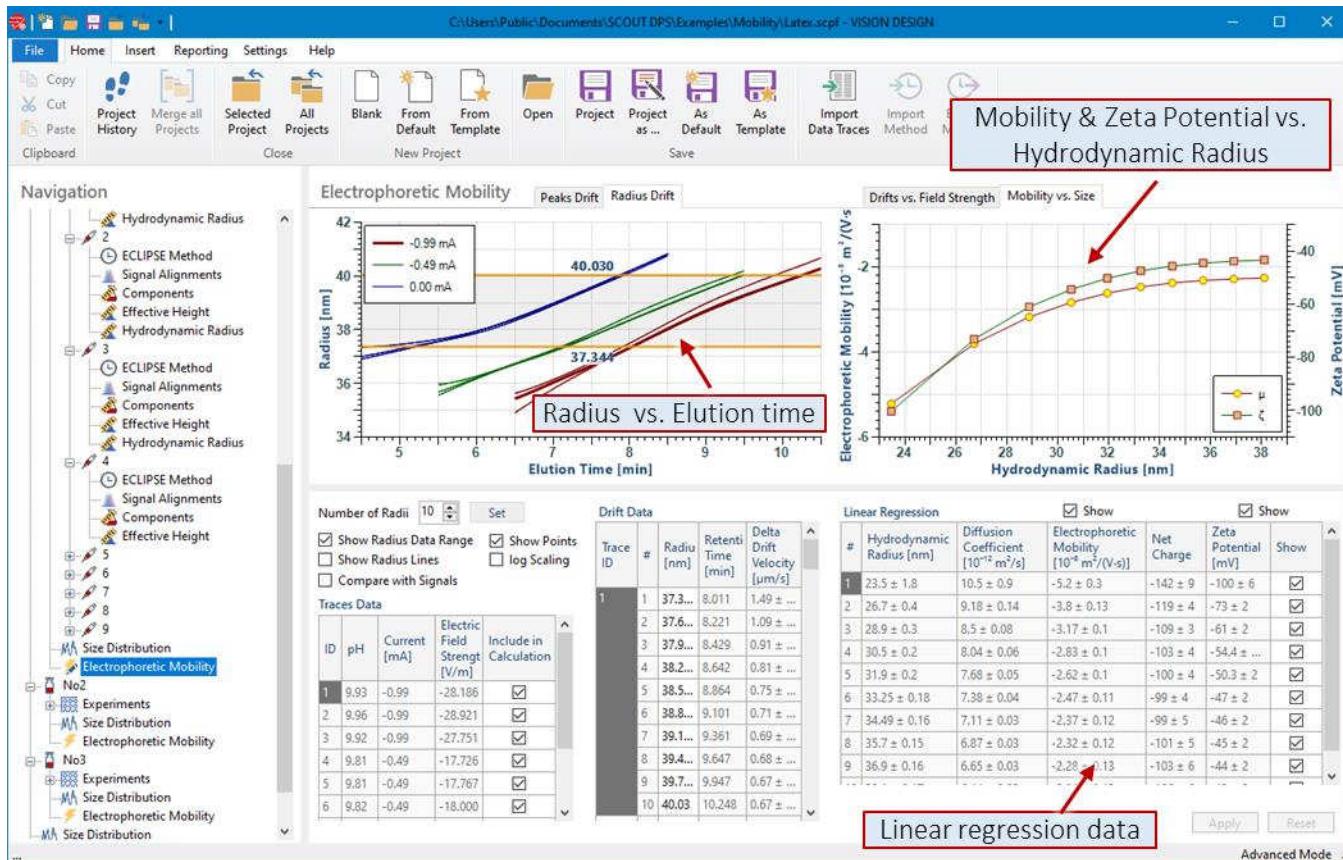


Figure 135. Electrophoretic Mobility level window in VISION DESIGN. **Radius Drift** is selected for analysis.

Comparing Electrophoretic Mobility Data

When mobility data is detected in VISION DESIGN, the option to compare electrophoretic mobility or zeta potential is available under the navigation panel (**Electrophoretic Mobility Comparison**). This allows for multiple sequences worth of mobility data to be easily compared in one view, along with a tabulated comparison of the various data. An example is shown in Figure 136.

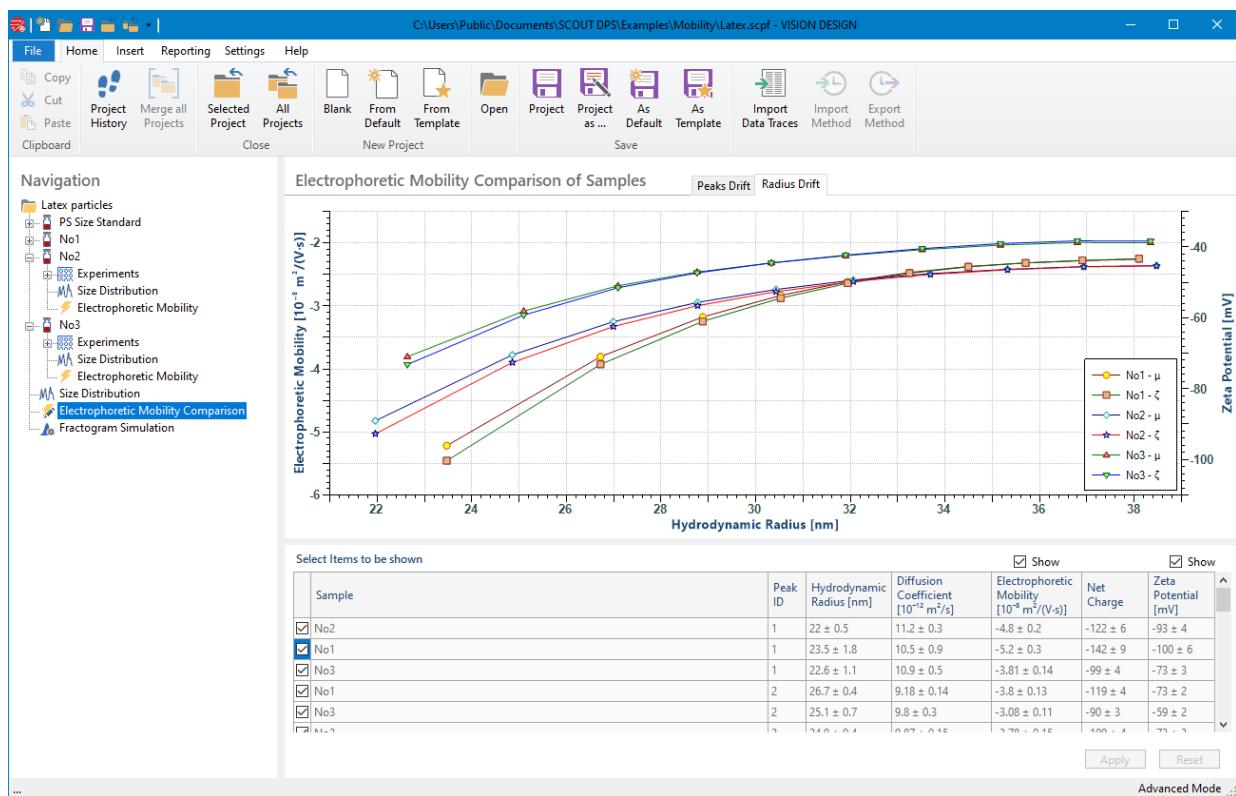
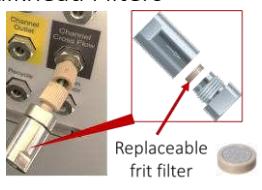
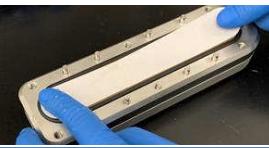


Figure 136. Electrophoretic mobility comparison of samples with Radius Drift view.

Maintenance Procedures

Please refer to Table 9 below for an overview of common maintenance procedures.

Table 9. Routine Maintenance Schedule for Eclipse FFF-MALS System

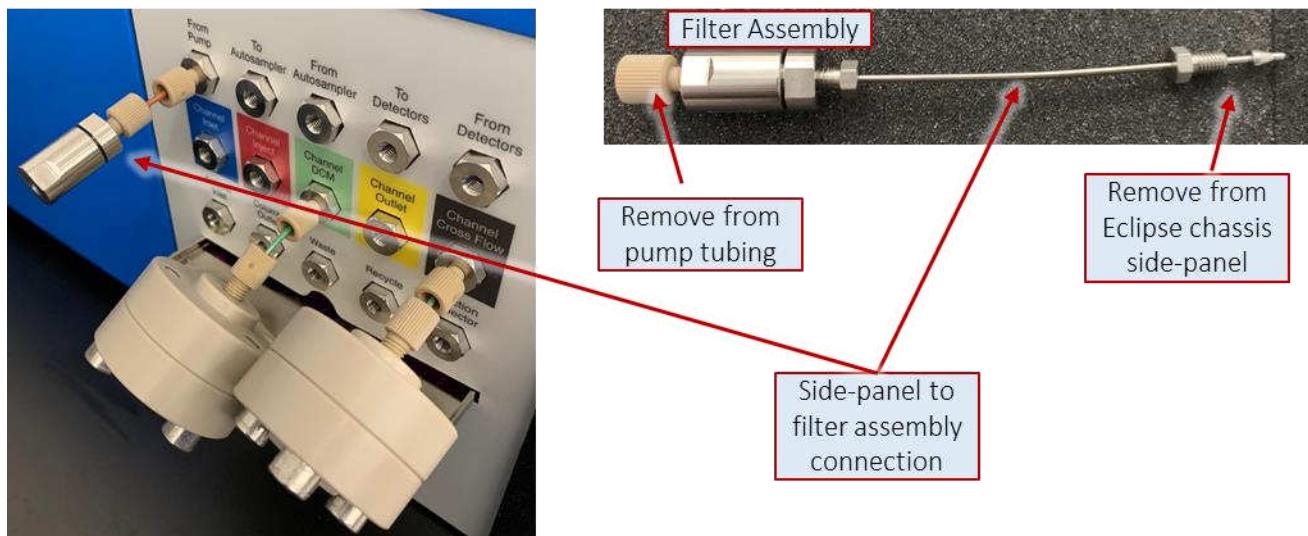
	Component	Recommendation	Estimated Frequency
HPLC Modules	Mobile Phase 	Always pre-filter into solvent reservoir with 0.1 – 0.22 µm filter. Replace daily if no preservative is used. If using sodium azide or other preservative, replace mobile phase every 1-2 weeks. Store system in alcohol solution (or pure organic solvent) for long-term shutdown.	Monitor mobile phase noise daily
	Solvent Reservoir Filter 	Microbial growth in the solvent filters can affect data quality even with fresh mobile phase. Always use a fresh bottle when replacing mobile phase. Sonicate to clean or replace entirely on a regular schedule. Replace every 3 months or if mobile phase has become contaminated.	As needed depending on mobile phase quality, or every 3 months
	HPLC Pump Frit Filter 	Replace frit filter on HPLC pump according to manufacturer recommendations (i.e., every 3 months).	3 months
	Inline Filter 	Common HPLC filters do not remove particulates or dust from mobile phase that are sensitive to light scattering. Strongly recommend at 0.1 or 0.2 µm inline filter with light scattering detectors. Replace the inline filter monthly. Bypass filter if flushing HPLC with any acids.	1 month
	Autosampler 	Recommended to regularly perform 10 – 20 injections of pure mobile phase or a cleaning solution (1% detergent) with the full sample loop volume (e.g., 100 – 900 µL) to clean out the sample loop and needle.	As needed (part of a monthly cleaning plan)
Eclipse Components	Eclipse Side Panel Bulkhead Filters 	Protects internal Eclipse flow meters at the Pump, DCM (if applicable), and Cross Flow ports. Replace if poor cross flow or DCM flow regulation is observed. The DCM inline filter may need to be replaced more frequently as it captures new membrane fibers during the membrane flush procedure.	As needed for Read/Set flow mismatch in the Inlet, DCM, or Cross Flow pathways, or every 6 months
	Flushing 	Run the Eclipse Flushing procedures once a week to remove solvent from the purge, SEC switching, and fraction collector pathways.	1 week
	Channel Membrane 	Replace the membrane every 2 – 4 weeks or when excessive noise is observed (especially compared to focus mode) when switching modes or when cross flow reaches 0 mL/min. When conditioned with a sample type (protein,	2 weeks

		nanoparticle, etc.), replace the membrane whenever switching applications.	
	Channel Spacer 	There are different types of spacers available. The laminated spacers (PTFE-coated polycarbonate) are semi-consumable and should be replaced every channel assembly as they can deform over time when compressed. The Mylar spacers, which are compatible with some organic solvents, does not seal as reliably but can be used for a year or more as long as they are not torn or damaged.	~ 2+ weeks for laminated spacers
Eclipse Mobility Components	Mobility Conductivity Cell 	The conductivity sensor can be checked with a standard monthly and calibrated as needed. It is recommended to calibrate the conductivity cell with 5 conductivity standards (Sigma-Aldrich KCl conductivity standards (500 µS/cm to 10,000 µS/cm) for best data quality.	Check every 2 to 4 weeks, calibrate as needed.
	Mobility pH Cell 	The pH cell can be checked with a standard monthly and calibrated as needed. It is recommended to calibrate the pH cell with 3 pH standards (Thermo Scientific pH variety pack) every 3 months, or more frequently for best data quality.	Check every 2 to 4 weeks, calibrate as needed.
	Mobility Module Storage 	When not actively being used for experiments, it is recommended to store the Mobility Module in KCl solution. Never store the cell dry or with DI water, as this can degrade the pH cell.	Daily; store in KCl solution when not in use

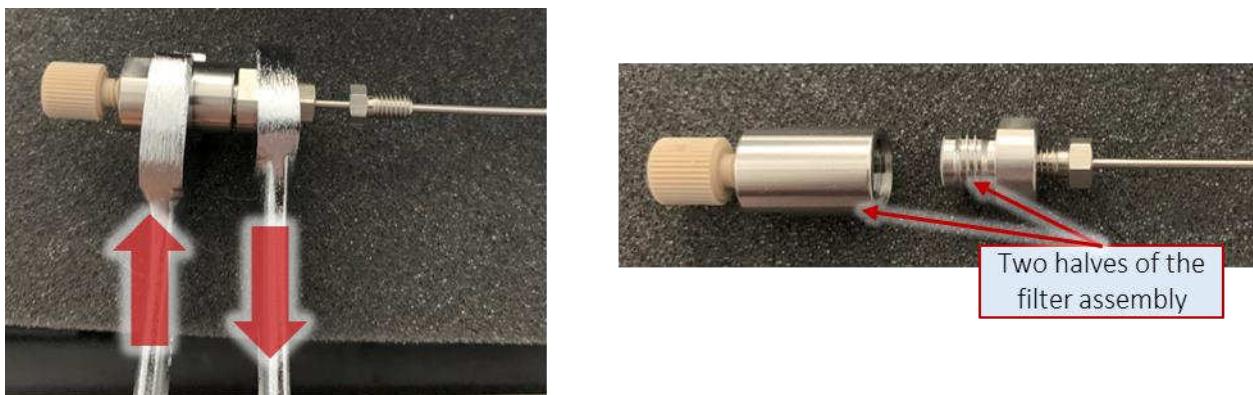
Replacing Bulkhead Frit Filter

The bulkhead frit filters or inline filters are intended to prevent particulates from clogging the sensitive internal flow controllers from the mobile phase. They are intentionally installed at ports that are not expected to be sample fluid pathways, thus should not come in contact with sample traveling through the system—these include the Pump flow inlet, the DCM flow outlet, and the Cross Flow outlet. Wyatt Technology recommends 20 µm stainless steel frit filters. The frit filters should be replaced whenever a clog is suspected, which will typically manifest itself as read flows for Pump, DCM, or Cross Flow not reaching set values. As part of your system best practices, replacing this frit regularly can maximize instrument uptime. If the system is well-maintained, it is recommended to replace the frits every 3 – 6 months depending on use. The DCM filter membrane may need to be changed more frequently due to the capture of membrane fibers from the new membrane flush. The instructions for performing this for the frit are provided below:

1. It is easiest to manipulate and handle the bulkhead frit filter by removing the filter entirely from the side panel. In the image below, the frit filter is installed with PEEK tubing; however, your assembly may be installed with 316 stainless steel. Use the wrench (P9014-0405; wrench, open-ended 1/4") provided in the Eclipse hardware kit to remove the stainless-steel fitting from the Eclipse side panel and the PEEK fitting extender tool (P9010-399) to remove the tubing from the channel entering the opposite end of the filter assembly.



2. Use the two wrenches (P9014-0708; wrench, open-ended 7/16" X 1/2") provided in the Eclipse hardware kit to loosen the filter assembly. You can then unscrew both halves of the filter assembly.



3. The frit filter is installed in a recess of the smaller half of the filter assembly. It may be possible to gently tap the filter assembly on a bench top to remove; however, if it does not come out easily, proceed with the step below.



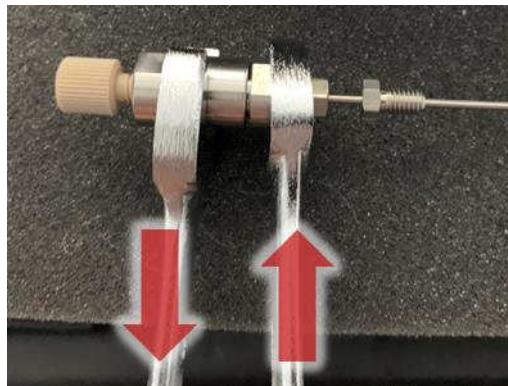
4. **(As needed)** If the filter cannot be easily removed, it is recommended to disconnect the fitting on the other end of the small half of the filter housing and use compressed air to push the filter out.



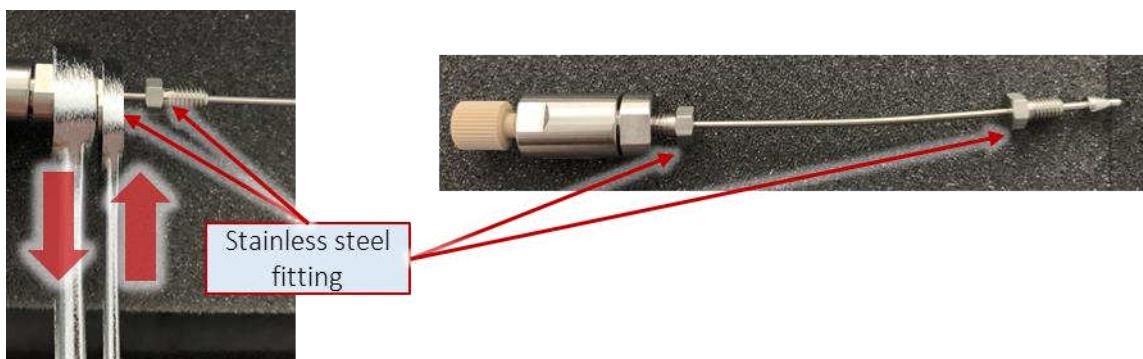
- Once the filter is removed, it is recommended to replace with a new frit filter. Discard the previously used frit. Always use gloves when handling the new frit filter and reassembling the filter housing.



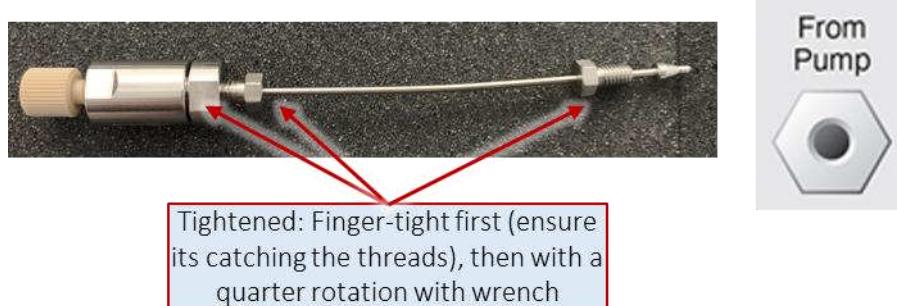
- Re-connect the two halves of the filter housing by hand while ensuring the threads are probably aligned. This can be done by rotating the smaller half of the filter housing counter-clockwise at first before tightening with a clockwise rotation. Once finger tight, inspect the filter housing to ensure it is flush.
- Once the filter housing is finger-tight, the two wrenches used above can be used to provide another quarter rotation to seal the filter housing.



- If the stainless-steel fitting was removed, it should also be tightened with fingers only. Then, a quarter rotation to seal the fitting can be applied. Never use a wrench to forcefully tighten a fitting, as this can damage the ferrule and lodge the ferrule inside.



- Once the two-halves of the filter housing are re-connected, and the stainless-steel tubing secured on the smaller half of the filter assembly, the bulkhead union can then then be re-installed between the Eclipse chassis side-panel and the channel.



Replacing the Inline Filter for DCM and Cross Flow Pathways

The DCM and cross flow pathways utilized an inline filter. Instructions for replacing the membranes for these types of housings can be found in [TN3504 – Wyatt Inline Filter Installation and Use](#). Compared to the traditional inline filter housing, the component that is shipped with Eclipse instruments (P/N 171035) comes with a Kalrez O-ring.

- Remove the fittings from the inline filter housing. Use an extender tool if necessary.



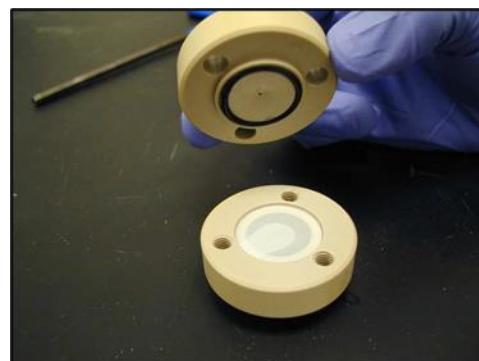
- Use a 4 mm hex driver or Allen key to remove the three screws from the filter holder.



3. Once you remove the screws, separate the housing to reveal the metal frit and O-ring.

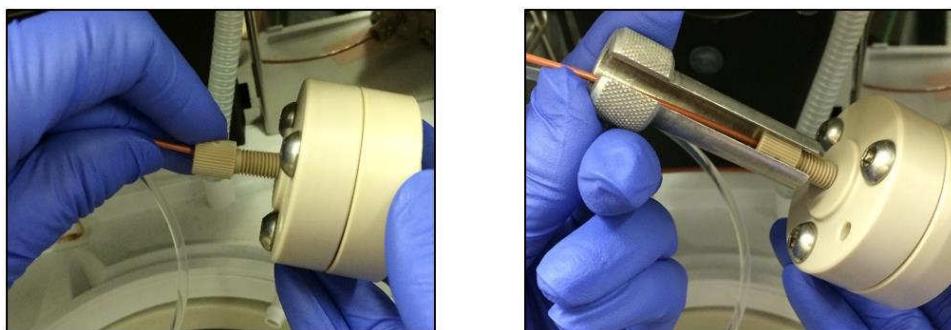


4. Rinse the housing with isopropyl alcohol and dry.
5. Using flat-tip tweezers, place a new membrane on the titanium frit. Be careful to avoid damaging the membrane. Add a few drops of alcohol or water to wet the membrane and keep it in place. Rinse alcohol away with pure water before exposing it to a salt-containing buffer.



NOTE: You may use a membrane suitable for your application and mobile phase. Wyatt Technology recommends hydrophilic PVDF for aqueous solutions. PVDF (hydrophobic or after conditioning in organic solvent) can also be used for THF. Consider PTFE for solvents such as NMP, DMAc, DMF, or DMSO.

6. Place all three screws back into the filter and tighten them with the hex driver. Tighten each screw a little at a time to ensure even and uniform sealing. A firm tightening is adequate.
7. The fittings can be re-assembled onto the filter housing. Assemble finger-tight first, and then an extender tool can be used to tighten firmly.



8. Hold the filter with the outlet side positioned upward and rinse the filter with the flow via VISION RUN to check that the outlet is free of air bubbles. Or that you no longer see air bubbles before connecting the tubing to the Eclipse.
9. Make the connection to the Eclipse. Finger-tighten the fitting and you may use the extender tool to tighten.



10. Change the membrane monthly for the pump and DCM pathways. The cross flow pathway, which is filtered by the channel membrane as well, does not need as frequent replacement.

Mobility Maintenance Procedures

For general storage, it is recommended to store the Mobility module—specifically the conductivity and pH cells—in a KCl solution. The Mobility module ships with KCl solutions for calibrating the conductivity cell. It is not recommended to store the cells in pure water. Having a spare set of tubing so that the Mobility module can be bypassed and storage in KCl solution separate from the rest of the analytical detectors is recommended.

WARNING: It is not recommended to store the mobility module in pure, deionized water. They should be kept in an appropriate storage solvent like pH storage solution or KCl solution when not used.

The Mobility module ship kit comes with a series of pH calibration standards and storage solution, as well as conductivity standards (Figure 137). The recommended maintenance schedule is provided below in Table 10.

Table 10. Mobility Module maintenance considerations

Module	Maintenance	Time Frame	Notes
Eclipse Mobility Module	Store in suitable salt-containing solution, such as KCl conductivity solution.	Daily	Do not store in pure water.
Mobility conductivity cell	Check with one of the conductivity standards, values should be within 5%.	2 – 4 weeks, calibrate if needed.	Calibrate with KCl conductivity solutions as needed.

Mobility pH cell	Check with pH 4.01, values should be within +/- 0.3. (pH 3.71 – 4.31).	2 – 4 weeks, calibrate if needed.	Calibrate with pH buffer solution standards.
Mobility channel	Compatible with aqueous solvents only. Replace membrane when elevated noise is detected or every 2 – 4 weeks.	2 – 4 weeks, or when noise is observed.	Can be disassembled and stored dry.

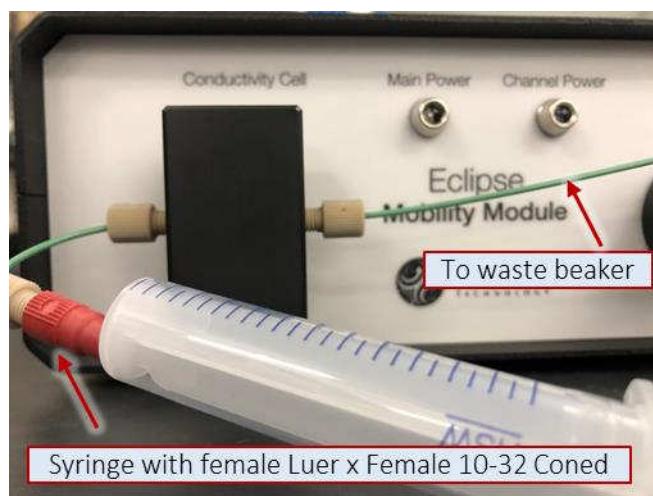


Figure 137. Thermo Scientific™ pH buffer variety pack (left) and Sigma-Aldrich™ KCl conductivity standards (right).

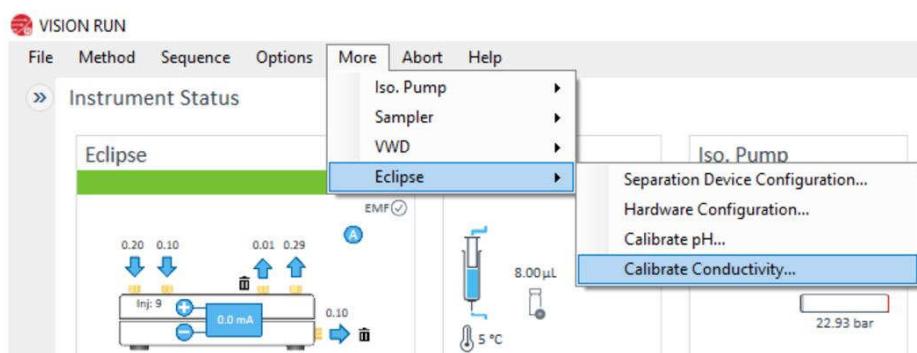
Procedure for Calibrating Eclipse Mobility Module Conductivity Cell

The conductivity cell can be checked with one of the standards every 2 – 4 weeks or more frequently for best data quality. If the conductivity cell measurements are within 5%, the cell may be fine. For most accurate data or when larger error is observed, it is necessary to calibrate the cell. This can be done with KCl conductivity standards. Check that the standards have been stored properly and are not expired. The procedure for checking the conductivity and calibration is as follows:

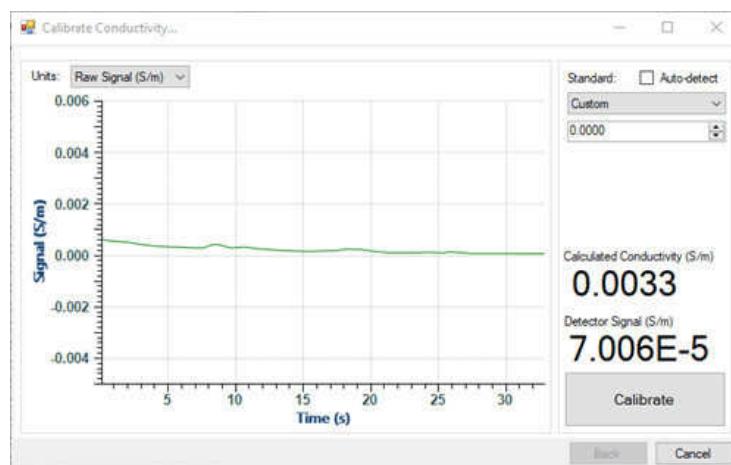
1. Take the Mobility module offline from the rest of the system. Solutions should be injected directly into the cell via syringe. It is recommended to use a syringe pump for most stable delivery of fluid. The Mobility Module hardware kit contains pre-swaged tubing for performing batch injections.



2. The monitoring, checking, and calibration of the conductivity cell is performed in VISION RUN. Launch the software and navigate to **More** → **Eclipse** → **Calibrate Conductivity**.



3. Syringe inject the first standard into the conductivity cell. The conductivity data should be displayed. If the values are expected, then you have successfully checked the conductivity and can proceed to use the same calibration. If you need to calibrate the conductivity, proceed with the following steps.
4. To calibrate, change the standard type to match the standard that is injected. If the standard is not in the drop-down menu, the “Custom” option can be selected and its conductivity entered manually. The injection of calibrants performed in this step will be repeated for subsequent calibrants. In this initial case, water (conductivity of 0.0 S/m) was used initially with a custom value inputted.
5. Once the signal is relatively flat while actively injecting standard, select the “Calibrate” button.



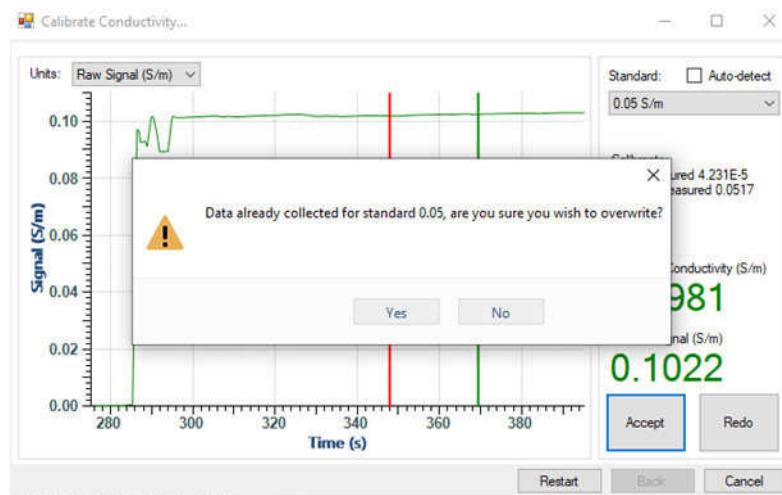
6. The button will change from “Calibrate” to “Waiting for stability...” and a vertical red line will appear as the signal stabilizes.



7. Once the signal has stabilized and the “Calculated Conductivity (S/m)” and “Detector Signal (S/m)” have turned green, you can select “Accept” if you are ready to move on to the next calibrant. If you aren’t satisfied with the calibrant reading or accidentally injected the wrong standard that was specified, you can click to “Redo” to re-collect the current calibrant.

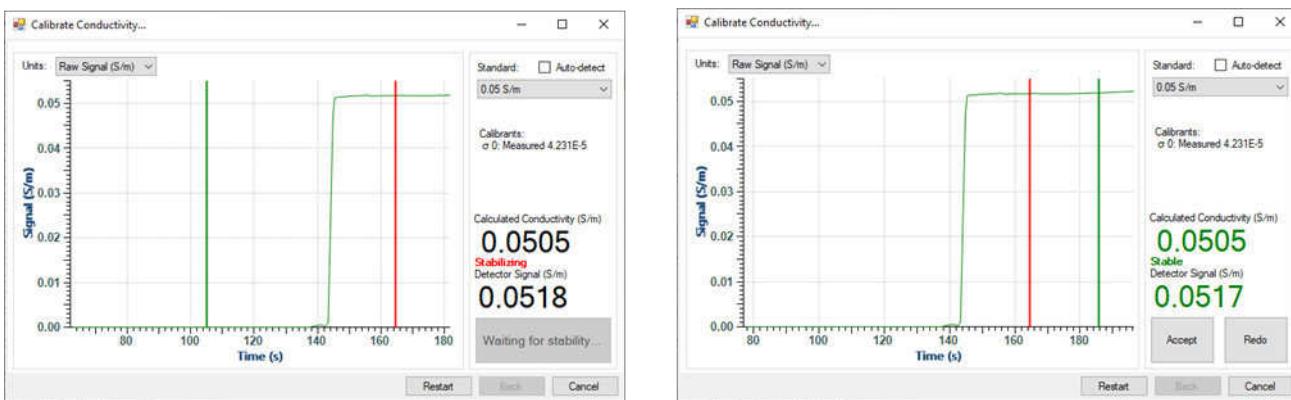


8. If you are redoing the measurement, you can select “Yes” to overwrite the incorrect value.

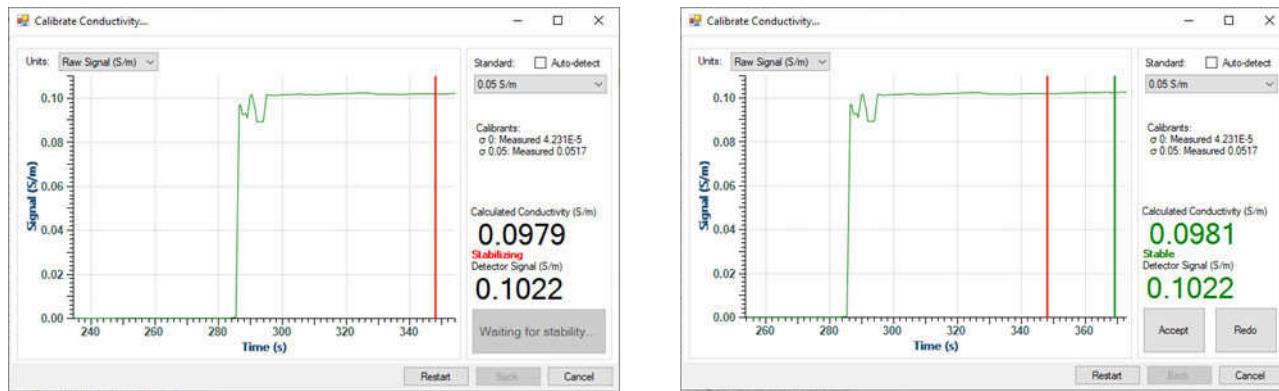


9. Repeat the steps by injecting the next calibrant into the cell and selecting the appropriate standard from the dropdown option or specifying the conductivity for a custom standard. These were steps 4 - 8 in this section.
10. It is recommended to inject at least three calibrants. In this procedure, we will perform calibration with 0.02, 0.05, and 0.1 S/m conductivity standards. An example of the window for 0.05 and 0.1 S/m is provided in the step below:

- a. 0.05 S/m



b. 0.1 S/m



11. Once three or more calibrants have been injected and the resulting signals recorded, you can click the “Finish” button to complete and update the calibration. However, before selecting “Finish,” you can perform the following additional commands:

- Add Standard:** Adds an additional standard to be measured for a more robust calibration curve.
- Restart:** If you want to re-perform the calibration, select this option.
- Cancel:** If you want to cancel the current calibration process, select this option.



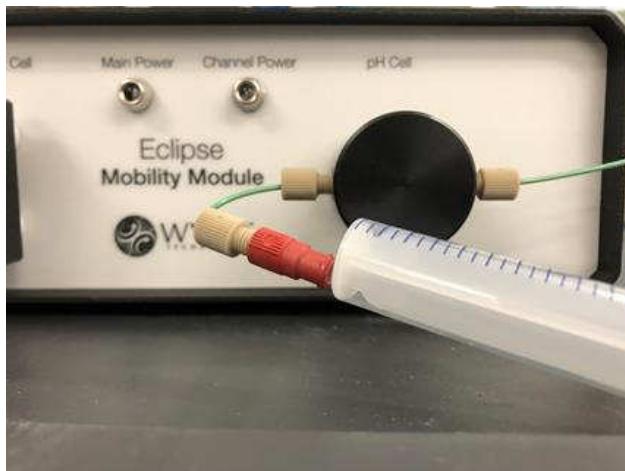
12. It is recommended to check the calibration every 2 – 4 weeks and update if one conductivity standard varies by more than 5%.

13. For large changes in calibration constant, the software will prompt you with a message. If your conductivity cell calibration drifts by a significant amount in a relatively short time frame, it suggests your cell meter may need to be cleaned.

Procedure for Calibrating the Eclipse Mobility Module pH Cell

The procedure for checking and calibrating the pH cell is similar to what has been described in [Procedure for Calibrating Eclipse Mobility Module Conductivity Cell](#). In this case, however, a syringe will be connected to the pH cell with syringe pump and you will navigate to More → Eclipse → Calibrate pH in VISION RUN. The following procedure can be followed for calibrating the pH meter:

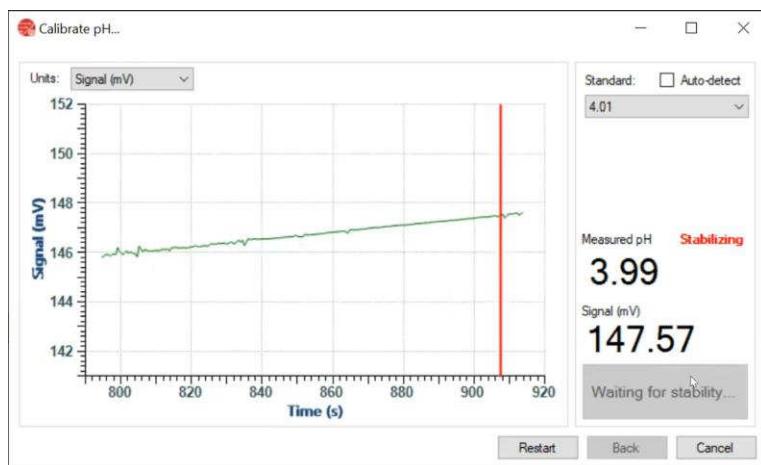
1. Take the Mobility module offline from the rest of the system. Solutions should be injected directly into the pH cell via syringe. It is recommended to use a syringe pump for most stable delivery of fluid. The Mobility Module hardware kit contains pre-swaged tubing for performing batch injections.



2. Flush the pH cell with DI water to rinse it out.
3. Navigate to More → Eclipse → Calibrate pH in VISION RUN.
4. Inject the first pH standard (i.e., pH 4.01 standard). You can check the reading. If the pH is within +/- 0.3, your results may be fine. For larger error or more precision, the calibration of the pH cell can be done by proceeding with these instructions.
5. To calibrate, change the standard type to match the standard that is injected. If the standard is not in the drop-down menu, the “Custom” option can be selected and its pH entered manually. The injection of calibrants performed in this step will be repeated for subsequent calibrants. In this initial case, a standard solution of pH 4.01 was used initially.
6. Once the signal is relatively flat while actively injecting standard, select the “Calibrate” button. The theoretical voltages are roughly as follows but may vary with temperature and other inherent variability:

pH Standard	Estimated Voltages
pH 4.01	140 to 155 mV
pH 7.00	0 ± 15 mV
pH 10.01	-160 to -177 mV

7. The button will change from “Calibrate” to “Waiting for stability...” and a vertical red line will appear as the signal stabilizes.



8. Once the signal has stabilized and the “Calculated pH” and “Detector Signal (V)” have turned green, you can select “Accept” if you are ready to move on to the next calibrant. If you aren’t satisfied with the calibrant reading or accidentally injected the wrong standard that was specified, you can click to “Redo” to re-collect the current calibrant.
9. If you are redoing the measurement, you can select “Yes” to overwrite the incorrect value.
10. Repeat the steps by injecting the next calibrant into the cell and selecting the appropriate standard from the dropdown option or specifying the pH for a custom standard. These were steps 4 - 8 in this section.
11. It is recommended to inject three calibrants, with pH of 4.01, 7.0, and 10.01.
12. Once three or more calibrants have been injected and the resulting signals recorded, you can click the “Finish” button to complete and update the calibration. However, before selecting “Finish,” you can perform the following additional commands:
 - a. **Add Standard:** Adds an additional standard to be measured for a more robust calibration curve.
 - b. **Restart:** If you want to re-perform the calibration, select this option.
 - c. **Cancel:** If you want to cancel the current calibration process, select this option.



13. It is recommended to check the calibration every 2 – 4 weeks and update if one conductivity standard varies by more than 5%.

Eclipse Cleaning Guide

An important part of obtaining high quality FFF-MALS data is maintaining a clean system. Contaminants in the system will contribute to a noisy or drifting light scattering baseline thereby decreasing the signal-to-noise ratio, obscuring meaningful signals, and decreasing the accuracy of the measurements. This guide will describe how to isolate the source of the noise in the FFF-MALS system in order to clean and remove contaminants.

Noise in the light scattering trace can be caused by particles or contaminants in one or more of the following components:

- Mobile phase
- HPLC System (pump, degasser or autosampler)
- Upstream detectors (e.g., UV detector)
- Eclipse separation channel (contaminated membrane)
- Eclipse (Rheodyne valve heads, fluid lines (especially tubing exposed to samples))
- Light scattering detector

In this section, discussions for identifying the root source of increased noise, and both basic and deep cleaning strategies will be discussed. The emphasis in this section will be aqueous systems, which are most commonly prone to contamination. Appropriate cleaning solutions for suspected contaminants may substituted based on your specific application.

Determining Source or Root Cause of Noise

1. To identify the root-cause of noise, you can take the following steps. The most sensitive instrument to noise is the multi-angle light scattering (MALS) detector. You can use the ASTRA LS Noise assessment methods to determine if you have elevated levels of noise or the front panel of the DAWN. RMS noise should be below 200 μV though lower noise may be needed for optimal signal-to-noise.
 - a. Compare noise levels between Elution and Focus mode. Compare Elution and Elution + Inject mode.
 - b. If Elution mode has an increased noise level but Focus mode has low noise, this indicates a contamination of the Eclipse separation channel. Changing the membrane will most likely solve the problem. Consider cleaning the channel if needed.
 - c. If only Elution + Inject mode has an increased noise level then most likely your autosampler is contaminated. Try to inject cleaning solutions 5 – 10 times with the full volume of the sample loop.
 - d. Increased noise in both Elution and Focus indicates a system contamination. Please proceed to Step 2.
2. Connect pump directly to your light scattering detector.
 - a. If noise level is still high, this indicates a contamination of mobile phase or pump (or flow cell). Use fresh mobile phase and exchange the membrane inside inline filter. Also, your light scattering detector could be the source. You can check this by taking the LS detector offline and inject fresh clean mobile phase with a syringe.

- b. If noise level is good, this indicates the mobile phase and pump are fine. Please proceed to step 3.
3. Plumb the system to include the existing upstream detectors
 - a. If noise level is increased again, your upstream detector or tubing may be contaminated. Can perform an in-situ cleaning of the upstream detectors.
 - b. If the noise level is still good, please proceed to step 4.
4. Set up your system again and perform the basic or intense cleaning procedures described below. If you suspect the Eclipse to be dirty, you can replace the downstream detectors with a short coil of red PEEK tubing (0.005" I.D.) to simulate detectors to avoid clogging the detectors.

Basic Cleaning

Running the basic cleaning procedure below may be necessary on a regular basis to keep the noise at a suitable level. The cleaning should proceed through the HPLC and all four modes of the Eclipse. Downstream detectors may be bypassed and replaced with a coil of tubing to avoid introducing clogs as contaminants are cleared from the HPLC. The following cleaning solutions and durations are recommended:

Step	Mobile Phase	Mixture	Flushing Duration
1	Water	100%	3 hours
2	Water/Ethanol	80%/20%	6 hours
3	Water	100%	3 hours
4	Buffer	N/A	N/A

The following considerations should be kept in mind:

- Flush always with pure solvent in between different mobile phases to avoid any precipitation of salt.
- We strongly recommend using a 5-way union instead of a separation channel for cleaning. The channel can be cleaned by hand as described in [Variable Height Channel](#) Cleaning Guide.
- For each step, if possible, purge the Eclipse by using the Flush options in VISION (right-click on Eclipse widget).
- Due to the higher viscosity of alcohol mixtures, the pressure on step 2 may be increased and the flow rate may need to be decreased to compensate. Slower flow rates for longer durations are acceptable.

Deeper Cleaning

If the noise does not improve or if noise is significantly elevated with large distributions of random spikes, it is recommended to perform a more aggressive cleaning. The channel should be bypassed with a 5-port union and the downstream analytical detectors should be replaced with a coil of PEEK tubing to avoid introducing downstream clogs. Analytical detectors can be flushed via syringe (using syringe pump) one-by-one to avoid introducing clogs. Running the basic cleaning procedure below may be necessary on a regular basis to keep the noise at a suitable level. The cleaning should proceed through the HPLC and all four modes of the Eclipse. An example cleaning method is provided in the next section. The following cleaning solutions and durations are recommended:

Step	Mobile Phase	Mixture	Flushing Duration
1	Water	100%	3 hours
2	SDS*	0.5%	6 hours

3	Water	100%	3 hours
4	Water/Ethanol	80%/20%	6 hours
5	Water	100%	3 hours
6	Water	100%	Eclipse Flush Procedures

* SDS may precipitate under lower temperatures. Disable autosampler cooling functions as a precaution.

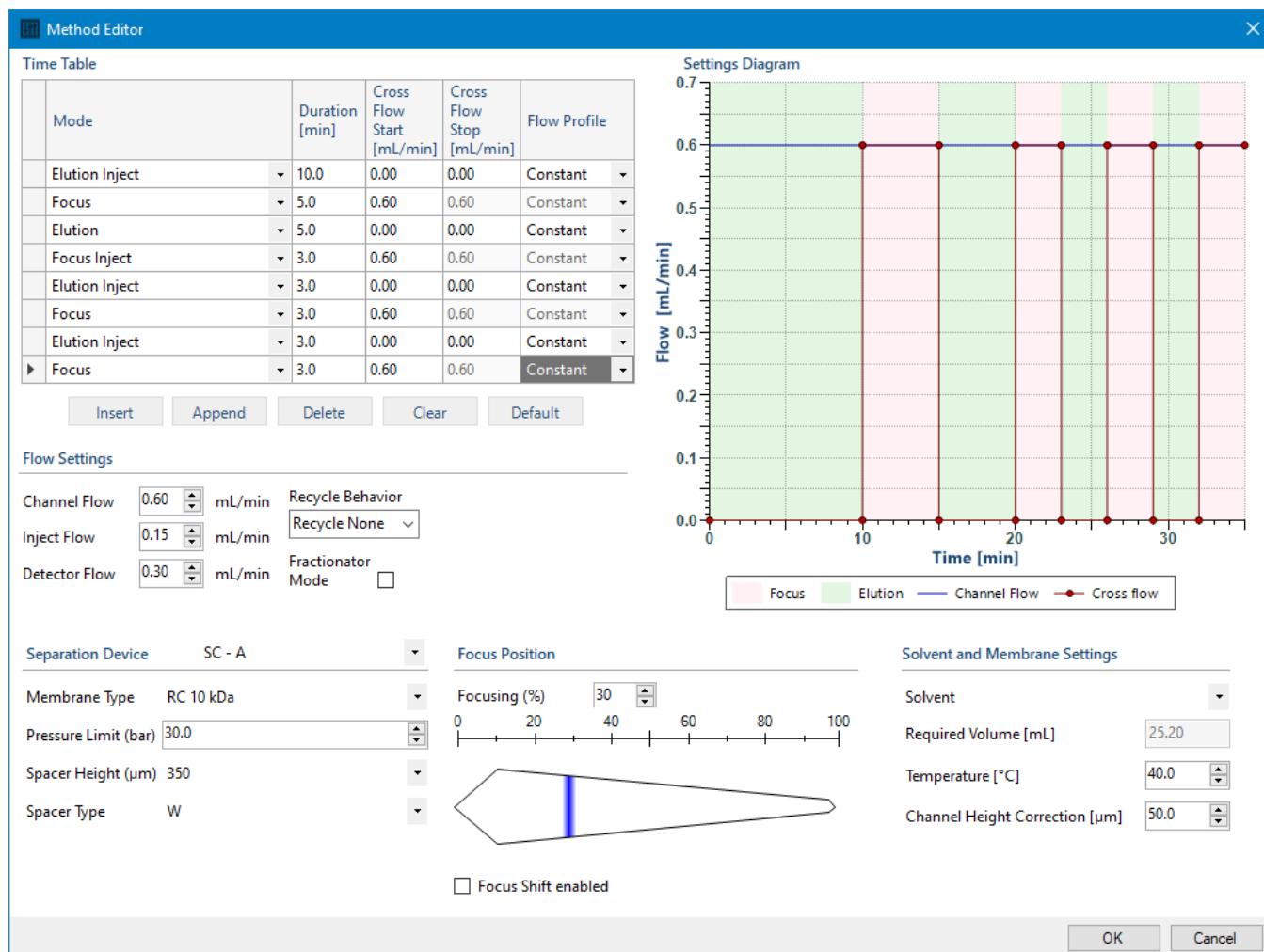
The following considerations should be kept in mind:

- Flush always with pure solvent in between different mobile phases to avoid any precipitation of salt.
- Use a 5-way union instead of a separation channel when cleaning. The channel can be cleaned by hand as described in [Variable Height Channel Cleaning Guide](#).
- For highly contaminated systems, please disconnect the detectors for cleaning to avoid introducing clogs into the detectors. Please attach some red PEEK tubing (0.005" I.D.) to the detector port in order to have enough pressure for the Eclipse to regulate cross flow.
- When using an isocratic pump, please run rinsing methods for each solvent manually. For quaternary pumps the entire rinsing procedure can be programmed in a sequence.
- During the cleaning steps it is recommended to inject the corresponding mobile phase several times, to also rinse the injection valve. A full sample loop volume can help flush the injection system.
- For each step, if possible, purge Eclipse using the Flushing functions in VISION 3 (right-click on Eclipse widget).
- Do not use higher SDS concentrations, as this will prolong the following rinsing step to clear the detergent.
- Due to the higher viscosity of alcohol mixtures, the pressure on step 2 may be increased and the flow rate may need to be decreased to compensate.
- Purge the Eclipse with water prior to returning the system to your normal mobile phase. Prepare fresh mobile phase and inject directly into the MALS detector to assess cleanliness.

Cleaning Method

A cleaning method that frequently switches between modes with low flow rates can be run for several hours via a sequence table with multiple injections to automate the cleaning. The method below cycles through the four modes while helping to remove residual contamination. Run the Eclipse purge options under the Flushing window to assist. This method can also be incorporated into a sequence. Please keep the following in mind:

- Method duration is 30 minutes
- Method consumes about 25 mL of solvent each time it is run and can be run at least 5 times
- Do not recycle solvent when cleaning
- Bypass channel with a 5-port union



If you have a binary or quaternary pump, methods for each solvent line can be created and the switching automated. An example sequence for cleaning where each method is pulling from a different solvent reservoir is provided below with multiple injections for each step:

Sequence Table						
	Ready					
	Enabled	Vial	Volume [μL]	Inj./Vial	Sample Name	VISION RUN Method
1	<input checked="" type="checkbox"/>	1	100.00	6	Water Cleaning	Cleaning Method from Bottle A (Water).M
2	<input checked="" type="checkbox"/>	2	100.00	12	SDS Cleaning	Cleaning Method from Bottle B (SDS).M
3	<input checked="" type="checkbox"/>	3	100.00	6	Water Flushing	Cleaning Method from Bottle A (Water).M
4	<input checked="" type="checkbox"/>	4	100.00	12	Ethanol Cleaning	Cleaning Method from Bottle C (EtOH).M
5	<input checked="" type="checkbox"/>	5	100.00	6	Water Flushing	Cleaning Method from Bottle A (Water).M

Note the following:

- In this example, the sample loop volume is 100 μL, so 100 μL injections were performed. You should inject the full volume of the loop for best flushing—if injecting 900 μL volumes, you may need more rows to account for the number of injections per vial column. In this case, a 2 mL autosampler vial is sufficient for twelve 100 μL injections.

- In this example, a quaternary pump was available for switching the solvents between rows. Each solvent has a unique method to ensure the pump is drawing from the correct solvent bottle. If using an isocratic pump, the solvent line will need to be switched between bottles.
- With an isocratic pump, the sequence can be used to do 6 injections (3 hours) for water before switching to 0.5% SDS and setting up a second sequence to do 12 injections (6 hours).

Although higher flow rates can be used for faster flushing, the mode switches and valve state changes can result in temporary pressure spikes that could overpressure the channel. This is especially true going to or from the elution with injection mode.

Variable Height Channel Cleaning Guide

During membrane installation, several of the components of the channel can be cleaned; however, it may be necessary to thoroughly clean the entire channel if the bottom block of the channel has been contaminated. If you suspect the entire system may need cleaning, it is always recommended to remove the channel and clean by hand. By pass the channel with a 5-port union to clean the Eclipse and other analytical detectors.

This can happen if the membrane has not been correctly centered or if the channel has been left in a solution that has formed microbial growth. This section will overlap with the section, [Replacing Channel Membrane and Spacer](#), but emphasize details related to completely disassembling the channel for cleaning. In addition to the tools listed in the membrane installation section, you will need a 2 mm hex driver to remove the channel temperature regulator from the bottom block of the channel.

To completely disassemble the channel and clean, please refer to the following instructions:

1. Follow steps 1-10 in [Replacing Channel Membrane and Spacer](#).
2. At this point, the top block of the channel will be removed along with spacer, membrane, the ceramic frit, and the O-ring. Handle the ceramic frit carefully to avoid damage. The O-rings from both channel blocks and the ceramic frit can be cleaned once removed.

WARNING: The ceramic frit can easily crack if bent or dropped. Be careful when removing or cleaning. Contact Wyatt Technology for ceramic frit replacements if you observe any cracks or damage to the frit. Do not remove the frit in Fixed Height channels!

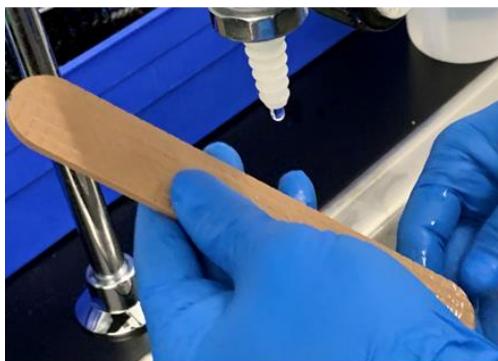


Remove O-rings with the plastic O-ring removal pick, which will eliminate risk of scratching or damaging the O-ring or block.

Be careful: the ceramic frit is fragile and can break if dropped.

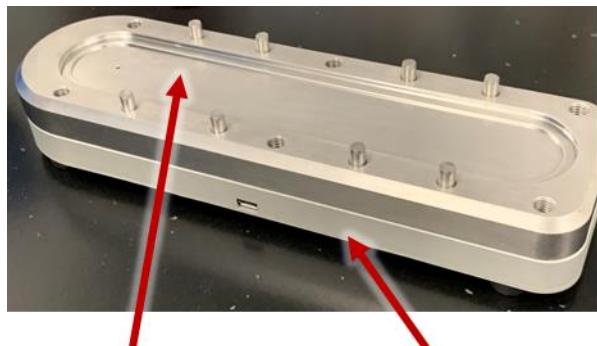
Start with the standard channel disassembly described earlier in this handbook. You can remove the two O-rings and the ceramic frit from the bottom block and set them aside.

- The O-rings can be scrubbed with 1% detergent solution, water, and/or alcohol. The ceramic frit can be rinsed to clean, or soaked in an appropriate cleaning solvent for the suspected contamination. If using 0.5 – 1% detergent solution, ensure the detergent is fully flushed from the ceramic. The material is quite porous and can retain residual solvent, fibers, or contamination.



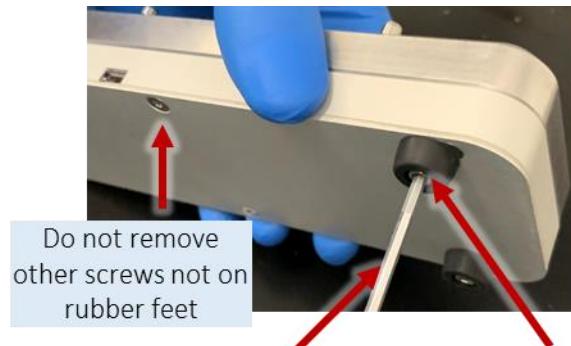
Residual fiber from membrane on frit

- The bottom block is installed with the temperature regulator, which is not water-proof. As a result, the channel temperature regulator will need to be removed before rinsing or submerging the bottom block of the channel. This is done by removing the four rubber feet on the bottom of the channel temperature regulator, which are screwed through the temperature regulator to secure it to the bottom block of the channel.



Bottom block of the channel

Channel temperature regulator (not waterproof)



Do not remove other screws not on rubber feet

2 mm hex driver

Only remove the 4 screws through the rubber feet

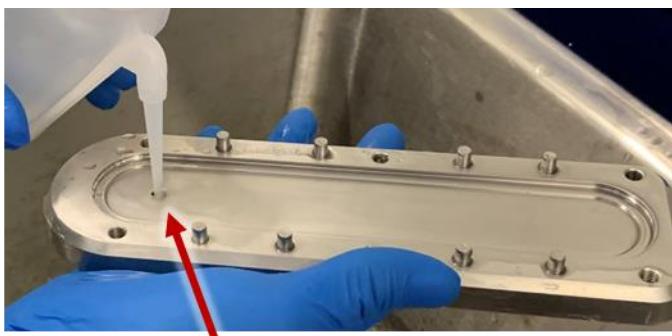
- With the four feet removed and the channel temperature regulator removed from the bottom block, the channel will be completely disassembled as shown below.



- The surface of the channel heater can be wiped with a damp cloth to remove dust but should not be submerged or rinsed with water.

WARNING: The channel temperature regulator is not waterproof. The channel temperature regulator should not be submerged or rinsed under running water. Clean and dry the top surface with a kimwipe or microfiber towel.

- Now that the bottom block of the channel has been removed from the channel temperature regulator, the channel block can be rinsed and cleaned. Similar to the top block of the channel, the channel can be scrubbed with 1% detergent, water, and alcohol. The ridges in the bottom block of the channel can also be cleaned.

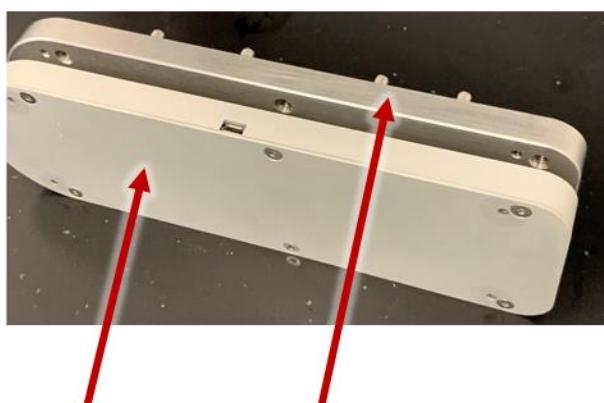


Clean fluid ports in the bottom channel block (and top block)



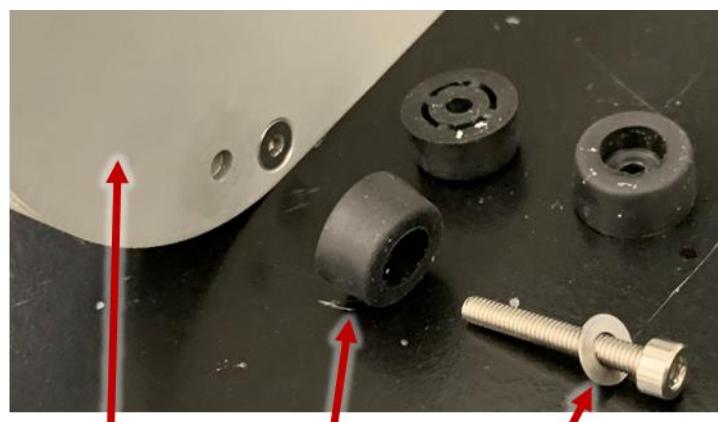
Clean inner edges

- Once cleaned, the channel block can be rinsed with alcohol and dried. A filtered air-gun can be used to dry the channel block more quickly. It is important to dry the channel block because the channel heater is not waterproof.
- The channel temperature regulator has four feet for the bottom block of the channel. The rubber feet have a direction and are secured to the regulator with a screw and washer.



Channel temperature regulator

Dried bottom block



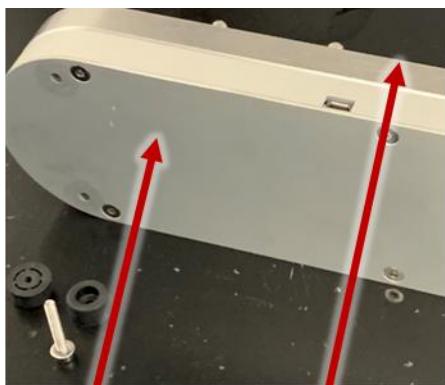
Bottom of channel temperature regulator

Rubber feet

2mm hex screw and washer

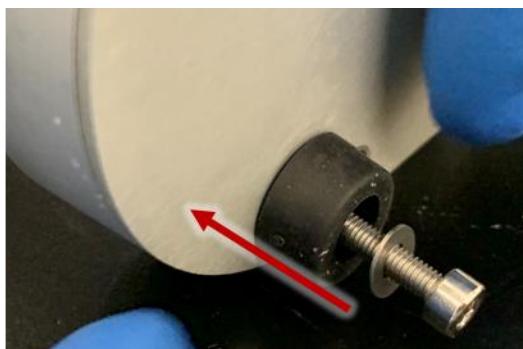
- Align the regulator (in addition to the 4 holes for rubber feet, the bottom of the channel temperature regulator will have 6 screws visible) with the bottom block of the channel. For the rubber feet, the flat side

will be flush with the channel temperature regulator and the side with a cavity (matching the size of the washer) will face outward.



Channel
temperature
regulator

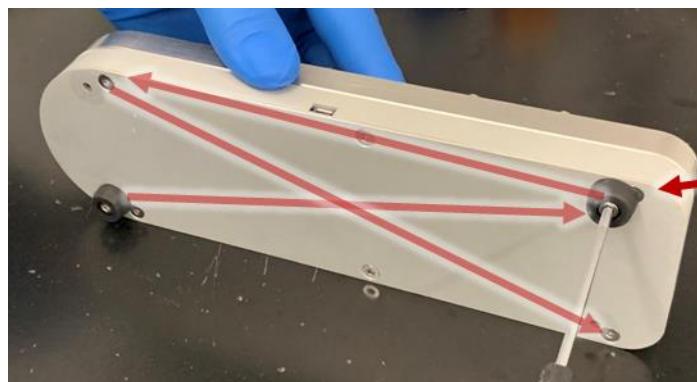
Dried bottom
block



Re-attach channel temperature
regulator to bottom block
through the rubber feet

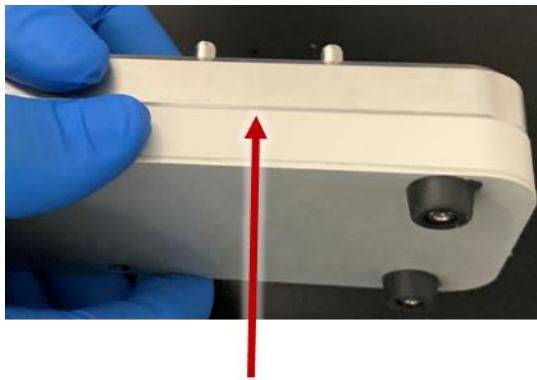


11. To apply pressure uniformly and ensure the channel temperature regulator is flush to the bottom block of the channel, the rubber feet can be screwed in a crisscross pattern. Please note that the rubber feet will likely continue to rotate even when screwed in. Do not overtighten the screw securing the rubber feet.

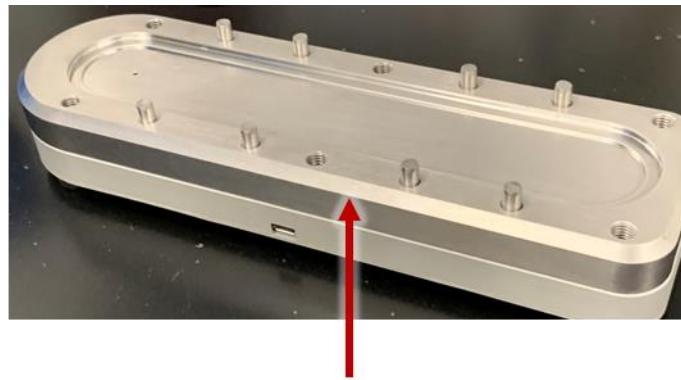


Don't over-tighten

12. Once secured, the rubber feet may rotate; however, the channel temperature regulator and the bottom block of the channel should be flush and secure.



Flush and secured



Ready for re-installation of O-ring, ceramic frit.

13. Once re-assembled, the bottom block is now ready for the re-installation of the ceramic frit and O-ring. The frit can be installed and centered, and the O-ring should be carefully re-inserted around the frit. Keep in mind that the textured surface of the ceramic frit faces down in order to distribute pressure evenly and

reduce damage to the frit. The smooth surface will be facing up and is ready for installation of the membrane.



14. Resume with step 11 in [Replacing Channel Membrane and Spacer](#).

Fixed Height Channel Cleaning Guide

During membrane installation, several of the components of the channel can be cleaned; however, it may be necessary to thoroughly clean the entire channel if the bottom block of the channel has been contaminated. If you suspect the entire system may need cleaning, it is always recommended to remove the channel and clean by hand. By pass the channel with a 5-port union to clean the Eclipse and other analytical detectors.

The fixed height channel has a permanently installed frit on the bottom block. Unlike the instructions shown above for the variable height channels, the fixed height channel frit should never be removed. As such, it is only recommended to clean the fixed height channel frit via in situ flushing with high flows under focusing, with the cross flow pathway diverted to waste.

Notable Differences between Eclipse (NEON) and Eclipse AF4 or DualTec

It is recommended to read this document carefully in order to fully understand and safely operate the Eclipse. Please contact Wyatt Technology if you are unclear about anything. In this section, a brief overview of the major changes from legacy Wyatt or Superon Eclipse instruments will be outlined. This is not a comprehensive list but is instead designed to capture some of the biggest changes:

Hardware:

- The hardware has been completely re-designed with an intuitive front panel and a greatly expanded list of alarms and system monitoring. It is imperative that the software be launched for complete alarm and system monitoring. Do not flow when the software is closed or if the instrument is powered off.
- The Eclipse has an internal system for recycling both detector and cross flow simultaneously or independently. It does not need to be paired with an ORBIT or external recycling system.
- (Optional) The Dilution Control Module (DCM) option enables increase in detector signals without loss of resolution up to 5x by removing excess solvent from the channel.
- (Optional) The Eclipse Mobility option enables the combination of AF4 cross flow and electrical fields for data analysis and potentially improved separation.
- (Optional) The SEC switching option (previously available in DualTec) is markedly improved with safeguards when switching to or from the column port. There is a dedicated column connection port.
- The Eclipse measures inlet, inject, cross flow, and detector flow (with DCM option). As a result, focus position is determined via flow meter.
- Hardware is both aqueous and organic compatible. There are no needle valves, all flow control is performed with CoriFlow and back-pressure regulators (with DCM option).
- There are 5 leak sensors, a vapor sensor, Rheodyne sensors, and solvent density measurements.
- The chassis temperature can be controlled and is recommended to be set a few degrees above ambient.
- The bulkhead has been clearly marked and color-coded, there is only one waste outlet. There is also a side-panel leak tray and connection for channel temperature regulator.
- Instead of small frit filters in the bulkhead unions, there are dedicated inline filters for the pump, cross flow, and DCM flow. With larger filter membranes, they protect the controllers longer.

Software:

- Plugins are no longer supported. All Eclipse and HPLC control is achieved through the VISION software. VOYAGER and SCOUT software programs are now called VISION RUN and VISION DESIGN, respectively.
- There is a new membrane flush procedure. With DCM, this can automate membrane flushing without needing to disconnect
- VISION RUN contains automated purge functionality, intelligent solvent recycling, a dedicated fraction collector port (with DCM option), and comprehensive system monitoring.
- Bronkhorst software such as FlowDDE and FlowPlot are no longer compatible with Eclipse. Functions are instead accessed via the “Performance & Tuning” window in VISION RUN. This includes “Learn Open,” “Capacity Adjustment,” and other settings. Due to digital control, there is not a need to input a conversion factor for cross flow capacity.
- Previously, cross flow regulation with default 6.67 mL/min capacity was poor below 0.12 mL/min; however, with the new Eclipse, the default capacity of 6.67 mL/min enables regulation down to 0.03 – 0.04 mL/min.

- Channel temperature is set in the Eclipse method editor and controlled up to 50°C.
- There are a number of other software improvements and diagnostic information. For troubleshooting, please send the EMDF to Wyatt Support, which contains information on the configuration, tuning settings, and other details. Collect all the Eclipse signals.

Channels:

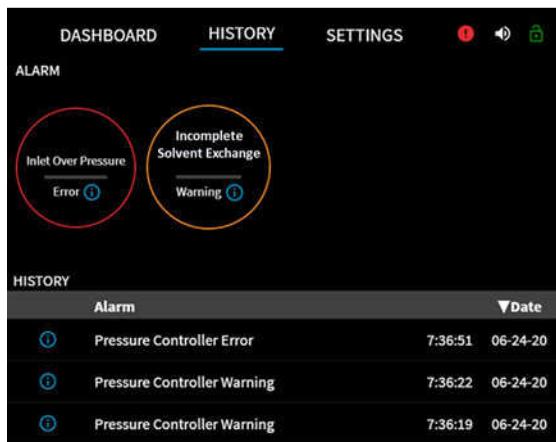
- The channels have been redesigned to be 316 stainless steel and all solvent compatible except for the spacers in the Variable Height Channels. They have enhanced reproducibility and mass recovery.
- The max torque setting for Variable Height Channels is 4 Nm, which is significantly less than with legacy channels. Avoid over-torque, which can damage the ceramic frit. Fixed Height Channels do not require a torque wrench and need only be tightened with an L-key until they reach a hard stop.
- Laminated spacers for Variable Height Channels provided a better seal but are consumable and should be replaced whenever the channel is disassembled for best performance. These are not compatible with organic solvents, which require either Mylar spacers or the Fixed Height Channels.
- All channels have a DCM port, if no DCM option is available, the port should be plugged. It can be loosened to remove any trapped air when flushing the membrane.
- All channels are color-coded for connections. The tubing will be either tan or green 0.01" I.D. or 0.03" I.D. tubing respectively, but with a reusable sleeve for identification. Cross flow pathways now use the green tubing instead of the 1/8" Teflon tubing used previously.
- All channels come with a temperature regulator. This unit is not waterproof and should not be exposed to water. The channel temperature is set in the Eclipse method editor and it is recommended to set the channel temperature a few degrees above ambient.

Additional Considerations:

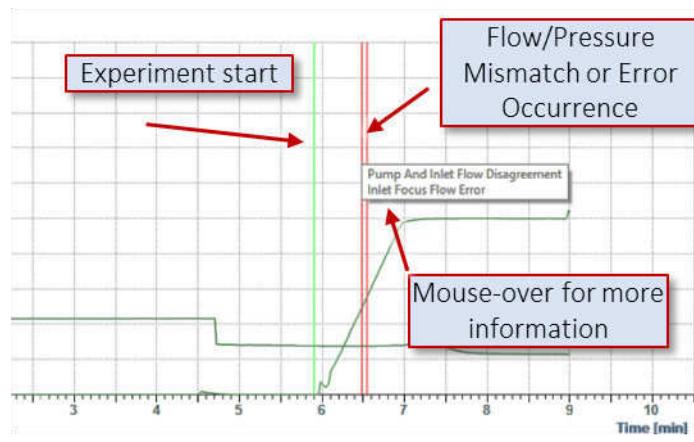
- It is recommended to increase the pump pressure with a coil of red PEEK tubing in order to improve the performance on the inject controller, which is sensitive to pump pulsations.
- Please consult the HPLC tips and tricks in this document for applying the best possible settings.
- Even with this list of notable differences, it is highly recommended to fully read and understand this document before operating the Eclipse.

Troubleshooting Tips & Tricks

The Eclipse features the Smart Services™ platform for monitoring the system and the VISION RUN online plot has alarm indicators. Selecting the badge on the Eclipse front panel or hovering over the vertical red line in the VISION online plot will provide additional information.



Eclipse Front Panel



VISION Online Plot

Figure 138. Smart Services™ platform and the VISION information prompts.

Pressing the ⓘ (information) icon on the Eclipse front panel (in the History tab or on a system health indicator) will show more information.

Eclipse Liquid and Vapor Leak Detection

The Eclipse has several internal leak sensors and a vapor sensor. There are leak sensors for the flow controller connections, the valve connections, the internal fluid matrix, and the side-panel (exterior) fluid matrix. There are individual alarms for each sensor to simplify the process of locating a leak. Internal leaks will be uncommon unless hardware changes have been performed.

If a leak has occurred, it may be the external fluid matrix, especially after exchanging tubing or performing a bulkhead frit filter replacement. Fluid that leaks from the side-panel will drain into the leak tray and enter the internal Eclipse leak drainage system. If an external leak is detected, identify the source of the leak and either tighten the fitting or replace the fitting and ferrule entirely. Once the leak has been identified and resolved, it may be necessary to dry the sensor. This can be done by rubbing the leak sensor with a cotton swab. The side-panel leak sensor can be accessed without removing the top cover; however, Figure 139 below shows where the leak sensor is located and where to access it with the cotton swab.

Solvents that are not conductive will not activate the liquid leak alarm. This includes many organic solvents. In the case of an organic solvent leak, the vapor sensor will activate. If the vapor alarm has been activated, check the external fittings and the surrounding area for organic solvents. It is possible that solvent vapor exterior to the instrument has been drawn in via the fans. If the vapor alarm activates even though only aqueous solutions are being used in the system and it is due to the environment, the vapor alarm sensitivity can be adjusted under the settings tab on the front panel of the Eclipse.

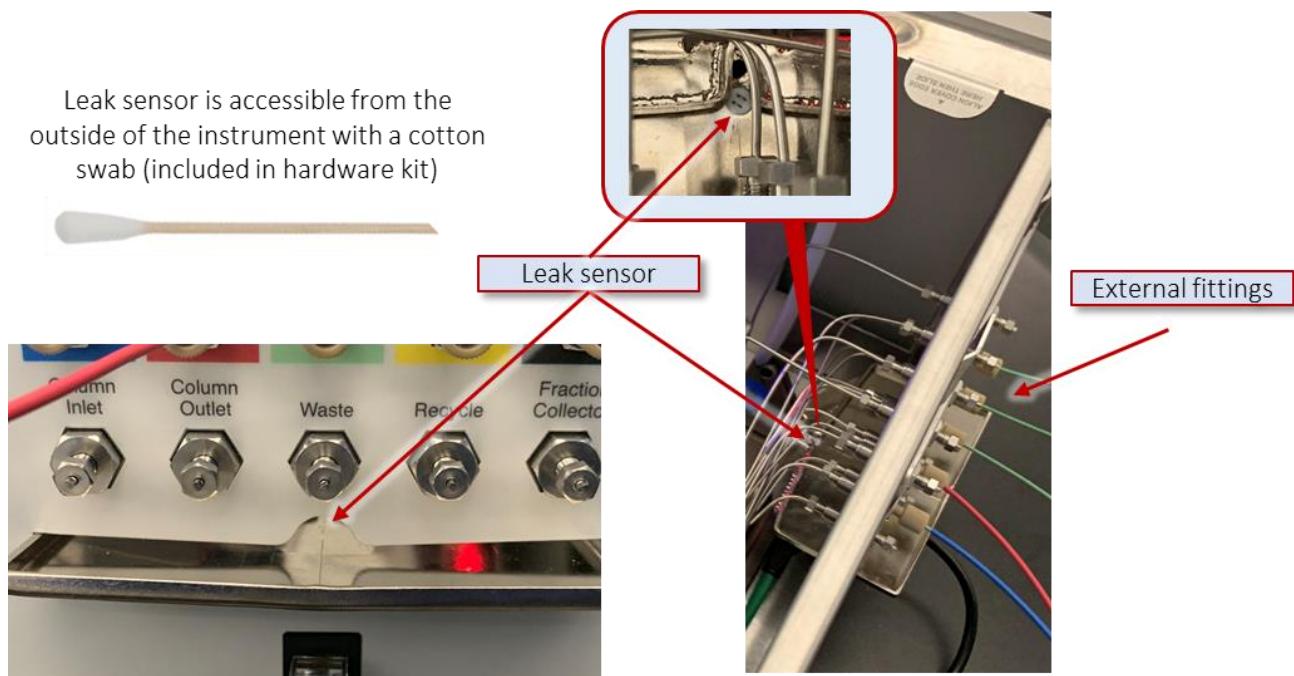


Figure 139. Side-panel leak sensor is located just inside the fluid matrix, which can be reached with a cotton swab included with the hardware kit in order to dry the sensor once a leak has been addressed.

Poor Flow Regulation

If you observe poor flow regulation in the Eclipse, there are a few possible causes. The most common cause is insufficient channel pressure. The flow controllers in the Eclipse require at minimum 5 - 8 bar of back pressure to regulate flow. At low detector flows (when using high split ratio) or without analytical detectors, the channel pressure may be insufficient. Narrow I.D. PEEK tubing, for example 0.007" (yellow) or 0.005" (red/pink), can be placed between the "To Detector" port and the first analytical detector to increase the back pressure to a suitable range.

If the channel pressure is in a suitable range, the next thing to check is for potential clogs or obstructions. The bulkhead frit filters or inline filters may clog over time. Replace the frit filters or filter membranes on the side-panel of the Eclipse and check the flows again.

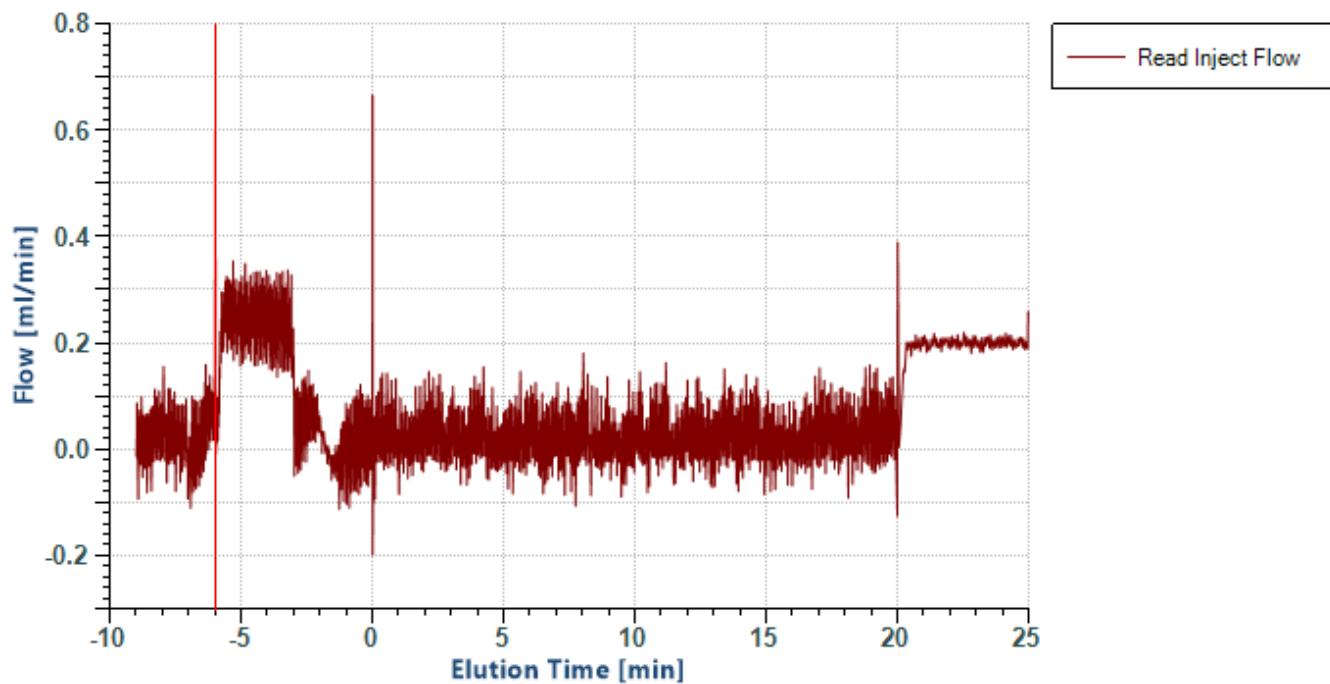
High System Pressures

High system pressures may be indicative of a clog. This will result in an overpressure alarm on the Eclipse front panel and the VISION software. For isolating a clog, the first place to check is the downstream detectors. Remove the tubing connecting to the inlet of the first analytical detector and see if the system continues to overpressure. If it does not, then likely there is an obstruction in the analytical detectors. The DAWN can be backflushed to remove clogs, or the inlet tubing can be replaced. The Optilab should never be backflushed—the only troubleshooting step would be to replace the 0.007" I.D. sacrificial tubing at the inlet. Contact Agilent for recommendations for isolating clogs in UV, FLD, or fraction collector modules.

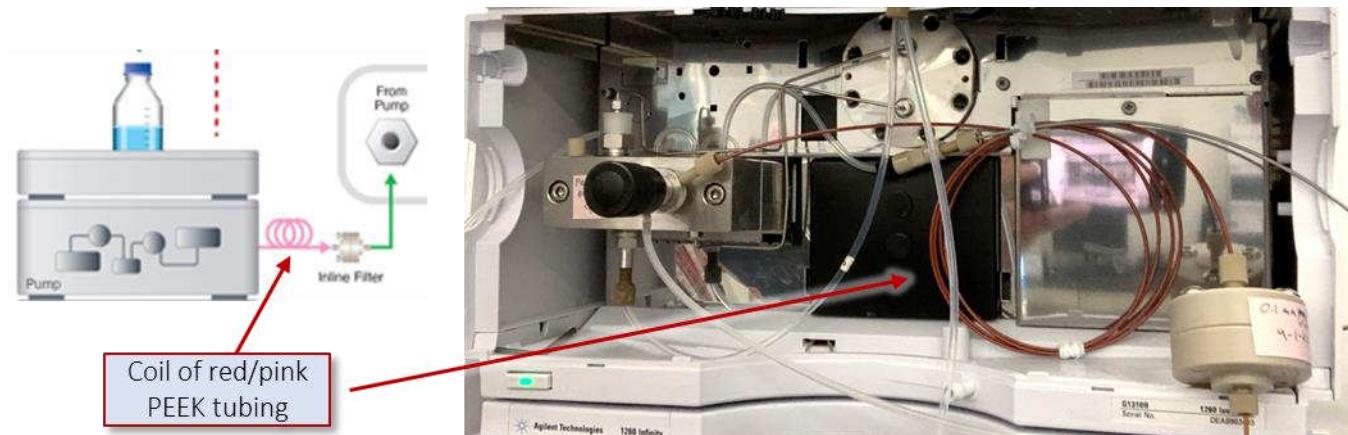
High system pressures may also be due to clog frit filters in the system or accidentally enabling fraction collector mode when the fraction collector port is plugged.

Oscillation in Inject Flow

When viewing the inject flow, you may see oscillations in the signal like so:

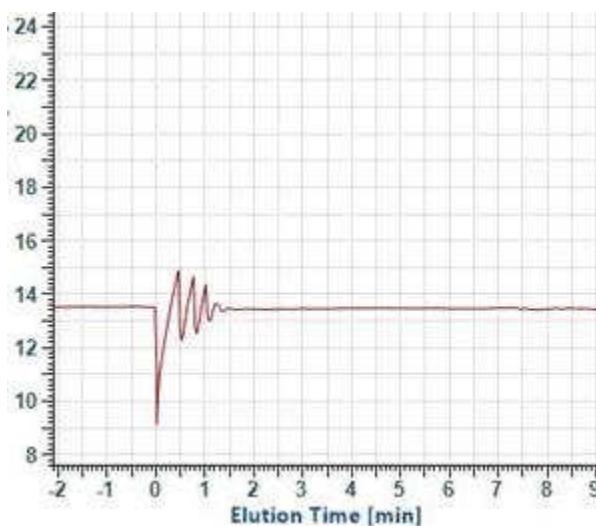


Because of how sensitive the inlet flow meters are to pump pulsations, this artifact is typically indicative of insufficient pulse dampening on the pump. In the case above, these oscillations disappeared when the red coil of tubing was installed between the pump and inline filter, increasing the pump pressure to at least 50 bar:

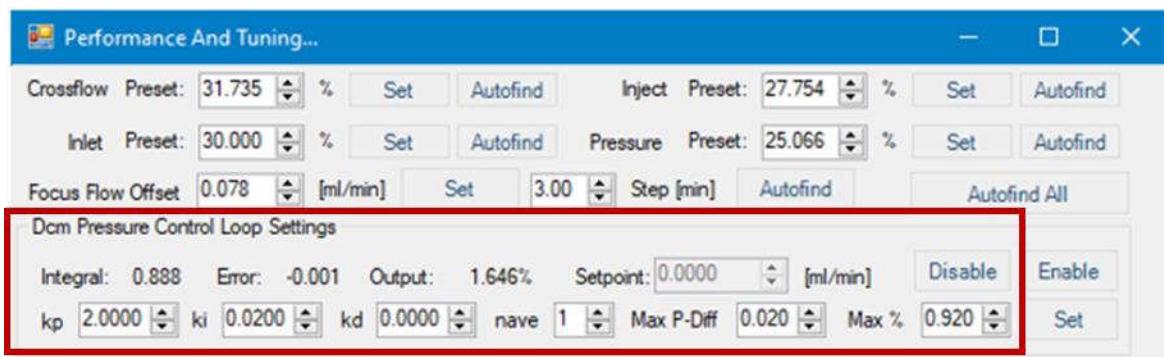


Oscillation in DCM Pressure

When viewing the DCM pressure, you may observe an oscillation in the DCM pressure:



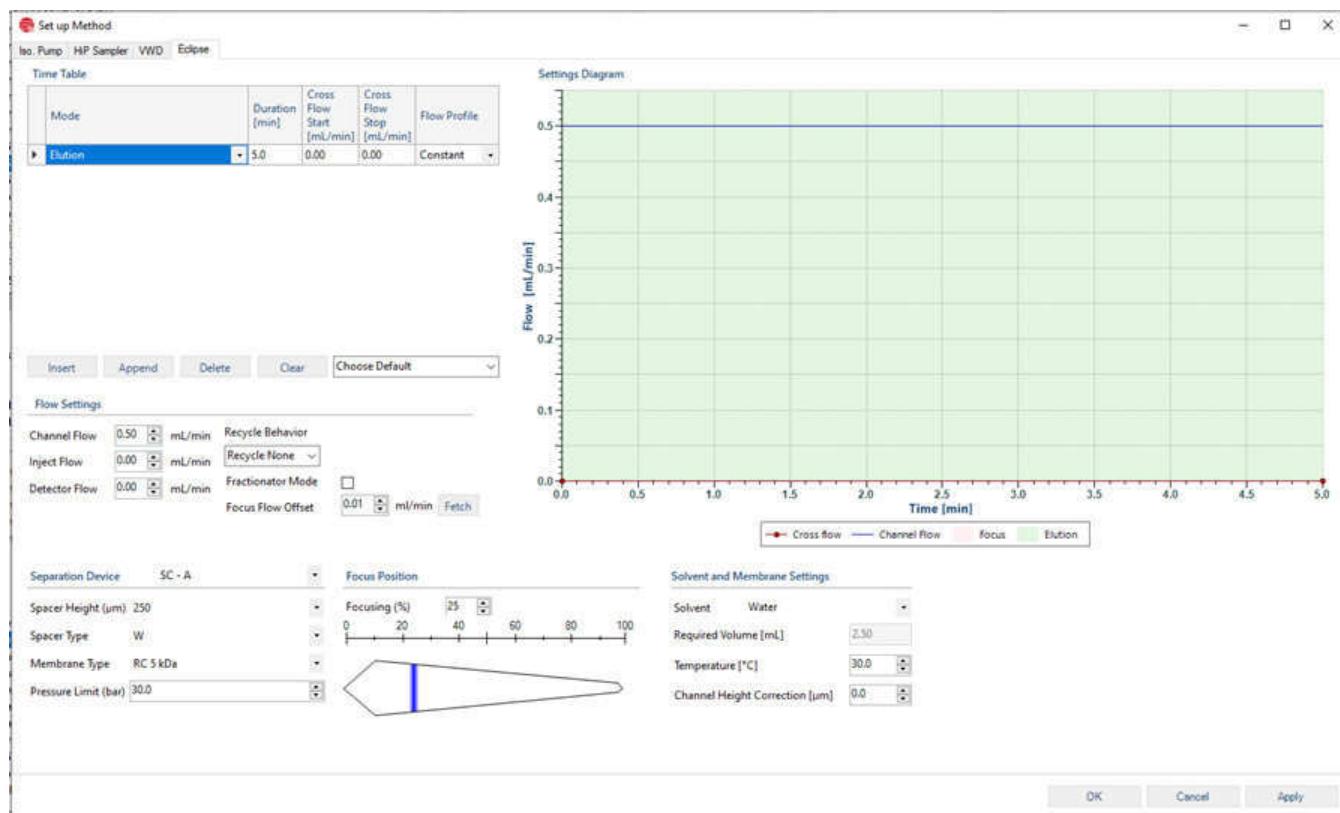
The most common root cause for this is an incorrect PID setting for the DCM pressure control loop settings. Please check that they match the values below:



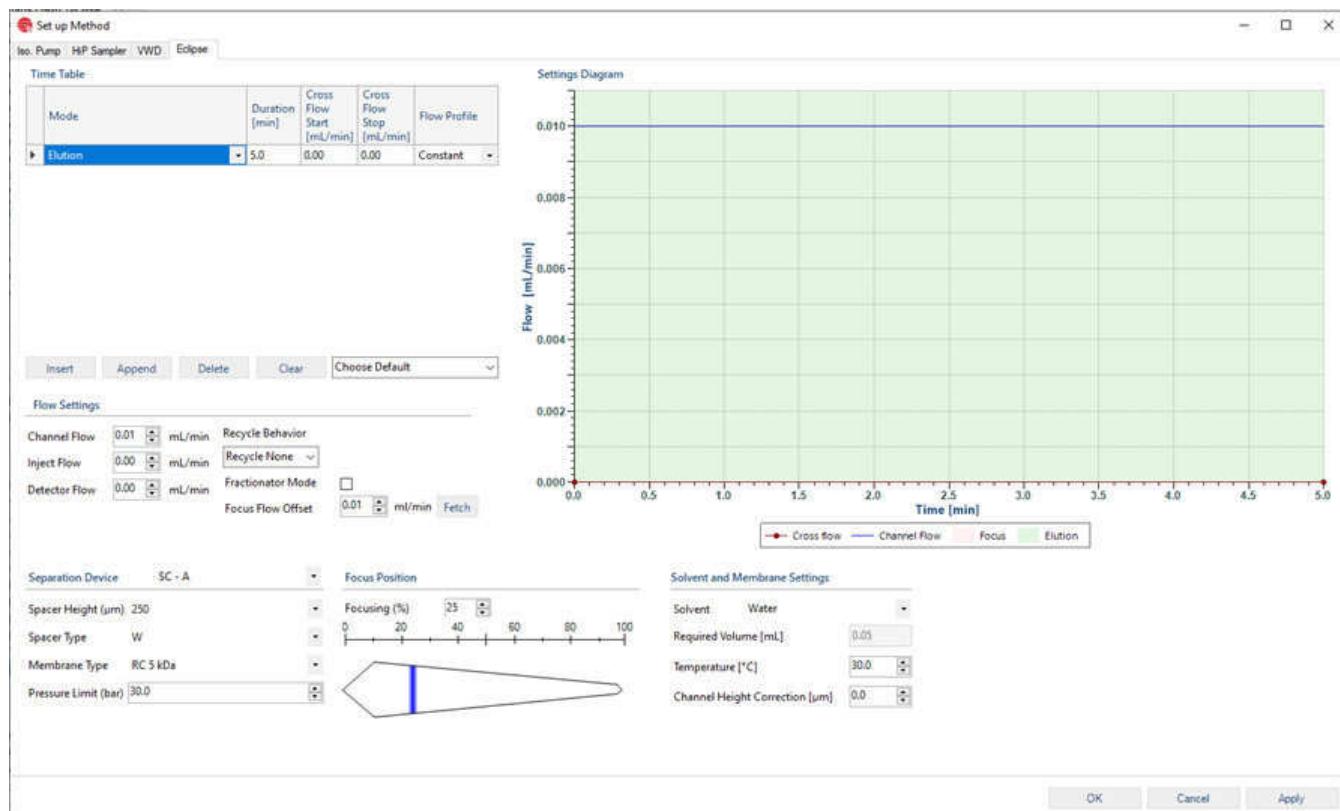
Zeroing the Bronkhorst Controllers

During troubleshooting, Wyatt Technology Support may recommended zeroing the controllers. Below are instructions for zeroing the Inject Coriflow; however, this procedure can be applied to zeroing other controllers as well. Please note that the CoriFlow is very sensitive and is prone to picking up environmental vibrations, so please do not bump or disturb the instrument during the zeroing procedure.

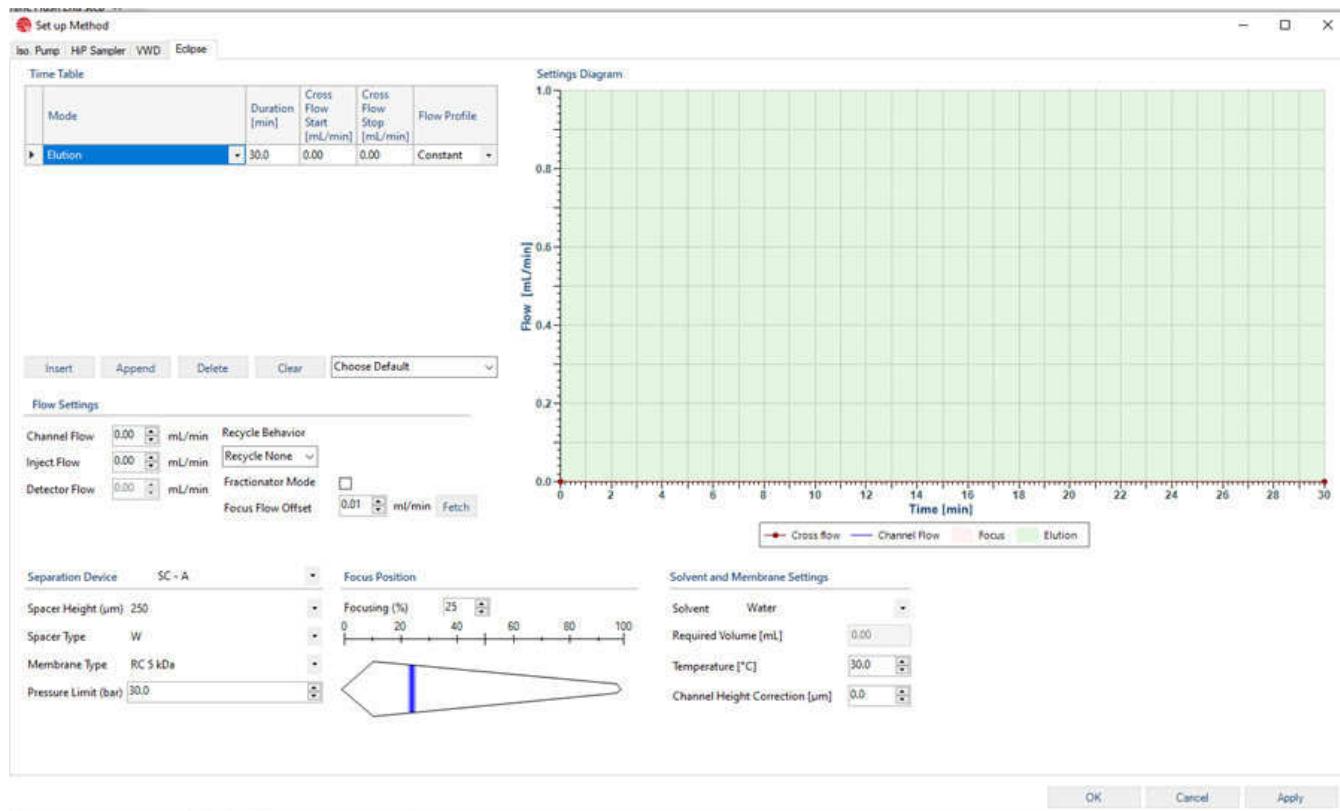
1. Create the following methods in VISION RUN.
 - a. Pre-Idle Step 1 Method



b. Pre-Idle Step 2 Method

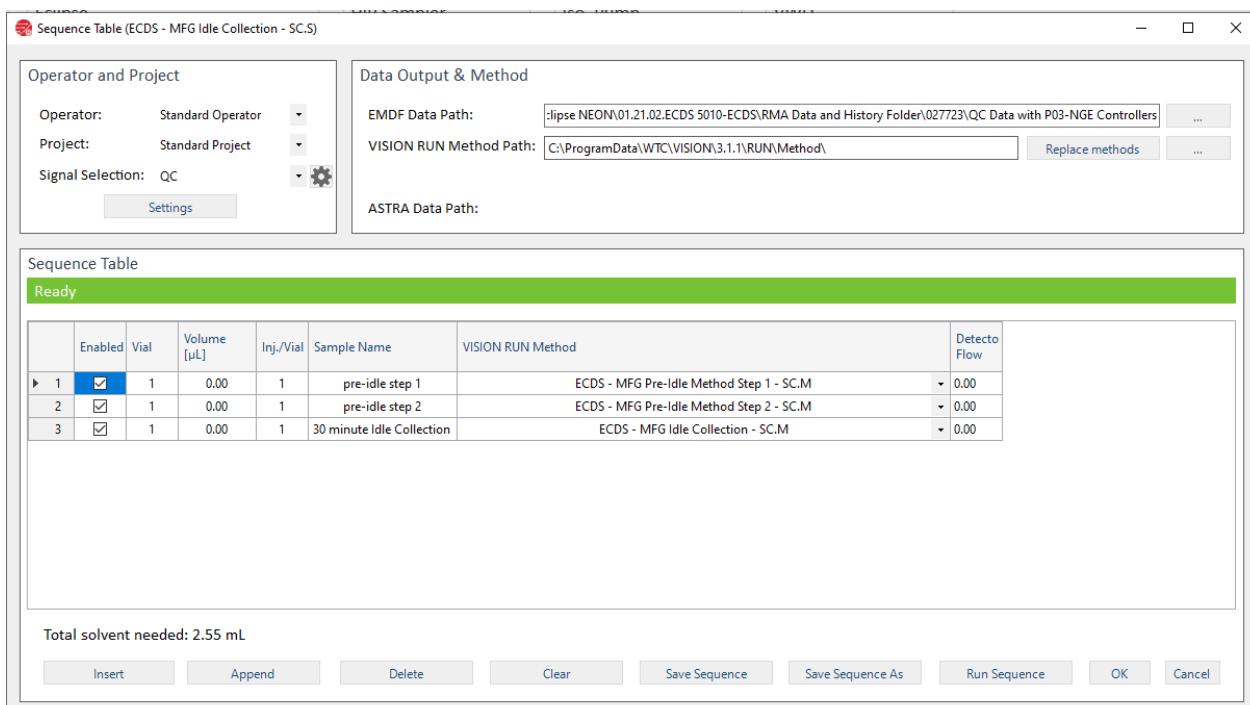


c. 30 Minute Idle Method

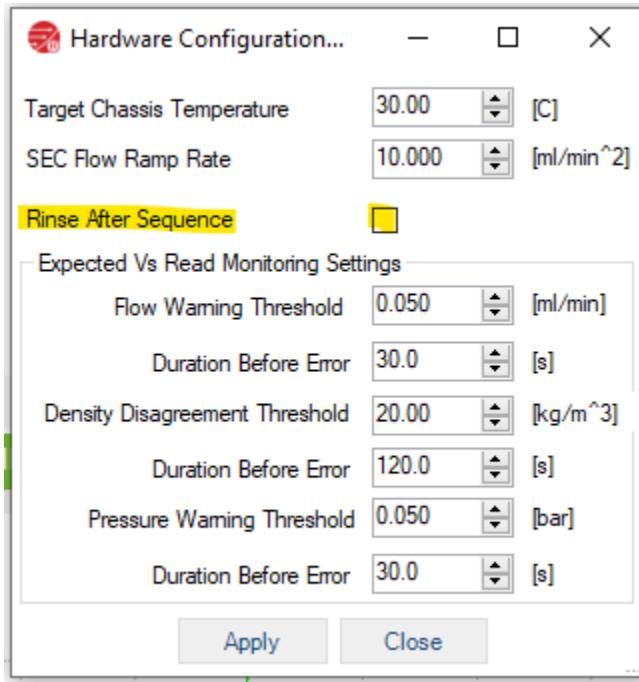


2. Create and save the following sequence:

a. Idle Collection sequence

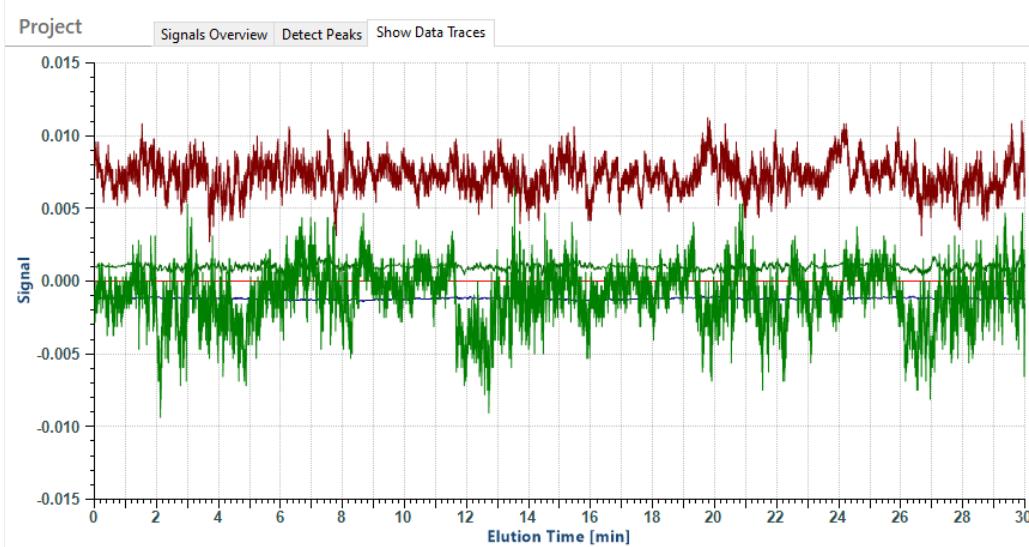


3. Disable “Rinse After Sequence” in More > Eclipse > Hardware Configuration

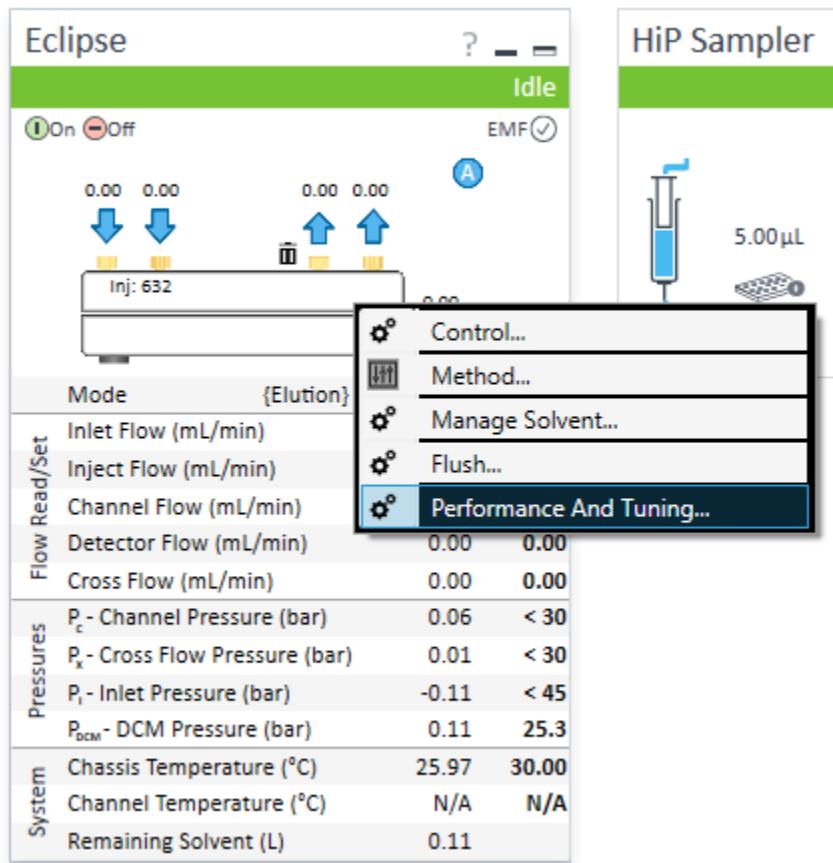


a.

4. Start the Idle Collection Sequence
5. Wait ~40 minutes until sequence has concluded
 - a. It is good practice to check that the flows are indeed stable and the system pressures have equilibrated before zeroing any of the Bronkhorst instrumentation. Please see a good example of an instrument that has equilibrated successfully prior to zeroing the Coriflows.

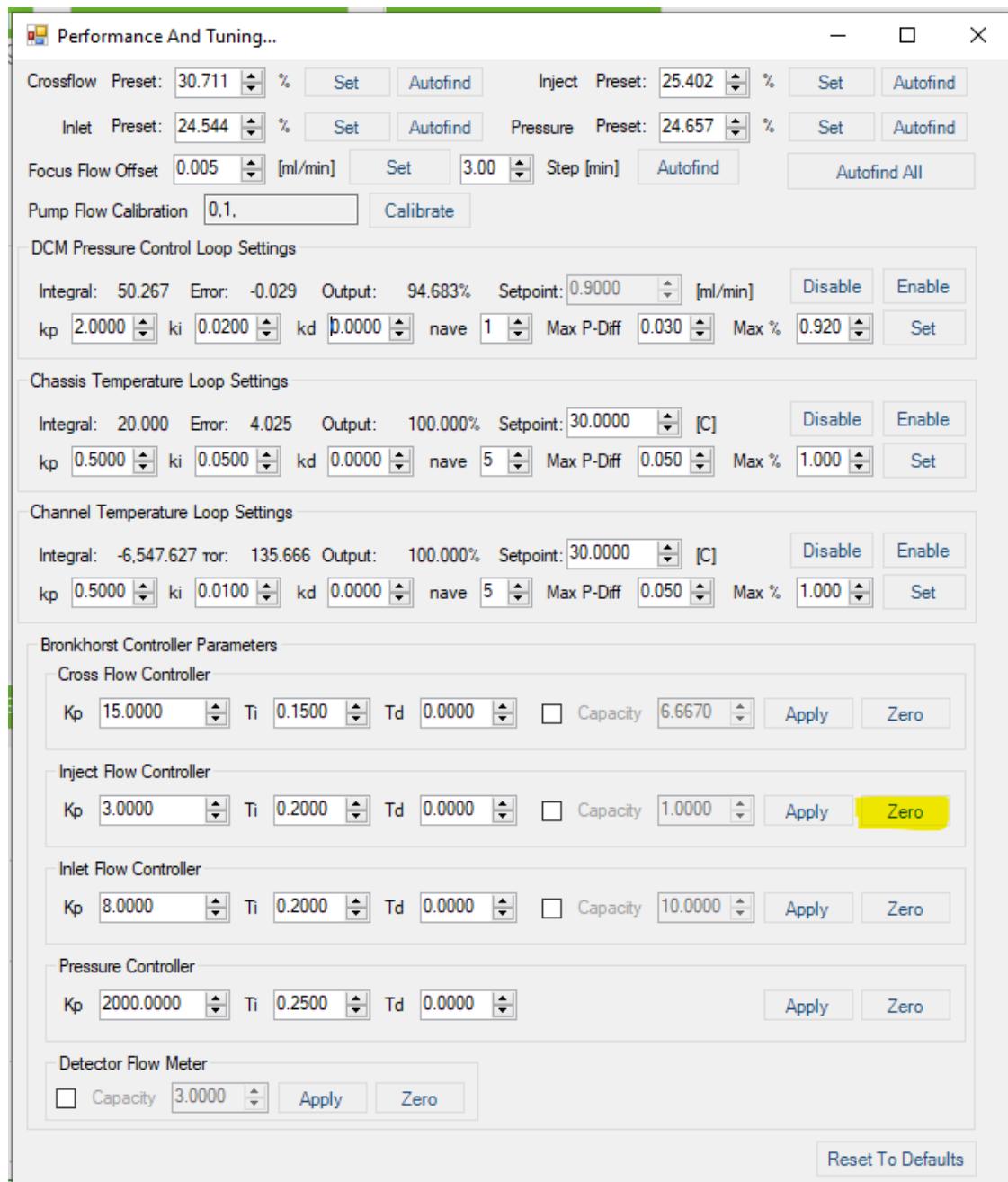


6. Open the Performance and Tuning Panel by right-clicking the Eclipse module in the main VR window and selecting “Performance and Tuning” in the dropdown.

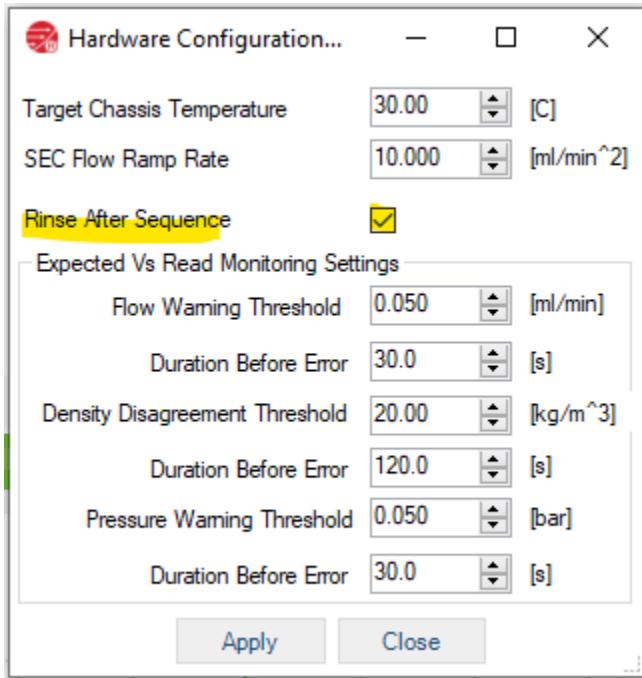


a.

7. Zero the Inject Coriflow by clicking “Zero” as highlighted below. The Eclipse module will enter a yellow “Not Ready” state and will return to a green “Ready” state once the Eclipse has finished zeroing the CoriFlow. If you need to zero a different controller, select the relevant controller.



3. You can confirm the Inject Coriflow has been properly zeroed by checking the inject flow data trace in the online plot and setting the minimum and maximum for that particular data trace to -0.05 and +0.05, respectively. You can also repeat the 30 minute Idle collection and inspect the inject flow reading in the data file.
4. Re-enable “Rinse After Sequence” if that is part of your workflow.



Instrument Connectivity Issues

Please refer to [TN1018 – Instrument Connection Guide](#) on the Wyatt Support Center for more information on instrument connectivity and troubleshooting instrument connections. Although written for ASTRA, the technical note covers PC-level troubleshooting that may help restore communications for the Eclipse with VISION. It will also guide you through re-connecting your other Wyatt analytical detectors that may be installed downstream of the Eclipse.

For communication issues with the Mobility module, the following special entries in the firewall might be needed when using a third party firewall:

- Outgoing/Incoming UDP Port 50001 should be open.
- Outgoing TCP Port 5000 should be open.

Additional Troubleshooting Resources

Wyatt Technology is committed to continually improving its resources. Please contact Wyatt Technology Support at support@wyatt.com for additional troubleshooting assistance and help.

Appendix A – Quick Reference Sheets

Please find the following quick reference sheets available in this handbook:

AF4 Simplified Theory Quick Reference

1

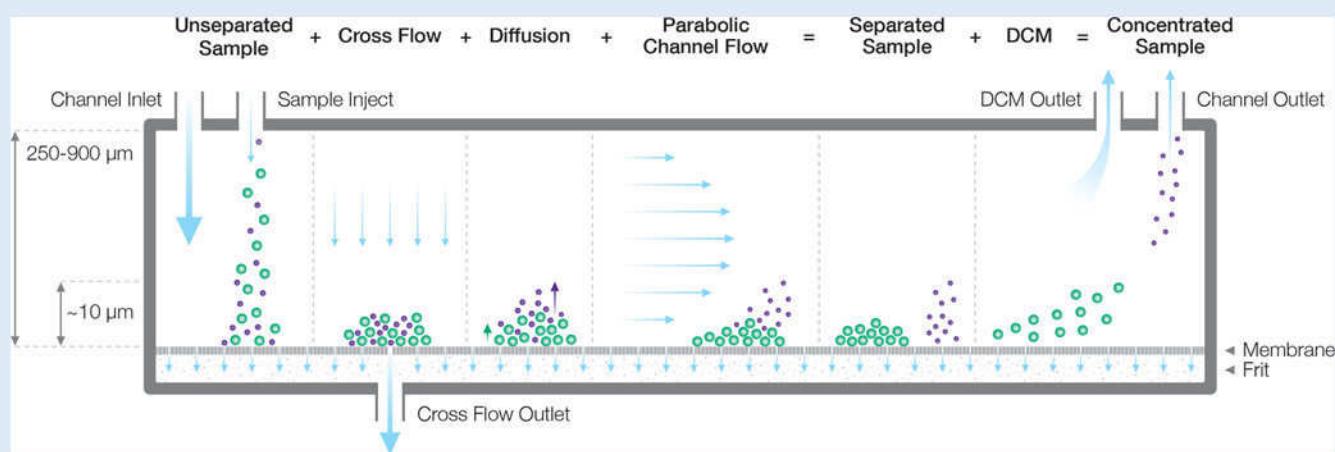


AF4 separation happens in a channel where flows are controlled by the Eclipse.

Variations and combinations of fluid flows enable separation by exploiting diffusion.

2

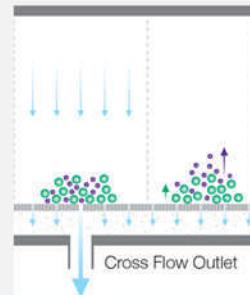
We'll look at a simplified breakdown of the separation process in the panels below. The figure below illustrates the key forces in play—which come together simultaneously to achieve separation.



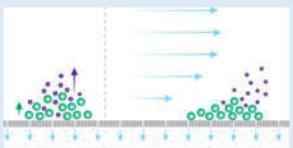
3

Sample is injected into the channel, where it may be concentrated into a narrow band in a step called "focusing." However, the main separation occurs during "elution." In the tunable elution step, cross flow pushes the sample to the channel's bottom, or "accumulation wall."

Although cross flow is pushing the sample down, the sample inherently moves against the force due to Brownian motion. Smaller species, which diffuse quickly, are statistically more likely to reach higher regions in the channel.



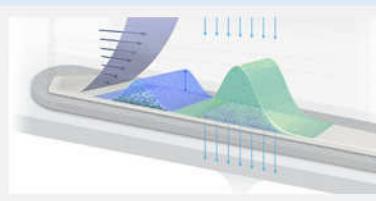
4



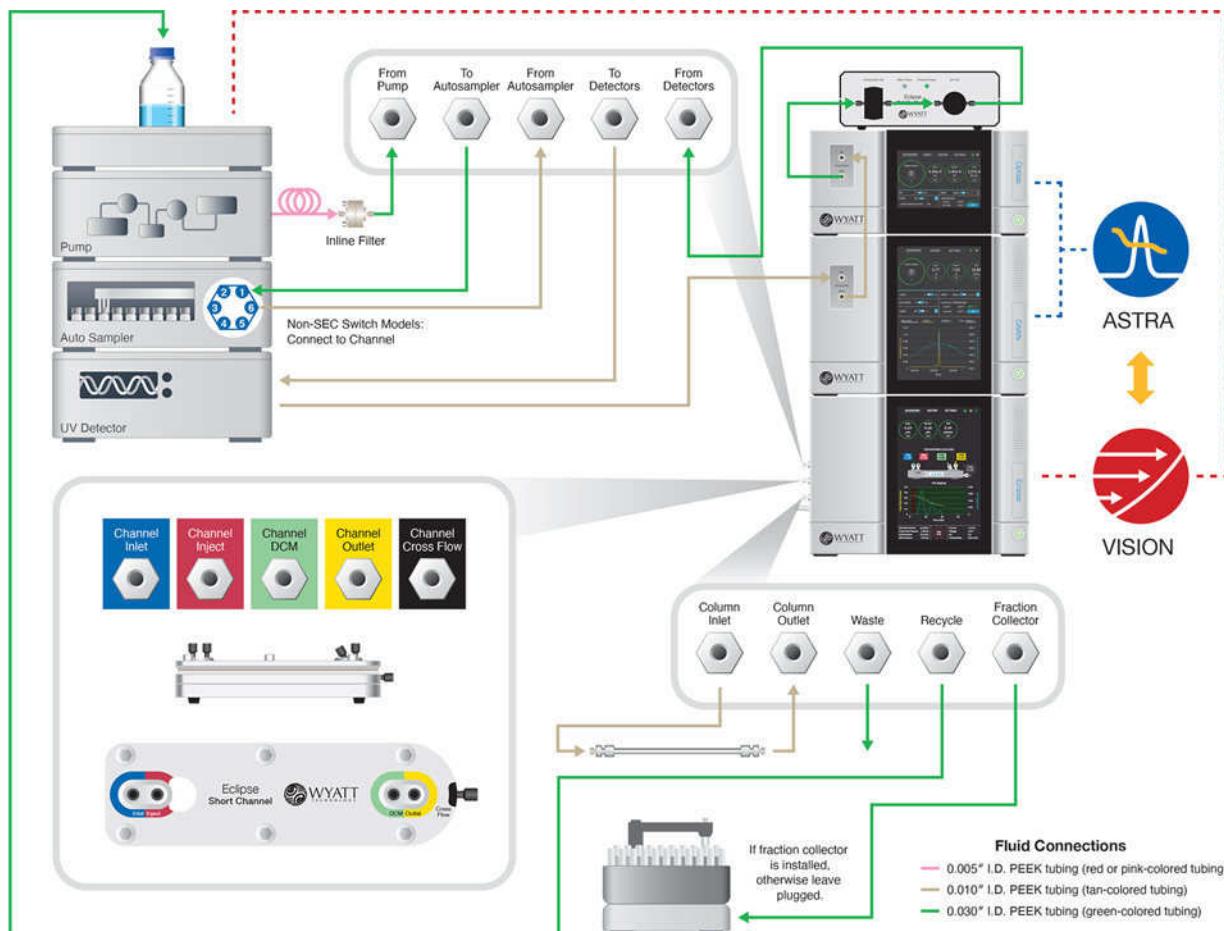
To exploit this equilibrium between cross flow and diffusion, channel flow is simultaneously applied. This laminar flow is parabolic in nature—flow near the edges is slower than flow near the middle. Smaller macromolecules, which diffuse more quickly, are statistically more likely to enter the faster fluid lanes, and so we achieve separation by hydrodynamic size.

5

In the fractogram, typically small macromolecules will elute first, and larger macromolecules elute later. This order is reversed compared to size-exclusion chromatography (SEC).



Eclipse Fluid Connection Quick Reference



	Eclipse Side Panel	Module	Tubing (1/16" O.D.)	Notes
HPLC & Detector (10/32 Coned Fittings)	From Pump	Inline Filter Outlet (or from Pump)	Orange (0.020" I.D.) or Green (0.030" I.D.) PEEK	Use coil of narrow tubing between pump and inline filter to put pressure on the pump to minimize pump pulsations and improve performance.
	To Autosampler	Port 1 for standard Agilent Autosampler Rheodyne	Green (0.03" I.D.) PEEK	Agilent autosampler: Port 1 for "Autosampler In"
	From Autosampler (SEC switch models)	Port 6 for standard Agilent Autosampler Rheodyne	Tan (0.010" I.D.) or Red/Pink (0.005" I.D.) PEEK	In non-SEC Eclipse models, connect directly from Port 6 (Agilent) to channel inject (port is color-coded red).
	To Detectors	First analytical detector (typically UV or MALS detector)	Tan (0.010" I.D.), Red/Pink (0.005" I.D.), or Yellow (0.007" I.D.) PEEK	Need to tune channel pressure based on detector flow rates. Narrower PEEK (0.005" I.D.) can be used to increase channel pressure for flow regulation, especially with DCM.
	From Detectors	Last analytical detector (typically RI detector)	If RI: Green (0.030" I.D.) PEEK If no RI and using fraction collector: Tan (0.010" I.D.) PEEK	Please see TN6504 for Fraction Collector configurations.
Eclipse (Super flangeless)	Channel Inlet	Super flangeless ferrule into Channel Inlet	Green (0.030" I.D.) PEEK	Color-coded blue sleeve
	Channel Inject (SEC switch models)	Super flangeless ferrule into Channel Inject	Tan (0.010" I.D.) PEEK	Color-coded red sleeve (from Autosampler if not SEC switching model)
	Channel DCM (DCM Models)	Super flangeless ferrule into Channel DCM	Green (0.030" I.D.) PEEK	Color-coded green sleeve
	Channel Outlet	Super flangeless ferrule into Channel Outlet	Tan (0.010" I.D.) PEEK	Color-coded yellow sleeve
	Channel Cross Flow	Super flangeless ferrule into Channel Cross Flow	Green (0.030" I.D.) PEEK	Color-coded black sleeve
Eclipse	Column Inlet (SEC switch models)	Inlet of guard column	Tan (0.010" I.D.) PEEK	
	Column Outlet (SEC switch models)	Outlet of the last analytical column	Tan (0.010" I.D.) PEEK	Max column pressure with Eclipse is 75 bar
	Waste	Waste reservoir	Green (0.030" I.D.) PEEK	
	Recycle	Solvent reservoir	Green (0.030" I.D.) PEEK	
	Fraction Collector	If applicable	Green (0.030" I.D.) PEEK	Remain plugged if FC not plumbed.

Variable Height Channel Membrane Installation Quick Reference Sheet

Replacing the Eclipse Channel Membrane

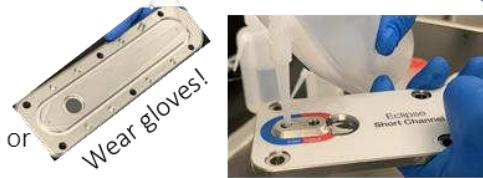
Supplies:

- Clean bench, gloves
- DI water (sink or squeeze bottle)
- Torque wrench
- New membrane
- New laminated spacer (if applicable)
- 1% detergent solution and/or alcohol for additional channel cleaning

- 1** Replace channel with 5-port union. Take to clean bench near sink and remove top block with hex driver or torque wrench.



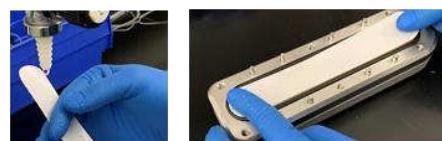
- 2** Remove top block, wash surface with soap, water, and/or alcohol. Clean ports or O-ring as needed.



- 3** (Variable Height) Remove spacer and discard membrane. You may optionally clean the frit as well with water (use O-ring removal pick to safely remove O-ring to access frit). Clean new (laminated spacers) or re-usable (Mylar) spacer with water, especially the inner edge. **Note:** Channel heater is not waterproof. Avoid soaking/rinsing bottom block itself.



- 4** Rinse new membrane (discard paper in packaging) to remove fibers and install centered on the supporting frit with *shiny side (wetted side) up*.



- 5** The spacer (variable height) has alignment pin holes to help with orientation, line up square side and round side with channel. Install the spacer and then place the top block on top, securing it with the alignment pins. Avoid shifting the membrane!



- 6** Set the torque wrench to 1 Nm and secure the bolts (following a crisscross pattern) across the channel.

Repeat for 2 Nm and then **again** for 3 Nm and 4 Nm to fully assemble the channel. There will be a “click” when the defined torque is reached—make small motions to avoid over-torqueing!

Note: Fixed Height channels only need to be assembled finger-tight, no torque wrench is needed.



- 7** Reconnect the channel to the Eclipse. The tubing sleeves are color-coded for convenience. Flushing steps varies by model:

Non-DCM: Disconnect **Channel Outlet** from Eclipse chassis (not channel) and flush to beaker with 2 mL/min channel flow in Elution + Injection (0.2 mL/min). Stop flow, reconnect **Channel Outlet**. Then Focus + Inject with the addition of 1.5 mL/min cross flow.

DCM Option: Right-click Eclipse widget in VISION, select “Flush Membrane,” and allow the system to automatically flush the membrane.

- 8** Run a conditioning run with a standard of similar chemical composition.

Replace membrane every 2-4 weeks, or when elevated noise is observed. Also replace when sample type is changed.

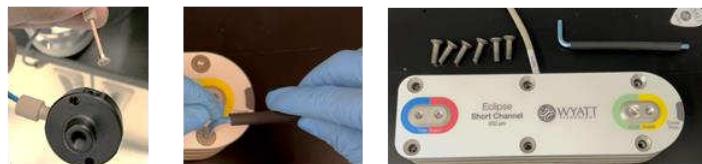
Fixed Height Channel Membrane Installation Quick Reference Sheet

Replacing the Fixed Height Channel Membrane

Supplies:

- Clean bench, wear gloves
- DI water (sink or squeeze bottle)
- Torque wrench
- New membrane
- New laminated spacer
- 1% detergent solution and/or alcohol for additional channel cleaning

- 1** Replace channel with 5-port union. Take to clean bench near and remove top block with L-key, incrementally loosen bolts.



- 2** Remove top block, wash surface with soap, water, and/or alcohol. Clean ports or O-ring as needed.

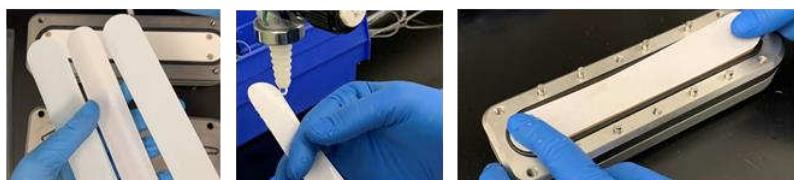


- 3** Discard old membrane.

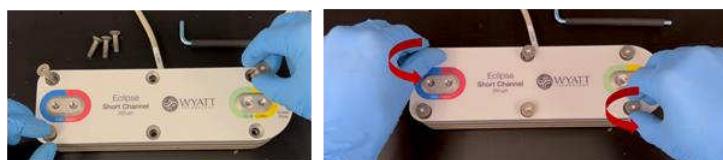
Note: Do not remove the channel frit.

Note: Channel heater is not waterproof. Avoid soaking/rinsing bottom block itself.

- 4** Rinse new membrane (discard two papers in packaging) to remove fibers and install centered on the supporting frit with *shiny side (wetted side) up*.



- 5** Position the top block over the bottom block so the holes are aligned. You can use the bolts to guide the assembly. Start with counter-clockwise rotation to ensure bolts catch the threads.



- 6** Use the L-key to tighten the bolts (following a crisscross pattern) across the channel. Repeat with quarter rotations until the bolts reach a physical stop and the L-key cannot be turned further. The fixed height channel has a metal-to-metal assembly. Ensure there is no visible gap between the top and bottom blocks.



- 7** Reconnect the channel to the Eclipse. The tubing sleeves are color-coded for convenience. Flushing steps varies by model:

Non-DCM: Disconnect **Channel Outlet** from Eclipse chassis (not channel) and flush to beaker with 2 mL/min channel flow in Elution + Injection (0.2 mL/min). Stop flow, reconnect **Channel Outlet**. Then Focus + Inject with the addition of 1.5 mL/min cross flow.

DCM Option: Right-click Eclipse widget in VISION, select “Flush Membrane,” and allow the system to automatically flush the membrane.

- 8** Run a conditioning run with a standard of similar chemical composition.

Replace membrane every 2-4 weeks, or when elevated noise is observed. Also replace when sample type is changed.

FFF-MALS System Best Practices Quick Reference Sheet

**Eclipse
Settings &
Maintenance
Checklist**
Solvent Reservoir:

- Change frequently!
- Replace daily if no preservative, or add NaN₃ (or alternative).
- Replace solvent frit filters (3m).
- Use fresh bottles.
- Pre-mix and filter (0.1 – 0.22 µm).
- Stir mobile phase (for RI stability).

Notes:**DAWN:**

- Monitor LS baseline noise (front panel).
- Keep clean and run COMET as needed.
- Run validation standards to check system constants and performance.

Pump & Degasser:

- Change frit (3m) & inline filter (1m).
- Pump compressibility settings.
- Disable Agilent bottle fillings.
- Ensure efficient degassing.
- Pulse dampening tubing coil.

**UV Detector:**

- Monitor noise
- Ensure matching settings between analog and ASTRA
- Validate vs. RI as concentration source.

**Autosampler:**

- Flush needle and sample loop.
- Influences Eclipse Focus + Inject time (time to flush loop)
- Avoid injecting bubbles (needle offset and vial inserts).
- Include as part of the solvent exchange.

**Optilab:**

- Purge when not running.
- Sensitive to temperature, pump pulsations, solvent homogeneity.
- Blank run to subtract RI baselines

**Eclipse:**

- Update solvent volumes (ISM).
- Replace bulkhead filters (3m).
- Flush valves regularly (1w)
- Membrane flush/reset injection counter.
- Monitor pressures and flows.
- Smart Services on front panel

**Eclipse Mobility Module:**

- Store in KCl solution
- Check pH, conductivity monthly—calibrate as needed.
- Use PSL or Mobility standards to check system.

Eclipse Channel:

- Replace membrane every 2-4 weeks, when system noise increases (especially relative to focus flow), or when changing sample types (i.e. proteins to particles).
- Replace laminated spacer when disassembling channel for best results, Mylar spacer may be re-used.
- Choose membrane, spacer, cross flow gradients based on sample type.
- Set channel temperature slightly above ambient for best reproducibility.

**Long-Term Storage:**

Flush to an appropriate solvent storage.

- HPLC: 20% alcohol solution
- Eclipse: 20% alcohol solution
- Channel: Clean, disassemble, and dry. Replace with 5-port union on system.
- Mobility Module: KCl solution

Channel Notes:

Bypass with 5-port union. Remove bolts/washers, replace membrane (always) and spacer (if needed). Rinse thoroughly. Assemble with 2, 3, then 4 Nm in crisscross pattern.

VISION: Running a Sequence



VISION RUN

Information for Sequence		Method/File Name	
VISION RUN Method			
ASTRA Method			
Channel:	Membrane:	Spacer/Height:	

System Preparation Prior to Sequence:

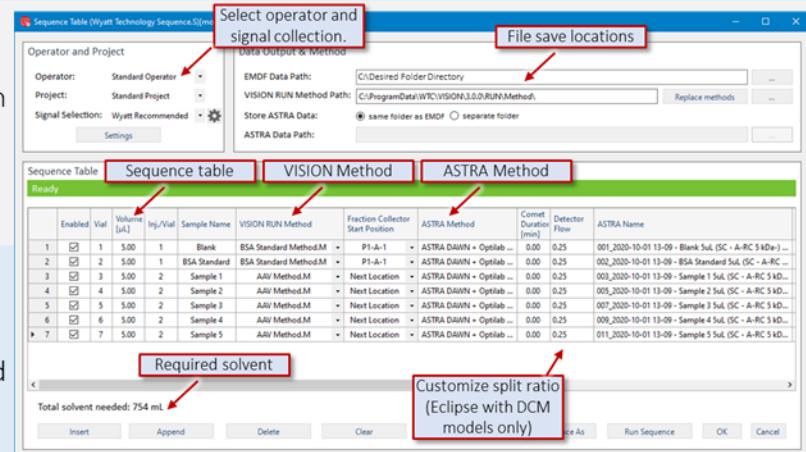
- Monitor system health on front panel of Eclipse (UV), DAWN (LS), Optilab (RI).
- Purge Optilab (if not already), Eclipse (right-click module). Check LS noise.
- Check mobile phase. Update Eclipse solvent volume if applicable.
- Double-click left panel to load your method. First line of the method will apply.
- Use manual control to adjust flows and set a diagnostic flow profile. Verify that set flows match read flows in the Eclipse panel.
- Compare to typical system pressures (pump pressure, channel pressure, etc.)

Good practices:

- Replace membrane every 2-4 weeks at least.
- Check system health
- Monitor noise
- Fresh mobile phase
- Maintenance Checklist

Test Flow Rate Setting:	Expected Pressure:	Maintenance	Last Done
Mode:	Pump:	HPLC Frit/Solvent Filter	
Channel Flow:	Inlet:	Pump Inline Filter	
Cross Flow:	Channel:	Eclipse Side Panel Frit	
Detector Flow:	DCM:	DCM Inline Filter	
Inject Flow:		Cross Flow Inline Filter	
		Other:	
		Other:	
		Other:	

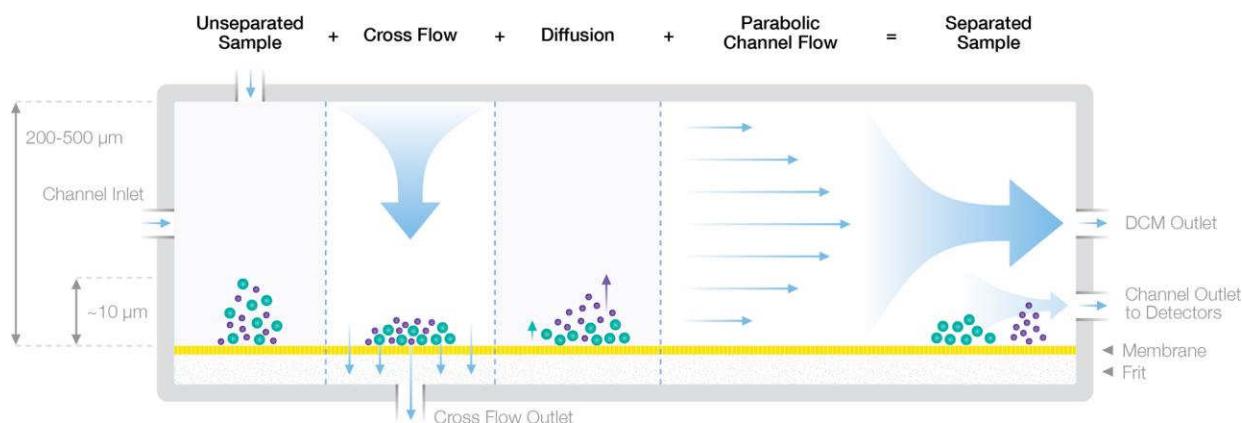
- Check that appropriate rinse settings (and Rinse After Sequence) are configured in Eclipse manual control (right-click). If you will need to modify or optimize the method, load an appropriate existing method and “Save Method As” to preserve HPLC settings for convenience. Then modify the Eclipse time table as desired or import time table from VISION DESIGN.
- Navigate to Method → Set Up Method to double-check appropriate settings for method. Check appropriate UV settings (wavelength, no reference, attenuation, etc.). Check other settings as appropriate, including FLD, fraction collector, vial cooling temperature, etc.
- Modify an existing sequence or create a new sequence. Check that operator, project, and signal selection is appropriate (recommended to collect all Eclipse and HPLC detector signals). Confirm file save location.
- Include a blank injection (baseline subtraction), a suitability standard (system check), and then your samples. End with blank and standard to verify system and membrane health.
- Input all sample parameters and confirm required solvent (bottom left). Settings to double-check would include vial position, number of injections, detector flow (DCM), and mobility settings (Eclipse Mobility module).



Appendix B – Basic Theory

The basic theory of AF4 is introduced in the section, [Asymmetric Flow Field Flow Fraction \(AF4\) Overview](#). It is expanded upon in this Appendix and the concepts of dilution control are also introduced.

Asymmetric-Flow Field-Flow Fractionation (AF4)



Asymmetric flow field flow fractionation (AF4), like size-exclusion chromatography (SEC), typically separates by exploiting differences in size between macromolecules; however, whereas SEC is a physical steric exclusion from a column packing material, AF4 is based on normal (Brownian) separation, where there is flow in parabolic nature under the force of cross flow. Different distances from the accumulation wall due to equilibrium of the cross flow field and diffusion led to separation.

However, for exceptionally large macromolecules, steric separation is even possible in AF4. This is caused by huge geometric dimensional differences which cause the macromolecule to protrude from the accumulation wall due its size rather than its diffusion. This is uncommon depending on the nature of the macromolecules and can be avoided by using a taller spacer height or promoted by using a shorter spacer height.

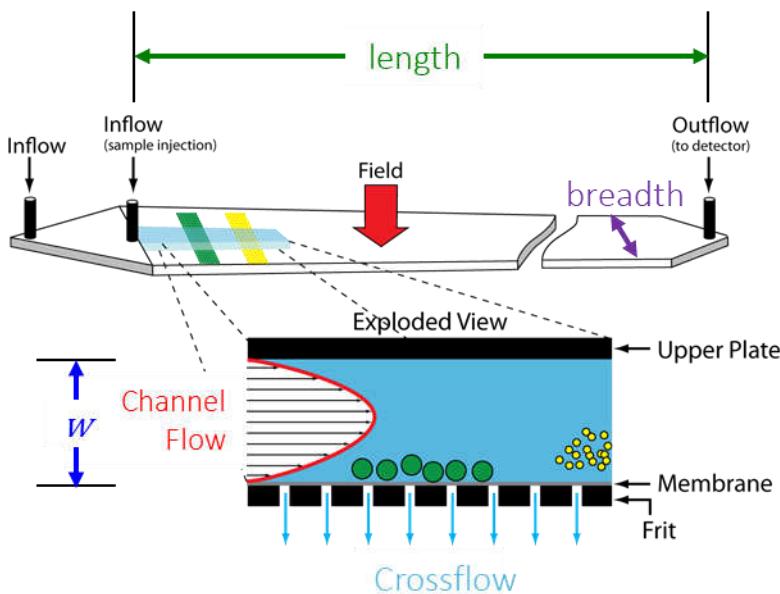
In this appendix, the theory will be expanded upon for the typical cases where steric exclusion is not a contributing factor. The retention time of a macromolecule with a certain diffusion coefficient in a channel with a defined thickness as it relates to the cross flow velocity is given by the following equation:

$$t_R = \frac{w^2}{6D} \ln\left(1 + \frac{V_c}{V}\right) = \frac{w^2 \pi \eta R_h}{kT} \ln\left(1 + \frac{V_c}{V}\right)$$

where

- w channel thickness
- D diffusion coefficient
- V detector flow rate (mL/min)
- V_c cross flow rate (mL/min)
- R_h hydrodynamic radius
- k Boltzmann constant
- T absolute temperature
- η viscosity

The channel thickness is defined by the spacer height that is used:



However, other factors like channel length and breadth can affect the fractionating power, as the cross flow is applied across the entire working area and so the total flux can vary:

$$F = S \cdot \left(\frac{384^{-1/2}}{bLD} \right) \cdot \left(\frac{w\dot{V}_{cross}^{3/2}}{\dot{V}_{channel}^{1/2}} \right)$$

where

F : fractionating power

S : selectivity

b : breadth of channel

L : length of channel

D : diffusion coefficient

w : thickness of channel

As a result, the following trends can be observed:

- Fractionating power increases with cross flow rate
- Fractionating power decreases with channel flow rate
- Fractionating power increases with channel thickness when flow rates are constant
- Fractionating power decreases with length and breadth of channel with the same flow rates

Channel Longitudinal Flow Velocity Profile

The laminar parabolic flow applied across the channel (longitudinal channel flow) is defined by the channel height and the channel flow rate. For a rectangular channel, the relationship is given by the following equation:

$$\langle v \rangle_z = \langle v \rangle_0 - \frac{u_0}{w} z$$

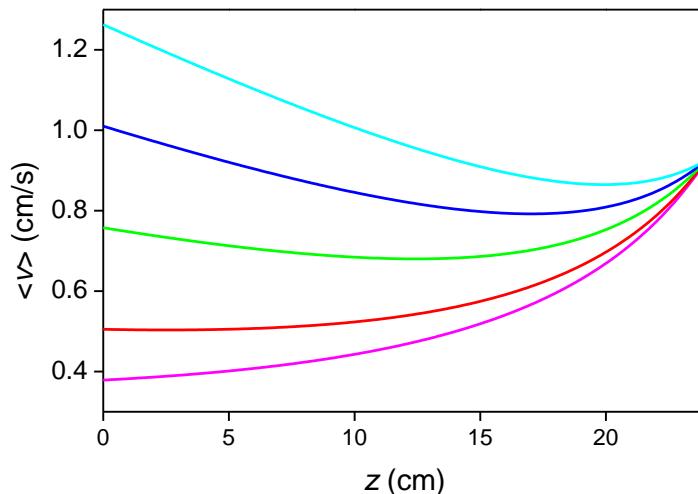
However, this can be further expanded to describe a trapezoidal channel by the following:

$$\langle v \rangle_z = \frac{V_{in} - u_0 \left(b_0 z - \frac{z^2(b_0 - b_L)}{2L} \right)}{w \left(b_0 - \frac{(b_0 - b_L)z}{L} \right)}$$

where

- $\langle v \rangle_z$ average longitudinal flow velocity (cm/s) at position z
- $\langle v \rangle_0$ average longitudinal flow velocity at the channel inlet
- V_{in} flow rate (mL/min) at the channel inlet, $V_{in} = V_c + V_d$
- V_c cross flow rate (mL/min)
- V_d detector flow rate (mL/min)
- u_0 cross flow velocity at the accumulation wall (cm/s)
- b_0 channel breadth at the inlet
- L channel length
- b_L channel breadth at the outlet
- z distance from the channel inlet

As a result, the flow velocity profile as a function of the distance from the accumulation wall for a long channel with a 350 μm spacer can be described by the following plot:



where the top cross flow rates from the top line (teal) to the bottom line (pink) are 0.5, 1.0, 2.0, 3.0, and 4.0 mL/min respectively. Flow near the accumulation wall is slower than flow further away from the accumulation wall.

Concentration in the Channel

The graphics presented thus far are illustrative but do not capture the true concentration profile in the channel. Most of the concentration of sample is in the bottom 10 μm of the channel near the accumulation wall even

though the channel height may be hundreds of microns. Nonetheless, the different channel heights play a critical role in the parabolic flow at the bottom 10 μm of the channel separation zone.

The concentration profile with respect to the distance from the accumulation wall is given by the following equation:

$$c_x = c_0 e^{-\frac{x}{l}}$$

where

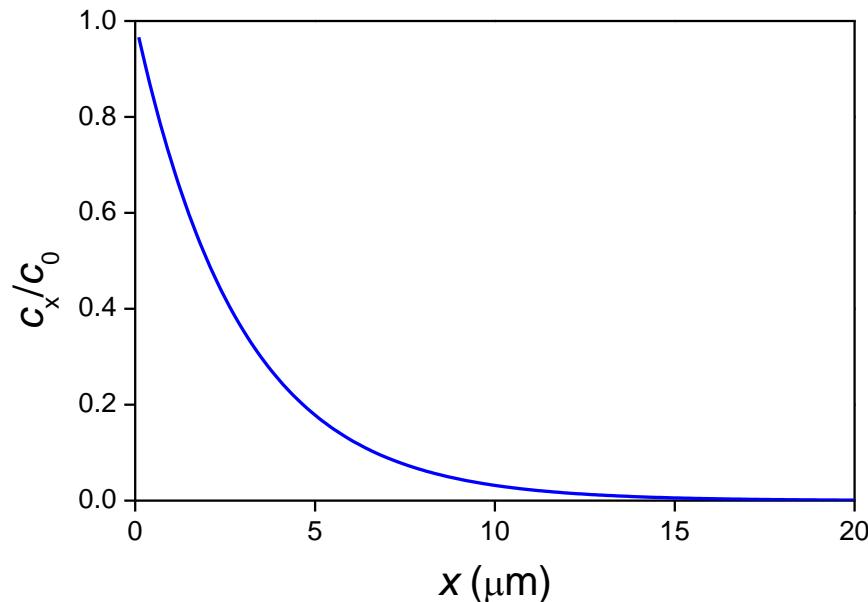
c_x concentration at distance x

c_0 concentration at wall, $x = 0$

x distance from wall

l distance of center of sample layer

When related to the distance, the correspond concentration profile is described by this graph below which is the concentration at some distance divided by the concentration at the accumulation wall, plotted against the distance from the accumulation wall:



The concentration profile with respect to the distance from the bottom wall and position along the channel can be further expanded by the following equation:

$$c_{x,z} = c_{00} \exp\left(-\frac{x}{l}\right) \exp\left(-\frac{(z-Z)^2}{2\sigma^2}\right)$$

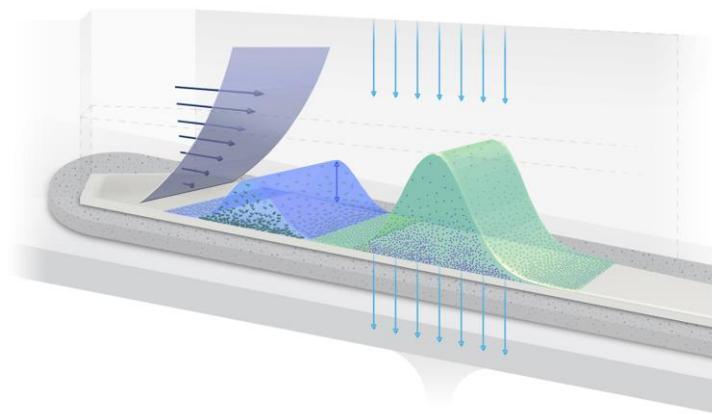
$$c_{00} \approx \frac{m}{b(z)w\lambda\sqrt{2\pi\sigma^2(z)}}$$

where

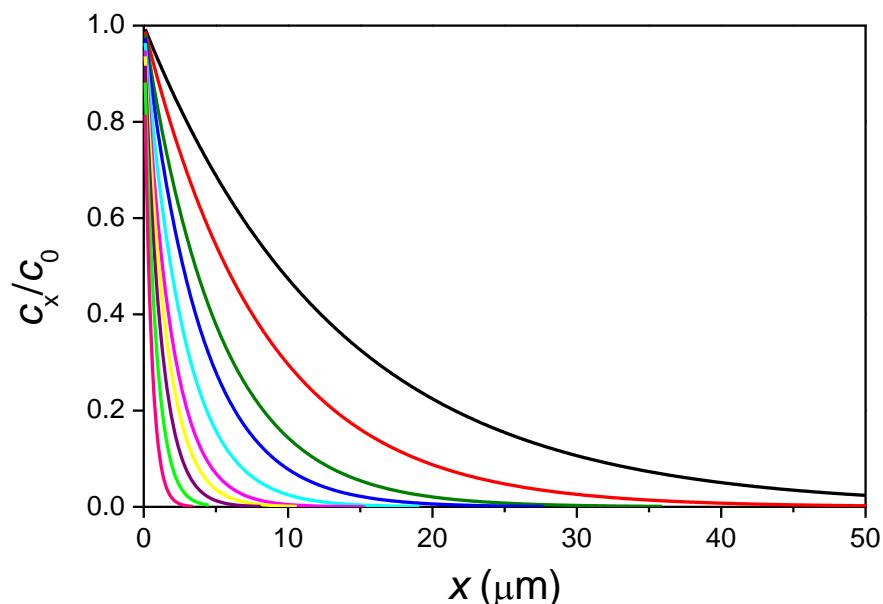
$c_{x,z}$ concentration at distance x from the wall and z along the longitudinal axis

- c_{00} concentration at wall, $x = 0$ and at the zone center ($z = Z$)
 x distance from wall
 z distance from channel inlet
 σ^2 variance at position Z , zone spreading mainly due to nonequilibrium effect
 m injected mass
 $b(z)$ channel breadth at position Z

This factors in the position along the accumulation wall as shown in the figure below:



Because of the role of diffusion coefficients, the quantity of sample at some distance from the accumulation wall can vary from 10^4 g/mol (black line) to 5×10^6 g/mol (inner most pink line):



As a result, large macromolecules tend to be in close proximity to the membrane whereas smaller macromolecules are typically within 50 μm of the accumulation wall. As a general rule of thumb, the majority of sample is typically found within 10 μm of the accumulation wall.

Focus Flow

There is a strong concentration of injected macromolecules during focusing. Injected sample is typically compressed into about 1 μL of volume with a channel breadth of about 2 cm, with a focusing line of 0.1 cm, and a sample layer thickness of 0.005 cm (i.e., $2 \times 0.1 \times 0.005 = 0.001 \text{ mL}$).

And injection of 100 μL of a 0.2% w/v solution would concentrate into 20 % w/v.

Cross Flow

In the figures thus far, the cross flow profile has been simplified. However even the cross flow velocity profile varies with distance from the accumulation wall. This is because all flow enters the channel at the inlet, and although parabolic laminar flow permeates through the channel, the cross flow velocity is zero at the upper (depletion) wall. This is given by the following equation:

$$u_x = u_0 \left(1 - \frac{3x^2}{w^2} + \frac{2x^3}{w^3} \right)$$

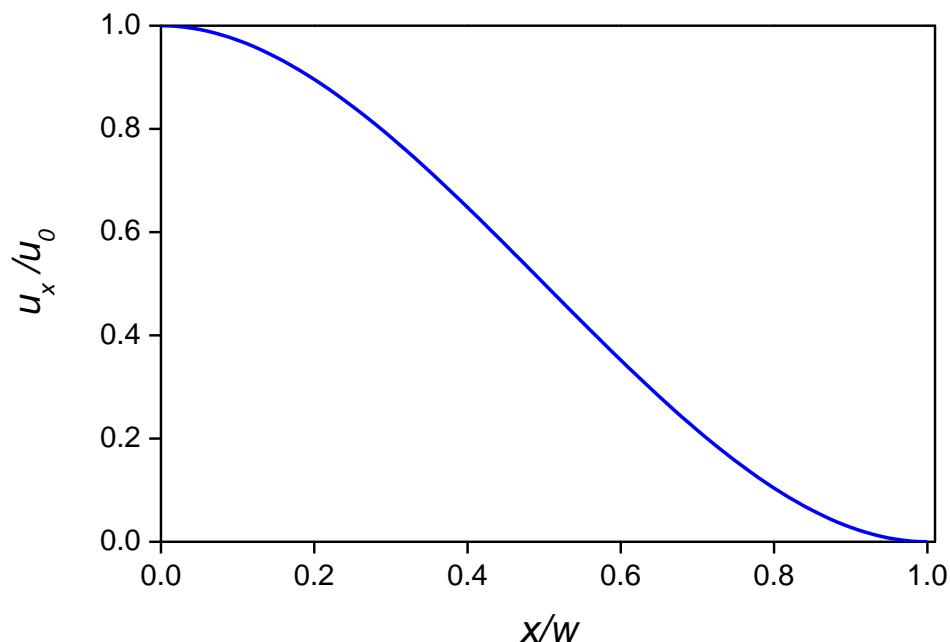
where

u_x cross flow velocity (cm/s) in distance x from the accumulation wall

u_0 cross flow velocity at the accumulation wall

w channel thickness

And related by this plot of the flow velocity ratio vs. distance from the accumulation wall divided by channel thickness:



SEC vs. AF4 Efficiency

A common orthogonal technique to AF4 is SEC, as both separate by exploiting the physical property of size. A natural question then is the comparison between both techniques as it relates to efficiency and selectivity. Selectivity can be defined by the following, while considering some of the retention and selectivity equations discussed above:

$$S = \left| \frac{d(\log t_R)}{d(\log M)} \right|$$

$$S = \left| 3 \left(\frac{R}{36\lambda^2} + 1 - \frac{1}{R} \right) \right| \left| \frac{d(\ln \lambda)}{d(\ln M)} \right|$$

where

- S selectivity
- t_R retention time
- M molar mass
- R retention ratio ($R = t_0/t_R$); t_0 = retention time of unretained component
- λ retention parameter

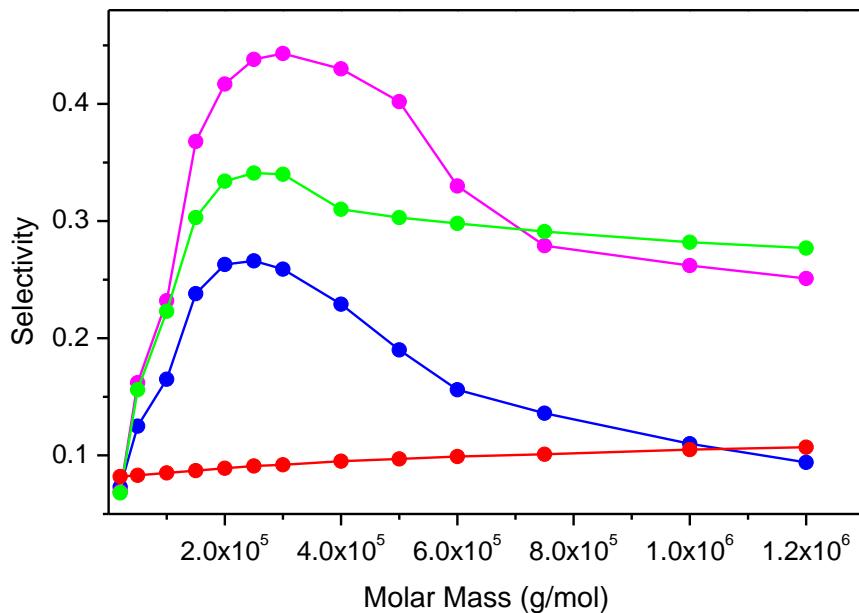
And the retention parameter is defined as:

$$\lambda = \frac{l}{w} = \frac{DV_0}{V_c w^2}$$

where

- λ retention parameter
- w channel thickness
- D diffusion coefficient
- V_0 channel volume
- V_c cross flow rate (mL/min)

Selectivity for AF4 is non-linear because the cross flow can be dynamically adjusted. A comparison of the selectivity between SEC and AF4 is illustrated in the plot below, where the red line (bottom) is for SEC and the AF4 runs have various cross flow gradient profiles:



In general, AF4 is much more selective than SEC. Next, we can look at efficiency, also referred to as plate heights. In SEC, the efficiency can be described by the following equation:

$$H = A + \frac{B}{u} + Cu$$

$$A \approx d; C \approx d^2$$

where

- H theoretical plate height (efficiency)
- A contribution of eddy diffusion (column particle size and packing quality)
- B contribution of axial diffusion
- C contribution of mass transfer
- u linear velocity of mobile phase (cm/min)
- d particle diameter

And the number of theoretical plates is defined as:

$$N = \frac{L}{H}$$

Conversely, the efficiency for AF4 is described by the following relationship:

$$H_n = 24\lambda^3(1 - 8\lambda + 12\lambda^2) \frac{w^2 \langle \bar{v} \rangle}{D}$$

$$H_n = 24 \frac{D^2 V_0^2 L}{w^4} \frac{V}{V_c^3}$$

where

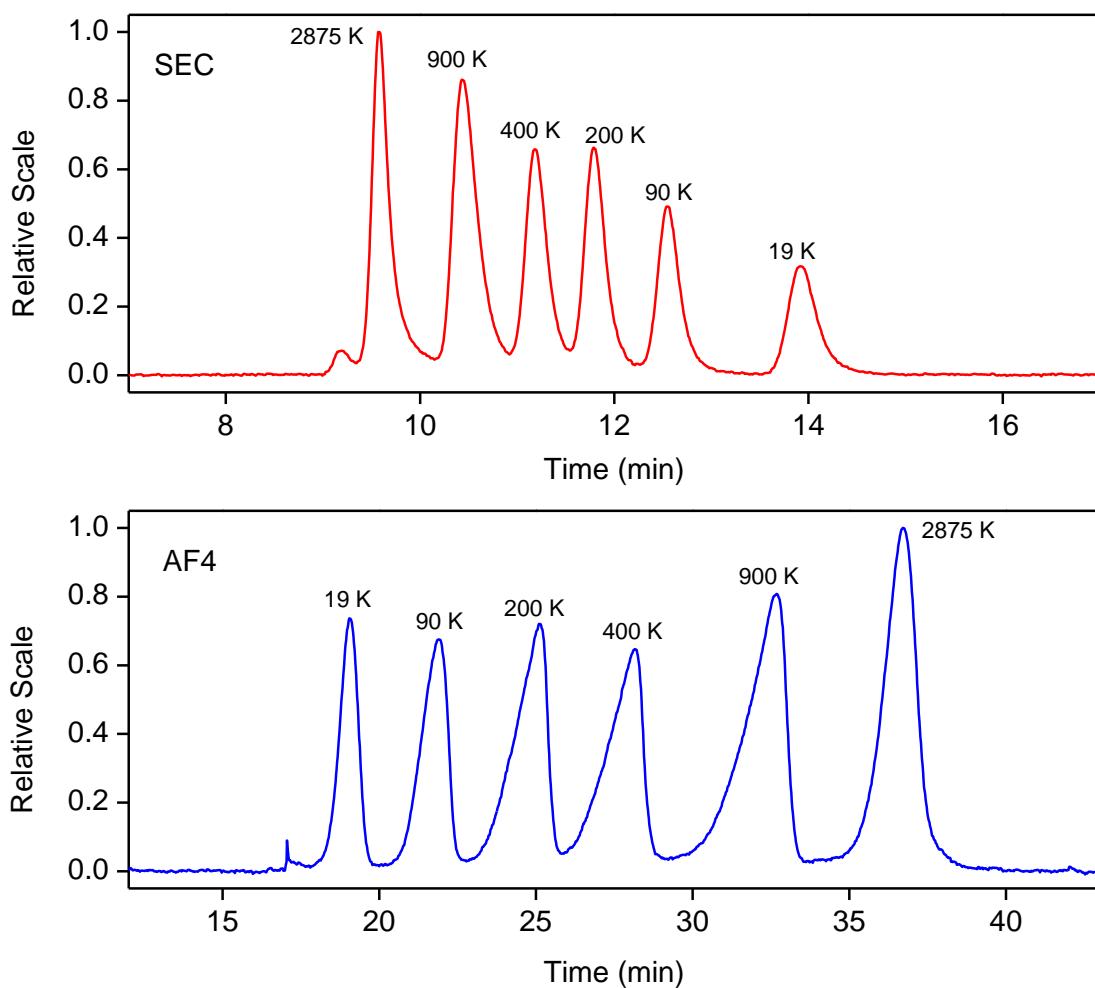
- H_n theoretical plate height (nonequilibrium contribution)

λ	retention parameter
w	channel thickness
D	diffusion coefficient
	time-average longitudinal flow velocity (cm/s)
V_0	channel volume
L	channel length
V_c	cross flow rate (mL/min)
V	average longitudinal flow rate (mL/min)

In general, AF4 is less efficient than SEC. Comparing the two techniques, we can derive the following observations:

1. Number of theoretical plates: $N_{\text{SEC}} \gg N_{\text{AF4}}$
 - N_{AF4} increases with molar mass
 - N_{SEC} decreases with molar mass
 - $N_{\text{AF4}} \approx 100$ per channel; $N_{\text{SEC}} \approx 18,000$ per column (for low molar mass compound)
2. Selectivity: $S_{\text{SEC}} < S_{\text{AF4}}$
 - $S_{\text{SEC}} \approx 0.1$ ($S_{\text{SEC}} \rightarrow 0$ at proximity of exclusion limit)
 - S_{AF4} increases with molar mass up to ≈ 0.6
(real measurements: $S \approx 0.3 - 0.45$)
 - No limit of molar mass in case of AF4

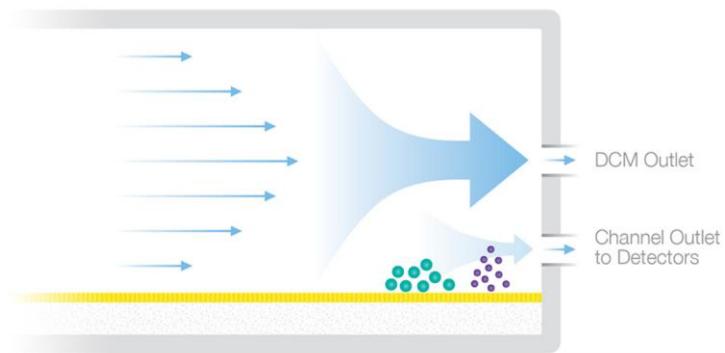
These differences can be illustrated in the following comparison of polystyrene standards via SEC and AF4:



The top, red trace is SEC and the bottom, blue trace is FFF. The FFF experiment is less efficient (wider peaks) but is more selective (retention time difference). As a result, SEC and FFF are orthogonal techniques uniquely suited for different situations, with FFF-MALS separations excelling for larger macromolecules and requirements for more gentle separations. Additionally, FFF-MALS avoids many of the limitations of column interactions that can occur with SEC.

Dilution Control Module

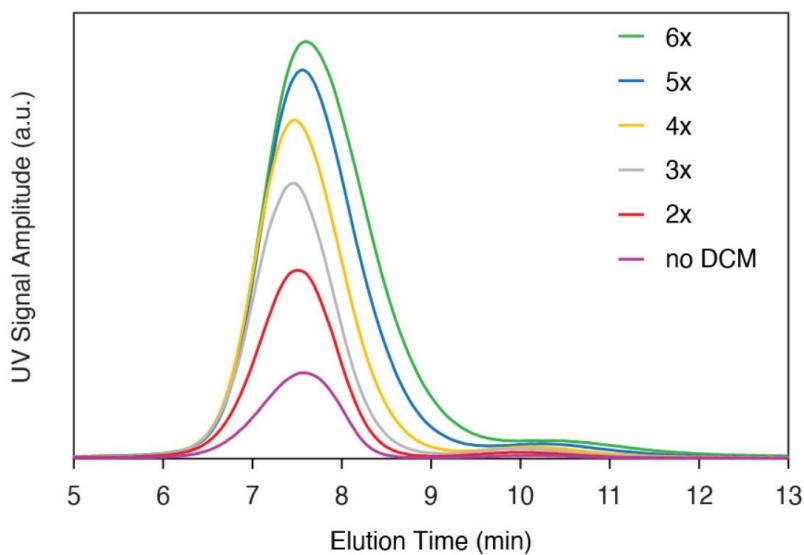
The Dilution Control Module (DCM) increases the concentration of sample eluting from the channel by a factor of up to 10x over standard FFF, with little to no loss in resolution up to 5x concentration enhancement. This is achieved by splitting away a fraction of the channel flow close to the upper wall, which does not contain sample, in a tightly regulated fashion. This exploits the earlier concentration profile, where the sample is located almost entirely within 50 µm of the accumulation wall. As a result, pure mobile phase can be siphoned off from the depletion wall.



The benefits include:

- Higher detection sensitivity for low-concentration samples
- Higher concentration of sample in collected fractions
- Improved dynamic light scattering measurements resulting from a lower detector flow rate
- Increase reproducibility of retention time, calculated recovery and collected fractions resulting from active control of the detector flow rate

The relative peak intensity with a UV detector is illustrated below:

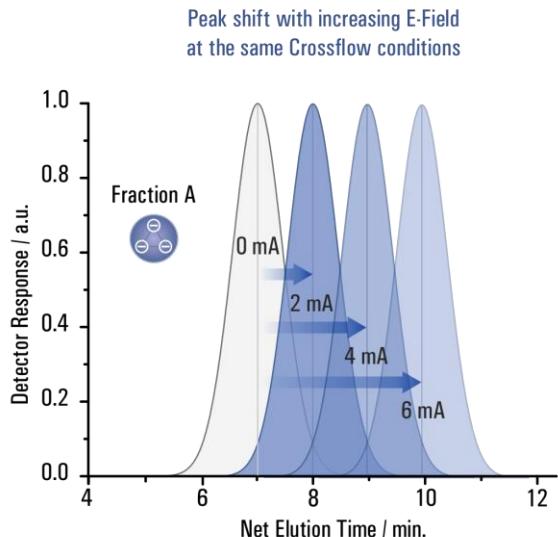
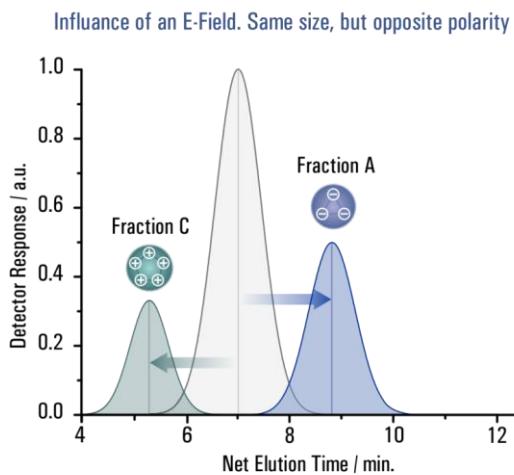
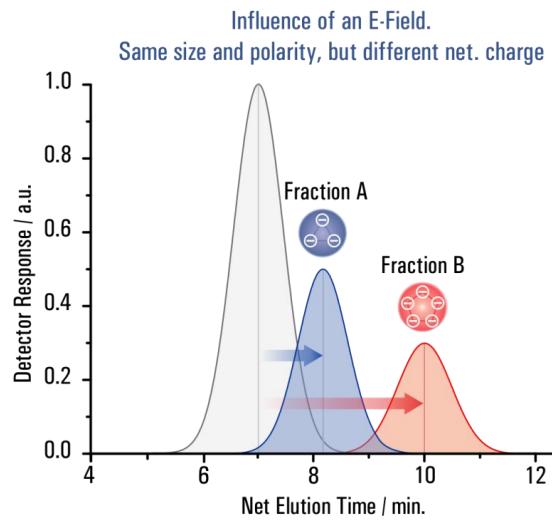
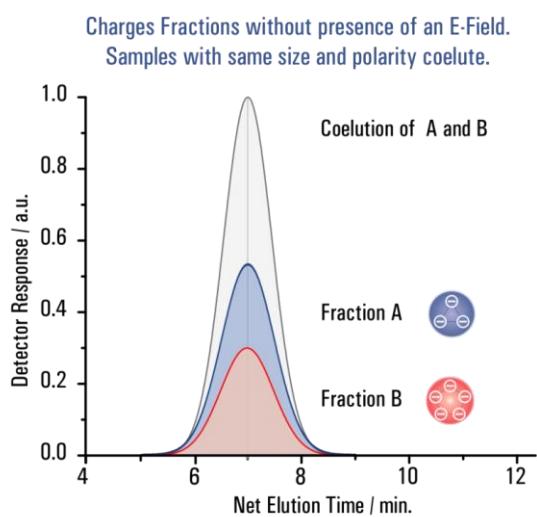


Mobility

Electrical/asymmetric-flow field-flow fractionation (EAF4) separates by both size and particle charge to determine zeta potential distributions, even of multimodal and polydisperse populations. Mobility combines an innovative EAF4 channel design with outstanding software control and analysis.

Use Mobility to:

- Quickly assess if your sample carries charge and if it is homogenous across the size distribution
- Improve resolution and peak shape for charged components
- Determine electrophoretic mobility of distinct populations within a fractogram with high precision



In order to determine electrophoretic mobility, μ , a modified AF4 experiment is applied. In AF4 the retention time depends on the drift velocity of sample components towards the accumulation wall, which is caused by a cross flow permeating through the accumulation wall.

The particle retention time t_R mainly depends on the channel thickness w , the particle diffusion coefficient D , the cross flow rate, and the detector flow rate. The channel thickness is determined by measuring the retention time of a sample with known diffusion coefficient. Care needs to be taken that the diffusion coefficient relates to the same temperature and solution conditions applied in the AF4 experiment.

Electrophoretic mobility (μ) and Zeta potential (ζ) of particles in dispersion are directly related to their colloidal stability and correlated to shelf life and function. The electrophoretic mobility allows deducing the particle surface charge by using models of the particle architecture and is defined as:

$$v_{ep} = \mu E$$

where

v migration velocity caused by the applied electric field

E electric field strength vector

The specific current, I , is applied, controlled, and measured. The absolute value of the electric field strength E between parallel arranged electrodes acting on the particle can be described as follows:

$$E = \frac{RI}{d} = \frac{I}{AK}$$

where

I specific current

d the distance between parallel arranged electrodes

R electrical resistance

A electrode area

K conductivity of the solvent

As described earlier, the retention time of sample components in AF4 experiments depends on drift velocity towards the accumulation wall, which is caused by a cross flow permeating through the accumulation wall perpendicular to the parabolic channel flow.

If an electric field is also applied, the total drift velocity of the sample components is the sum of the drift velocities from the combined electric field and cross flow:

$$v = v_{ep} + v_c$$

where

v the total drift velocity

v_c the drift velocity induced by cross flow

By measuring the retention time (t_{Ri}) of experiments with applied electric field at a current (i) and the retention time (t_{R0}) of experiments without applied electric field as well as the process parameters channel flow rate (V_i) and cross flow (V_c), the total average drift velocity of each experiment can be calculated.

$$v = \left[e^{\left(\frac{t_{Ri}}{t_{R0}} \right) \ln \left(1 + \frac{V_c}{V_d} \right)} - 1 \right] \frac{Vd}{A_m}$$

where

V_c cross flow rate

V_d channel flow rate

A_m membrane area

This is done with multiple measurements at different electrical field strengths. The total average drift velocities are plotted as a function of the electric field strength. The slope of the linear regression directly indicates the value of the electrophoretic mobility. That is given by:

$$v = \mu E + v_0$$

where

v_0 cross flow drift velocity of the experiment without electric field

As described earlier, a radius calculation can be performed which is critical for deriving properties like net charge and Zeta potential from electrophoretic mobility. This requires the effective channel height to be calculated with a size standard of known diffusion coefficient. The effective channel height is calculated via:

$$w = \left(\frac{6t_{R_g}D_0}{\ln\left(1 + \frac{v_c}{v_{out}}\right)} \right)^{\frac{1}{2}}$$

where

w effective channel height

t_{R_g} retention time of a size standard

D_0 known diffusion coefficient of a size standard

The diffusion coefficient of a sample fraction at a certain retention time can then be derived and the hydrodynamic radius can be computed using the Einstein-Stokes relation:

$$R_h = \frac{k_g T}{6\pi\eta D}$$

where

k_g Boltzmann constant

T absolute temperature of the solvent

η viscosity of the solvent

If the hydrodynamic radius is determined, the net charge (Z) can be derived by:

$$Z = \frac{\mu_{ep} 6\pi\eta R_h}{e} \frac{(1 + \kappa R_h)}{f(\kappa R_h)}$$

where

Z the number of effective charges

e the elementary charge

κ^{-1} the reciprocal of the Debye length, which is defined as:

$$\kappa^{-1} = \sqrt{\frac{\epsilon_0 \epsilon_r k_B T}{2e^2 N_A I_c}}$$

where

ϵ_0 the permittivity of free space

ϵ_r the liquid dielectric constant

N_A Avogadro's constant

I_c ionic strength of the solvent.

The Henry function can then be described as follows:

$$f(\kappa R_h) = 1 + \frac{(\kappa R_h)^2}{16} - \frac{5(\kappa R_h)^3}{48} - \frac{(\kappa R_h)^4}{96} + \frac{(\kappa R_h)^5}{96} + \left(\frac{(\kappa R_h)^4}{8} - \frac{(\kappa R_h)^5}{96} \right) e^{(\kappa R_h)} \int_{\infty}^{(\kappa R_h)} \frac{e^{-x}}{x} dx$$

which can be simplified as:

$$f(\kappa R) = \frac{16 + 18\kappa R + 3(\kappa R)^2}{16 + 18\kappa R + 2(\kappa R)^2}$$

If flow rates are varied during the elution phase (e.g. cross flow gradient), an adjustment based on the channel geometry into finite volume elements along the channel is needed, with constant cross flow in each volume slice. The Zeta potential (ζ) can be derived from electrophoretic mobility by applying the equation below, which is valid for Zeta potentials < 50 mV. It includes the Helmholtz-Smoluchowski equation as well as the Debye-Hückle via application of the generalized Henry function:

$$\zeta = \frac{3\eta\mu_{ep}}{2\varepsilon_0\varepsilon f(\kappa R)}$$

References

- [1] B. Kirby, Micro- and Nanoscale Fluid Mechanics, 2010.
- [2] K. G. Wahlund and J. C. Giddings, Anal. Chem., vol. 59, pp. 1332-1139, 1987.
- [3] A. Litzen and K. G. Wahlund, Anal. Chem., vol 63, pp. 1000-1007, 1991.
- [4] A. Litzen, Anal Chem., vol 65, pp. 461-470, 1993.
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- [6] C. Johann, A. Kaltenborn, H. Schuch and M. Schumacher. Patent WO: PCT/EP2008/008184, EP:2104853, 2010.
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Appendix C – Eclipse Specifications

Various Eclipse specifications are outlined in this section.

Dimensions

The Eclipse has the following dimensions:

58 cm (L) x 36 cm (W) x 26 cm (H) (Eclipse Controller)

Wetted Materials

The wetted materials in the instrument flow path are summarized below for the Eclipse, channels, and Eclipse Mobility module.

Eclipse (Base Model, with DCM, with SEC, and with DCM + SEC)

- 316 stainless steel (internal tubing)
- Fluoroelastomers (Markez Z1352, Kalrez 4079, FFKM Perfluoroelastomer)
- FR3 stainless steel
- Vespel (Rheodyne valves)
- External PEEK tubing if applicable

Channel (short, long, semi-preparative)

- 316 stainless steel
- Inconel 625, alumina ceramic, or METAPOR ceramic (frit for membrane support)
- Fused silica (glass viewing window)
- Membrane material (ultrafiltration polyethersulfone or regenerated cellulose on polypropylene backing)
- Aqueous Spacer (polycarbonate with PTFE-coated surface with 9461P glue)
- Organic Spacer (Mylar)
- PEEK tubing and Super flangeless™ ferrules

Mobility Module and Mobility Channel (Aqueous-only):

- Platinum
- Titanium
- pH probe (polyetherimide (PEI), PTFE, glass)
- Silicone (O-ring)
- PEEK tubing

Specifications

Front End: Recommend Agilent 1260 Infinity II modules including DAD

Eclipse: Cross-flow regulation from 6.67 to 0.03 mL/min. Precision of detector flow rate: 0.1% (with DCM). Dedicated Fraction Collector output port for minimal peak broadening (DCM models only).

Software: VISION software suite. Comprises VISION DESIGN module for *in silico* method development and optimization, and VISION RUN for running sequences and coordinating ASTRA data acquisition.

Communications: Ethernet RJ-45 cable

Inputs/Outputs: Safety overpressure and leak pump shutdown, Inject signal out, 24-bit A/D input

Dilution Control Module: Factory installed option. Split-flow ratio is controlled by VISION RUN and increases sample concentration at the detector by up to a factor of 10 (up to 5 with negligible loss of resolution).

Mobility Module: Includes Mobility module with pH sensor and conductivity sensor with software supported calibration, and Mobility channel. Maximum operating voltage +/- 30 V, maximum current 50 mA; constant current or constant voltage mode.

SEC Switching Option: Factory installed option allows for automated software-controlled switching between SEC mode and FFF mode.

Channels: Short, Long, Dispersion Inlet and Semi-Prep channels available. All channels are solvent universal, maximum 30 bar with software overpressure protection and come with DCM port. Temperature regulated from ambient to 50 °C.

Channel Spacers: A variety of spacer thicknesses are available for each channel, ranging between 250 and 1000 µm (depending on channel selection). Available materials are FFKM perfluoroelastomer-coated polycarbonate (aqueous solvents) and Mylar (organic solvents).

Channel Membranes: Pre-cut polyethersulfone and regenerated cellulose membranes with molecular weight cutoffs between 2 and 30 kDa. Templates available to cut other materials to size.

Appendix D – Eclipse Flow Diagrams

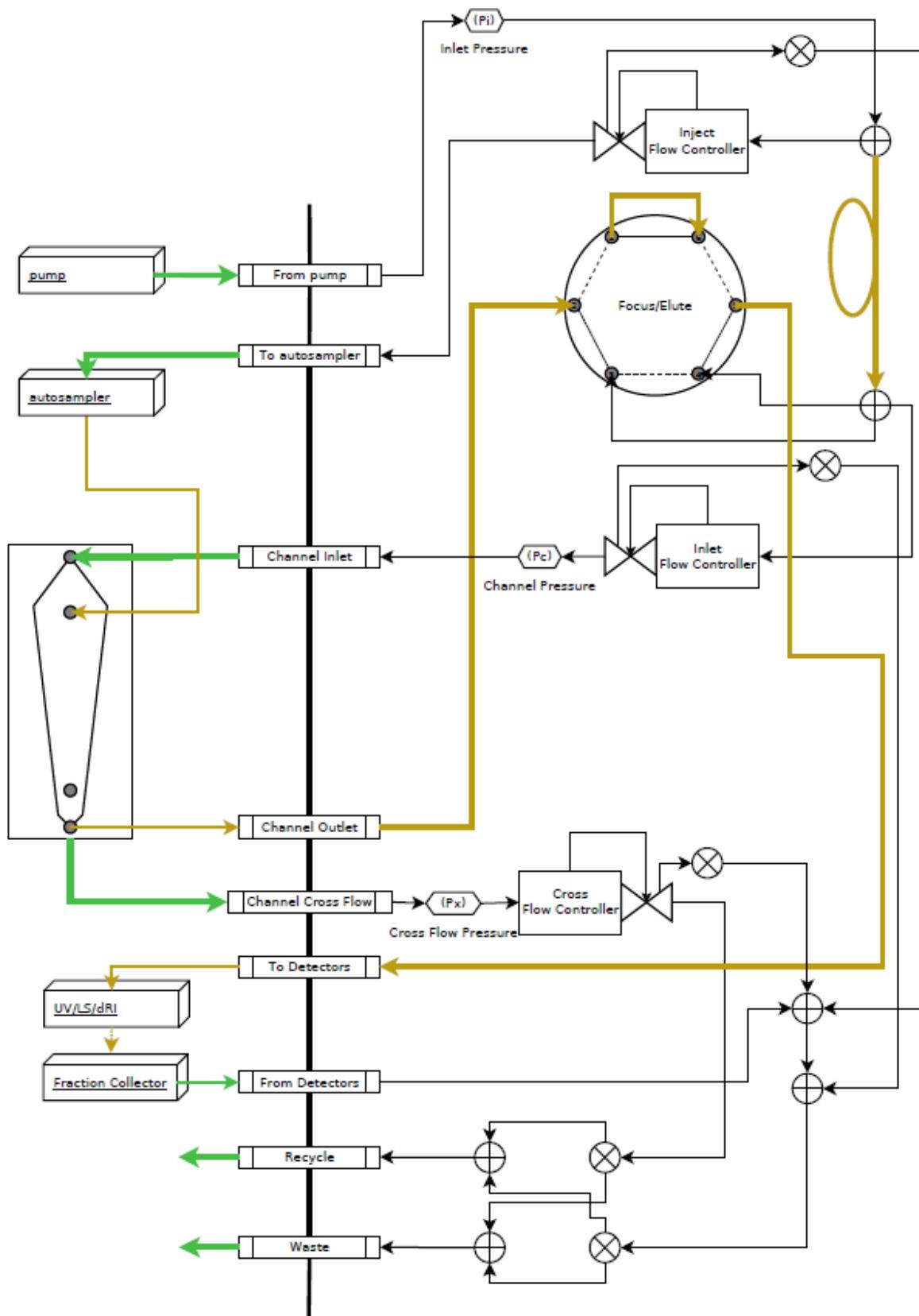
The flow diagrams for various Eclipse models are shown in this section, including the Eclipse base model, the Eclipse with DCM, the Eclipse with SEC switching option, and the Eclipse with DCM and SEC switching option. In the flow diagrams below, the connections on the left are external connections to the HPLC, channel, or downstream detectors. The divider represents the bulkhead unions. The right-side refers to internal Eclipse connections. The color and tubing indicators are as follows:

Location	Color	Size	Material
External	Green	0.03" I.D., 1/16" O.D.	PEEK (typical)
External	Beige/Tan	0.01" I.D., 1/16" O.D.	PEEK (typical)
Internal	Beige/Tan	0.01" I.D., 1/16" O.D.	316 stainless steel
Internal	Black	0.04" I.D., 1/16" O.D.	316 stainless steel

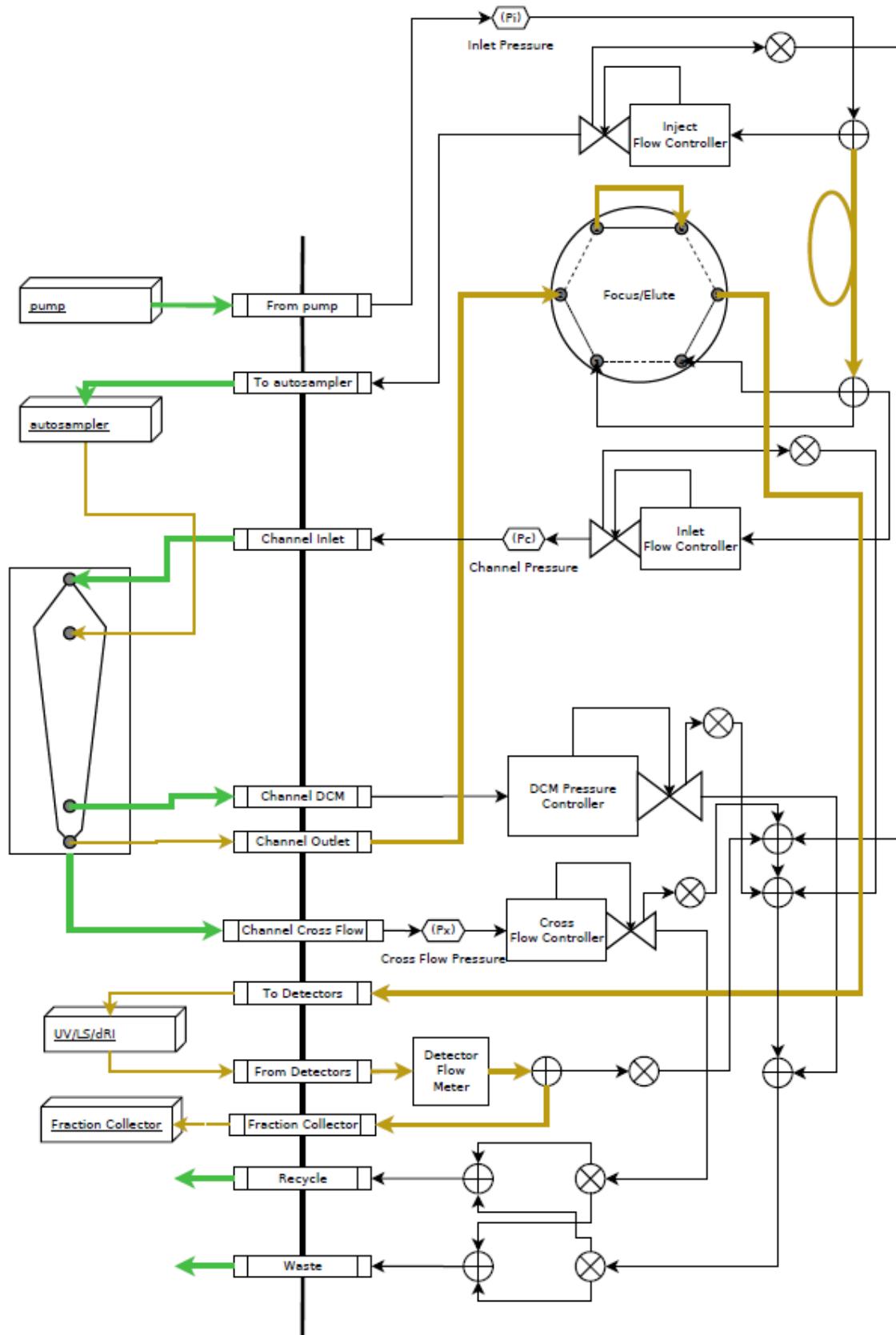
Legend:

Symbol	Description
	Valve
	Union
	Needle valve
	Side panel bulkhead union of Eclipse; left is exterior and right is interior for these flow diagrams
	Module and the tubing that enters or exits.
	Pressure sensor responsible for reporting the pressure for various pathways
	Rheodyne valve, a switching valve that can connect fluid lines via the solid lines or the dashed lines

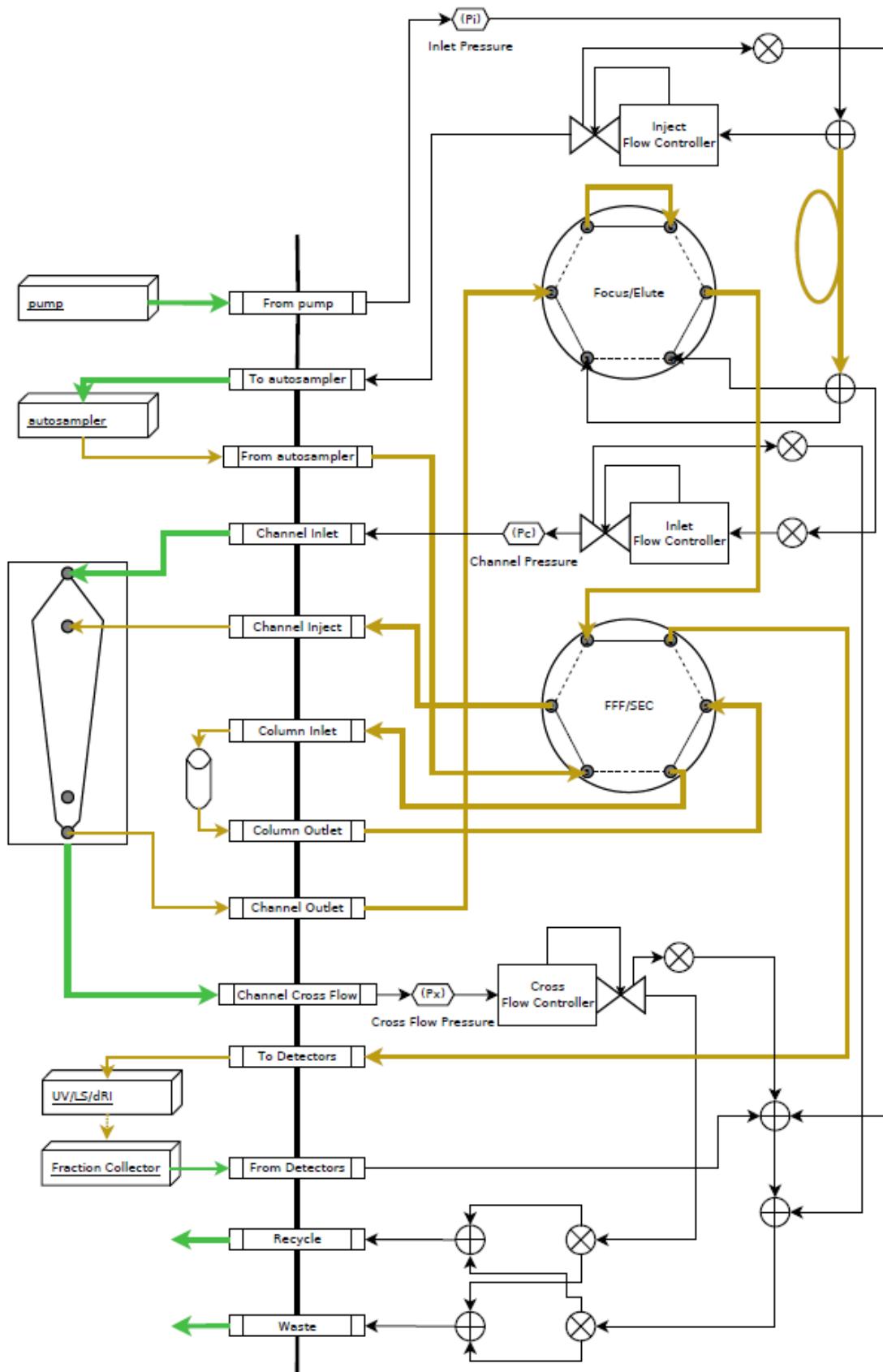
Eclipse Base Model (WEC) Flow Diagram



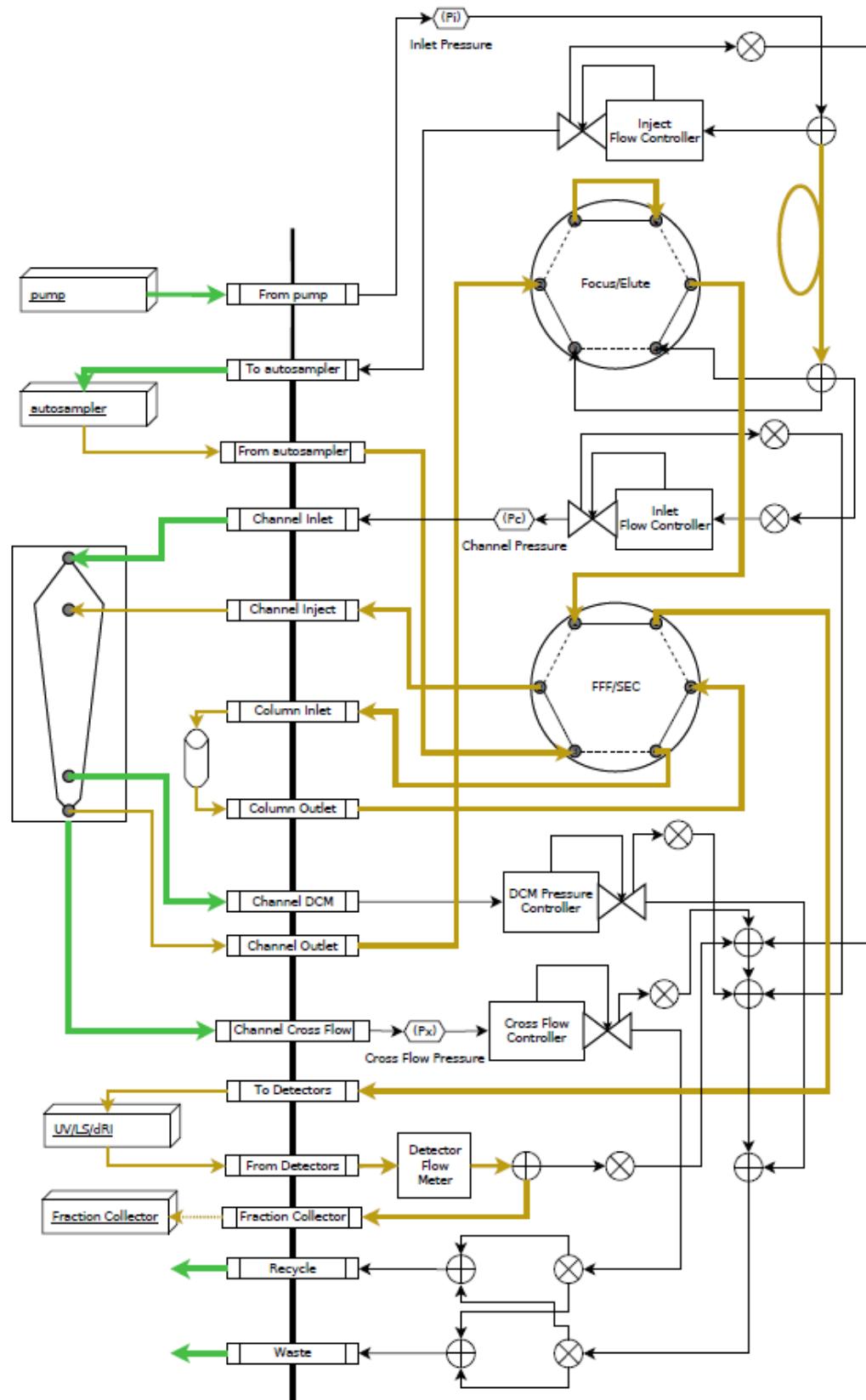
Eclipse with DCM Model (WECD) Flow Diagram



Eclipse with SEC Switching Model (WECS) Flow Diagram



Eclipse with DCM and SEC Switching Model (WECDS) Flow Diagram

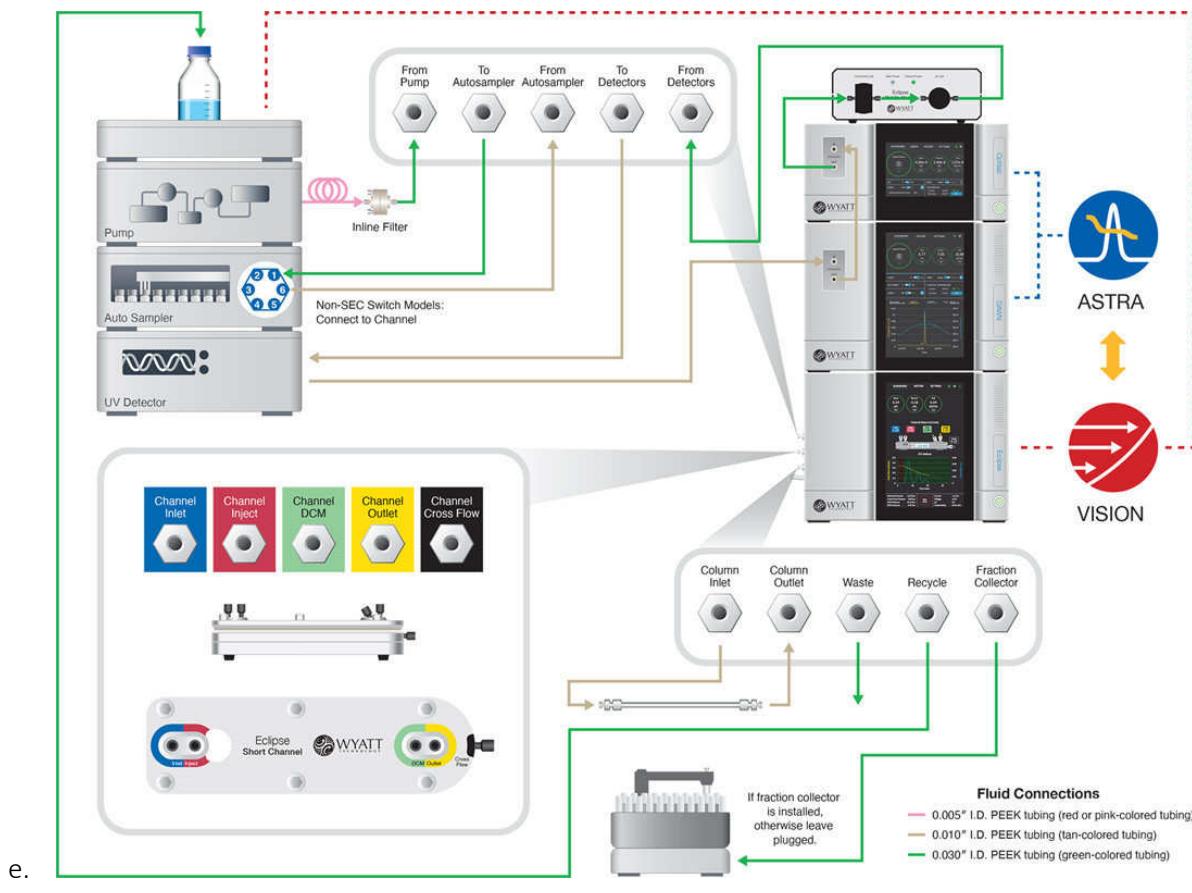


Appendix E – Eclipse Installation Quick Guide

The following section describes the basic workflow for installing an Eclipse and configuring the software.

1. Place detectors, depending on UV module location, can arrange to minimize tubing volume
 - a. e.g., Eclipse, DAWN, Optilab, Mobility from bottom to top
 - b. Install elbow drain tubing connection on bottom detector
 - c. Prepare all auto-inject, alarm out, Ethernet cables and connect to back panels
 - i. Analog: Eclipse Analog In (Red(+)/Green(-)); Analog In 2 (White (+)/Black (-))
 - ii. Alarm: Pump to Eclipse (Eclipse = Active High)
 - iii. Alarm: Pump to DAWN to Eclipse (DAWN = Active Low; Eclipse = Active High)
 - d. Configure instrument IP addresses, recommend:
 - i. Computer Adapter: 192.168.254.100
 - ii. Agilent HPLC: 192.168.254.11
 - iii. Eclipse: 192.168.254.101
 - iv. DAWN: 192.168.254.102
 - v. Optilab: 192.168.254.103
 - vi. Mobility: 192.168.254.50 (Default; can only be changed if connected)
 - e. Install & configure ASTRA
 - f. Assess MALS and Optilab, miscible solvent flush if necessary
 - g. Perform IQOQ tests for MALS and Optilab, if necessary
2. Prepare ASTRA for FFF-MALS Experiments
 - a. (Only for ASTRA 8.0 or older) ASTRA SDK applied to ASTRA feature activation (not needed in ASTRA 8.1)
 - b. Create ASTRA FFF methods for analytical detectors, include “VISION UV” for digital UV
3. Prepare VISION for FFF-MALS Experiments
 - a. Input license keys, ensure ASTRA module is enabled
 - b. Click VISION RUN tile, then Add and Configure. Select Agilent 1100/1200/1260/1290 LC and Auto-Configure with Agilent IP address.
 - i. Check individual module configurations (cooler, seal wash, etc.)
 - c. Select Eclipse, then “>” to move to right panel. Configure with Eclipse IP address and Mobility IP address if applicable, hit Apply first.
 - i. Confirm serial number and model displays
4. Launch VISION
 - a. More > Eclipse > Separation Device
 - i. Configure with desired channel type and/or column—will physically install later
 - b. More > Eclipse > Hardware Configuration
 - i. Set chassis temperature (slightly above ambient), SEC flow ramp limit, Rinse mode.
5. Plumb the Eclipse (using red, green (orange also acceptable alternative), and tan PEEK tubing):
 - a. Control pump flow rate with VISION. Install red PEEK tubing on pump head to acquire 150 bar of pump pressure at 4 mL/min pump flow.
 - b. Install inline filter after red PEEK tubing, label with membrane, date, and if contains Viton O-ring

- c. Flush out air then connect inline filter to stainless steel frit filter to “From Pump” port of Eclipse
- d. Work backwards for tubing connections to assist with routing:
 - i. Connect waste bottle to “Waste” port
 - ii. Connect waste bottle to “Recycle” port until recycle pathway is flushed with new solvent
 - iii. For SEC models: leave bridge tubing between inlet and outlet
 - iv. Connect detectors together, green PEEK for Optilab and Mobility
 - v. Connect “From Detectors” to last detector; create “bypass” tubing for UV/MALS to “From Detectors” if fraction collector is present, as to bypass Optilab and Mobility for FC experiments.
 - vi. Connect tan PEEK from “To Detectors” to first detector inlet, may replace with red PEEK later for channel pressure tubing.
 - vii. Connect “To Autosampler” to Agilent sampler Rheodyne #1
 1. For SEC models: connect tan tubing from Rheodyne #6 to “From Autosampler” on Eclipse.
 2. For non-SEC models: connect tan tubing from Rheodyne #6 to 5-Port union.



- e.
- f. Plug DCM ports if not applicable. Check estimated length of channel placement, can remove tubing sleeves and custom cut tubing for appropriate length.
- g. Connect channel tubing (inlet, inject, outlet, cross flow, and DCM) to 5-port union from corresponding Eclipse ports.
 - i. Install DCM inline filter (if applicable), label with membrane and date.
 - ii. Install cross flow inline filter, label with membrane and date.
- 6. Organize tubing via inline filter holder and route tubing as needed to keep everything organized

7. Either manually flush or use method to perform a solvent exchange
 - a. Purge HPLC pump first at high flow rates to save time
8. Confirm good baselines, low noise and solvent exchange is complete.
9. Set up initial HPLC settings for “Template Method.”
 - a. HPLC Pump
 - i. Disable Agilent bottle fillings for Eclipse bottle fillings
 - ii. Input appropriate compressibility setting in method
 - iii. FFF typically uses the highest flow gradient possible (100 mL/min/min)
 - b. Autosampler
 - i. Define cooler and cooler settings
 - c. UV Detector
 - i. Wavelength (280, 260 nm for proteins/nucleic acids; or 254 nm for polymers)
 - ii. Acquire signal without reference
 - iii. Use bandwidth of 2 nm; peak width > 0.1 min (2 s response time; 5 Hz)
 - iv. Analog output (even with digital UV for backup)
 - v. Zero Offset of 5% and Attenuation can be customized by 1000 mAU to start
 - d. FLD Detector
 - i. Gain value of 12
 - ii. Excitation of 280 nm for proteins or 260 nm for nucleic acids
 - iii. Emission of 340 – 350 nm for proteins or 320 – 330 nm for nucleic acids
 - e. Fraction Collector
 - i. Use 3rd party pump and disable pump linking (for accurate vial collection)
 - ii. Calculate delay volume if using peak triggers
10. Create an Eclipse method
 - a. Use BSA default flows
 - b. Focus at 25% regardless of channel
 - c. Determine whether you want to recycle flows or not during methods
 - d. Calculate FFO using Performance & Tuning or within method
11. Save this “BSA” method as a template method called “Template Method”
 - a. This method can be used to create future methods with appropriate UV settings, etc.
 - b. After “Save As” Template Method; ‘save as’ again to generate “BSA Method”
 - c. Create template method without fraction collector enabled as needed.

12. Create a sequence with blank injections, samples, and optionally a conditioning method or shutdown methods.
 - a. Save As to create a “Template Sequence”
 - b. This sequence can be used to create future sequences more quickly.
13. Assemble the Fixed Height or Variable Height Channel with roughly 300 – 450 µm spacer or equivalent channel height equipped with a 10 kDa RC membrane.
14. Run BSA Method with conditioning run for the membrane, then analytical runs.
15. Process ASTRA data to determine all system parameters like normalization, alignment, and band broadening with the best/last analytical BSA run.
16. Create a duplicate experiment for method creation.
 - a. In duplicate, delete peaks and baselines
 - b. Save this as an ASTRA method for future experiments (no pre-populated peaks or baselines in future sample runs)
17. Update VISION RUN sequence “template” with new ASTRA method with system parameters.

Appendix F – Agilent HPLC Connection Instructions

Hardware Connections from Agilent Detector to Wyatt Instruments

This information is derived from TN3600 – Agilent HPLC Connection Guide for Wyatt Instrumentation and is presented here as a convenience. Please refer to TN3600 or contact Wyatt Technology Support for the latest information.

NOTE: The UV signal from the Agilent 1220 Infinity LC VWD cannot be exported since analog out ports are not available on this HPLC model.

1. Locate a BNC/Banana Plug Adapter (Wyatt p/n P3795-04) and a RJ-12 cable with flying leads (Wyatt p/n P4045-19).

NOTE: One BNC/Banana Plug Adapter is required for each auxiliary channel that will be connected. Wyatt provides one adapter in the HPLC connection kit for your instrument. If you require a second BNC adapter, the adapter is available from Pomona Electronics® (p/n 1296, BNC Adapter, Male to Binding Post).

2. Connect two of the flying lead wires of the flat grey RJ-12 cable to the red and black binding posts of the BNC adapter, refer to Table 11.
3. One possible configuration is to wire the *black wire* from the RJ-12 cable to the black binding post on the BNC adapter, and the *white wire* from the RJ-12 cable to the red binding post on the BNC adapter. Plugging the RJ-12 into the **AUX IN 1&2** port on the rear of the DAWN will transmit the analog voltage to **AUX channel 1** of the Wyatt instrument. Alternatively, if instead the RJ-12 cable is connected to the **AUX IN 3&4** port on the rear panel, then the analog voltage will transmit to **AUX Channel 3** of the Wyatt instrument. Some Wyatt instruments have different ports and wiring. Please refer to Table 1 and Figure 140 below.

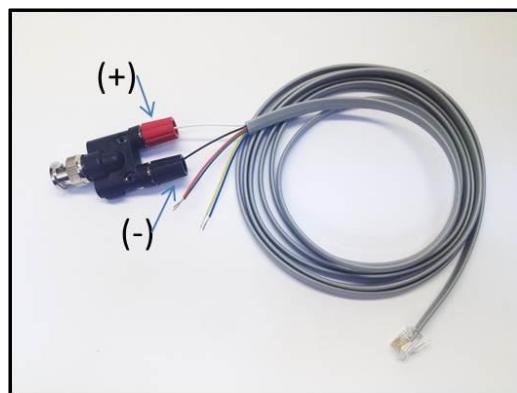
	BNC Adapter Analog Output 1		BNC Adapter Analog Output 2		BNC Adapter Analog Output 3		BNC Adapter Analog Output 4	
	(+) RED Binding post	(-) BLACK Binding post	(+) RED Binding post	(-) BLACK Binding post	(+) RED Binding post	(-) BLACK Binding post	(+) RED Binding post	(-) BLACK Binding post
AUX IN 1 & 2	White RJ-12	Black RJ-12	Red RJ-12	Green RJ-12	Not used	Not used	Not used	Not used
AUX IN 3 & 4	Not used	Not used	Not used	Not used	White RJ-12	Black RJ-12	Red RJ-12	Green RJ-12

*The Wyatt Optilab (T-rEX™, UT-rEX™ and NEON) or Wyatt ViscoStar® II instruments have only one Analog Input port, labeled “**Analog IN**”. This port has only one active channel, which uses the green (-) and red (+) wires of the RJ-12 cable. When wiring to connect to either of these ports, one must connect the green wire from the RJ-12 cable to the black binding post of the BNC connector, and the red wire from the RJ-12 cable to the red binding post of the BNC connector. In contrary to miniDAWN® TREOS® I and all the DAWN detectors (HELOS I/II and NEON) with two Aux In ports (Aux In (1&2) and Aux In (3&4)), the miniDAWN TREOS II/ miniDAWN (NEON) have one **Aux In** (1&2) port where the active channel depends on the wiring in the above table. The ViscoStar III/ViscoStar (NEON) have one **Analog In** port where the active channel depends on the wiring and is different than the above table (2 channels with either white-black (Analog In 2) or red-green (Analog In 1) wiring pairs).

Table 11. Wire color conventions used when wiring together the BNC adapter to the Wyatt RJ-12 cable.



a) BNC connector necessary for the auxiliary cable connection.

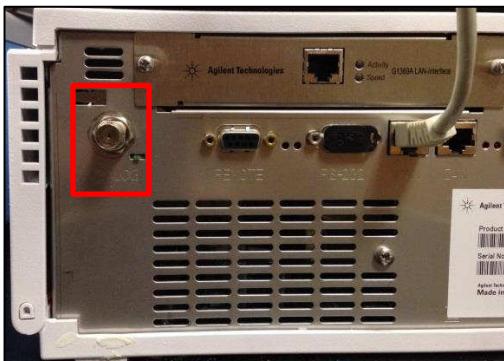


b) BNC adapter connected to the white (+) and black (-) wires of the RJ-12 cable. In this configuration the signal will be transmitted from the auxiliary channel to either AUX IN Channel 1 or 3 depending on which port it is plugged into on the Wyatt Instrument.

Figure 140. BNC adapter connection to RJ-12 cable.

NOTE: The blue and yellow wires from the RJ-12 cable are ground wires and are not necessary for any connections. It is recommended to tape off any unused wires on the RJ-12 cable with electrical tape unless excessive noise pickup is observed.

4. Connect the BNC adapter of the assembled cable to the “ANALOG 1” port on the back of the Agilent detector as shown in Figure 141.



a) The red circle indicates the open port on the rear of the Agilent detector “ANALOG 1”.



b) BNC adapter connected to an Agilent detector “ANALOG 1” port.

Figure 141. Auxiliary channel connection on the rear panel of the Agilent Variable Wavelength Detector (VWD).

NOTE: The Agilent Variable Wavelength Detector (VWD) only has one available analog output BNC available on the rear panel (refer to Figure 141). An “ANALOG 2” port may be available depending on the type of Agilent concentration detector being connected. For example, the Agilent Diode Array Detector (DAD detector) typically has two analog out ports (Figure 142 below). In this later case, a second BNC adapter may be used to output a second signal to the DAWN or miniDAWN instruments. Please refer to Table 11 and follow the directions above to connect a second set of wires at the end of the RJ-12 cable with the corresponding colors to the second BNC to use the second AUX channel.

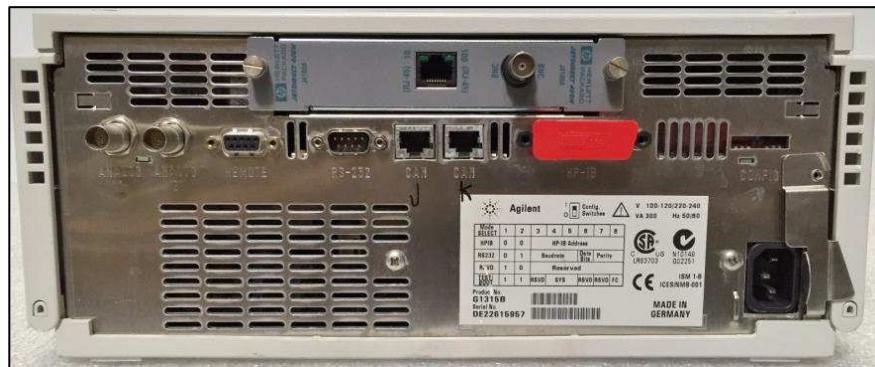


Figure 142. The rear panel of an Agilent Diode Array Detector (DAD) with two available analog output channels.

5. Plug the RJ-12 end of the cable to the correct port on your Wyatt instrument as shown in Figure 143.

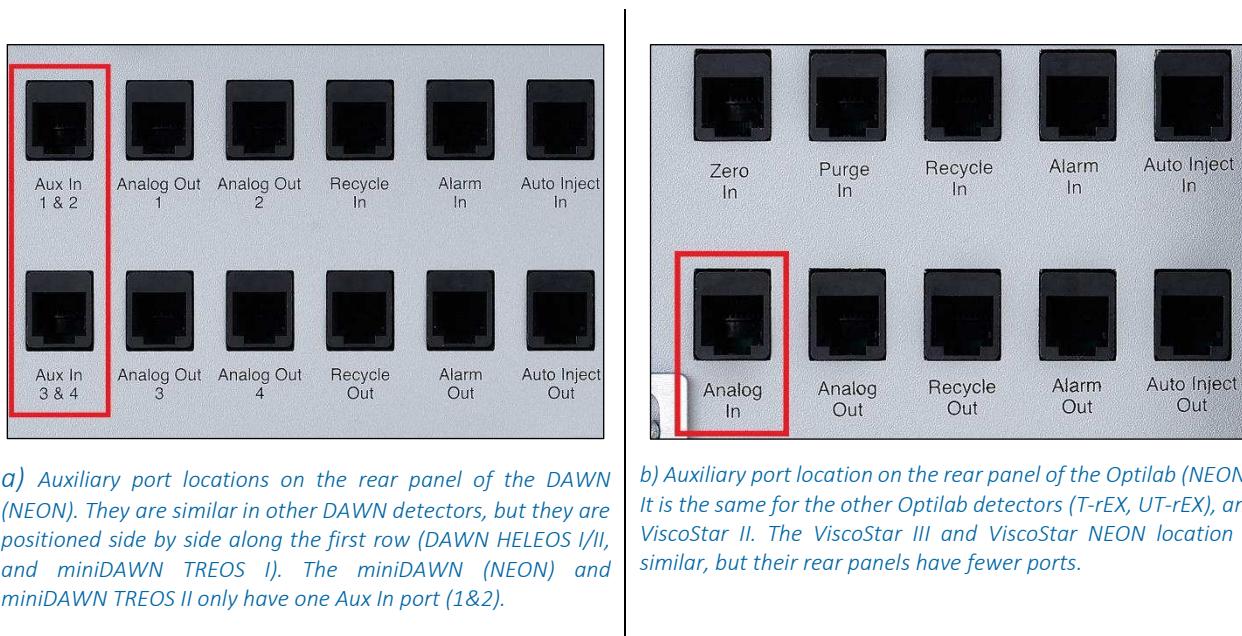


Figure 143. Auxiliary channel inputs on the rear panel of the Wyatt Technology instruments.

Configuring ASTRA to Accept Auxiliary Voltages from Agilent Detectors

UV Detector:

1. Select the appropriate ASTRA method and instrument configuration. Refer to the *ASTRA User’s Guide* for assistance with selecting the appropriate ASTRA method and configuration for your particular detector configuration.

2. Once the appropriate ASTRA method and instrument configuration has been chosen, expand the “[Configuration](#)” tree and double-click on “[Generic UV Instrument \(UV\)](#)”. This will expand the instrument profile, and allow you to enter the necessary instrument information (refer to Figure 144 below):
- [Cell Length \(cm\)](#) - The length of the UV cell in cm (this value is often 1.0 cm).
 - [Channels](#) - Enable relevant Aux Channels that have been physical connected in the procedure detailed above.
 - [Wavelength](#) - the wavelength to be monitored (typically 280 nm for proteins).
 - [UV response](#) - the UV calibration constant for the AUX channel (typically 1 AU/V full scale) which is used to convert the volts of the analog signal to absorbance units). The Agilent VWD allows settings between 0.98 mAU to 4000 mAU at discrete values for either 100mV or 1V full scale, while the Agilent DAD allows settings between 0.96 and 2000 mAU at discrete values for either 100mV or 1V full scale. Hence using 1000 mAU with a 1V full scale output will achieve 1AU/V for the UV response.
 - [Active Channel](#) - Allows user to select the channel that will be used to perform the data processing and calculation of the concentration from the UV signal.

NOTE: For UV detectors that have multiple analog UV output ports (i.e., Agilent DAD) the multiple wavelengths can be exported to ASTRA by enabling these Channels here. Refer to the Agilent Instrument User Guides for details.

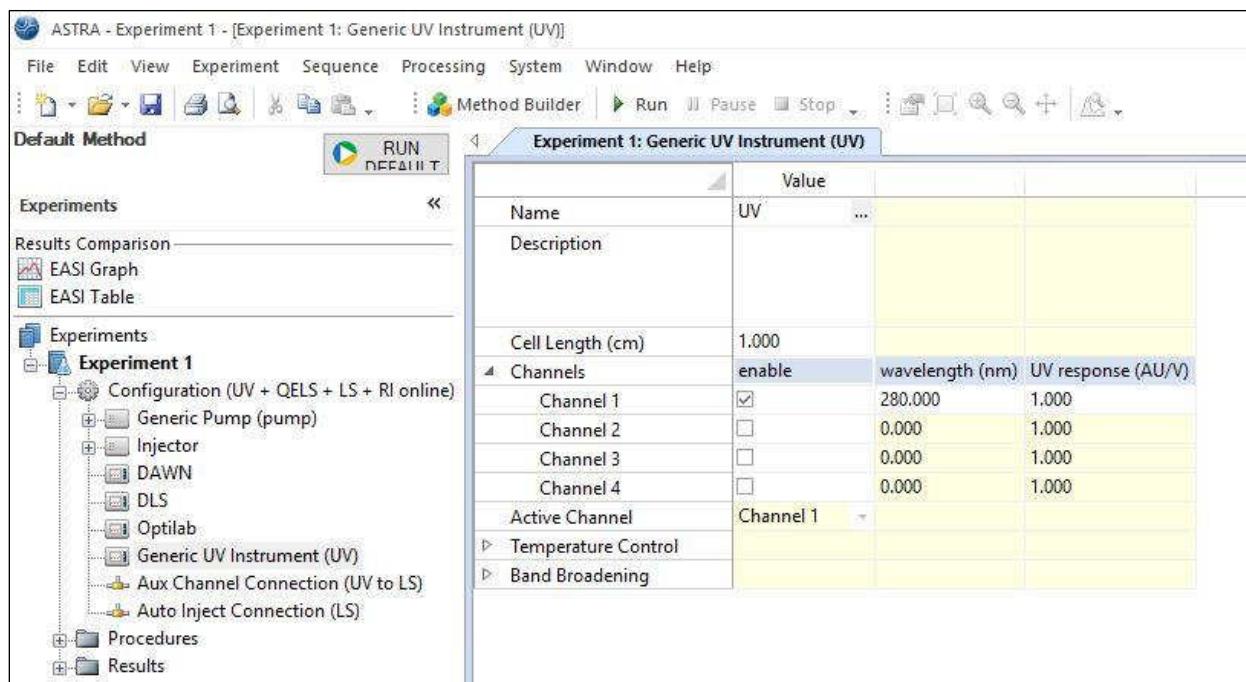


Figure 144. Screenshot of the ASTRA software showing an example experiment method which has the “[Generic UV Instrument](#)” profile expanded under the “[Configuration](#)” sub-menu.

3. Select “OK” to save changes and exit this window.
4. Expand the “[Aux Channel Connection \(UV to LS\)](#)” configuration by double-clicking on the heading (refer to Figure 145). This window will allow you to define the hardware connections you have previously made in the software, such as:

- **Source Device** - the instrument that sends analog data over this connection, this should be left as is. If multiple channels are selected under the “[Generic UV Instrument](#)” configuration window, the drop-down menu will list all the selected channels. Select with the channel with the wavelength you wish to display.
- **Destination Device** – the instrument that receives analog data over this connection, this should be left as is.
- **AUX Channel** - The input AUX channel number directly corresponds to the AUX port that is connected from the UV detector via the RJ-12 cable to the rear panel of your Wyatt instrumentation (refer to Table 11). For example, an RJ-12 cable with a BNC adapter attached to its black and white wires that is plugged into the Analog 1 port on the rear of the Agilent detector and plugged into the AUX IN 1&2 port of the Wyatt instrument will have an input AUX channel number of 1. If multiple signals are imported, input the AUX channel that corresponds to the selected channel under Source Device.

NOTE: For the Wyatt Optilab (T-rEX, UT-rEX and NEON) or Wyatt ViscoStar II detectors, the input AUX channel is always 2 since they are using only one active channel with the green (-) and red (+) wires of the RJ-12 cable for one Analog Input port. For the DAWN (HELEOS I/II and NEON), miniDAWN (TREOS I/II and NEON), and ViscoStar III/ViscoStar (NEON), the input AUX channel depends on the wiring as described in Table 11.

- **Calibration Constant** - This number is a correcting constant that can be used to adjust the value by which the AUX signal should be scaled. Under most circumstances, this value should be left at 1. If the calibration constant is changed to 2, then the calculated molar mass will be doubled. If this value is changed to 0.5, then the calculated molar mass will be halved, if the concentration source is chosen to be UV signal.

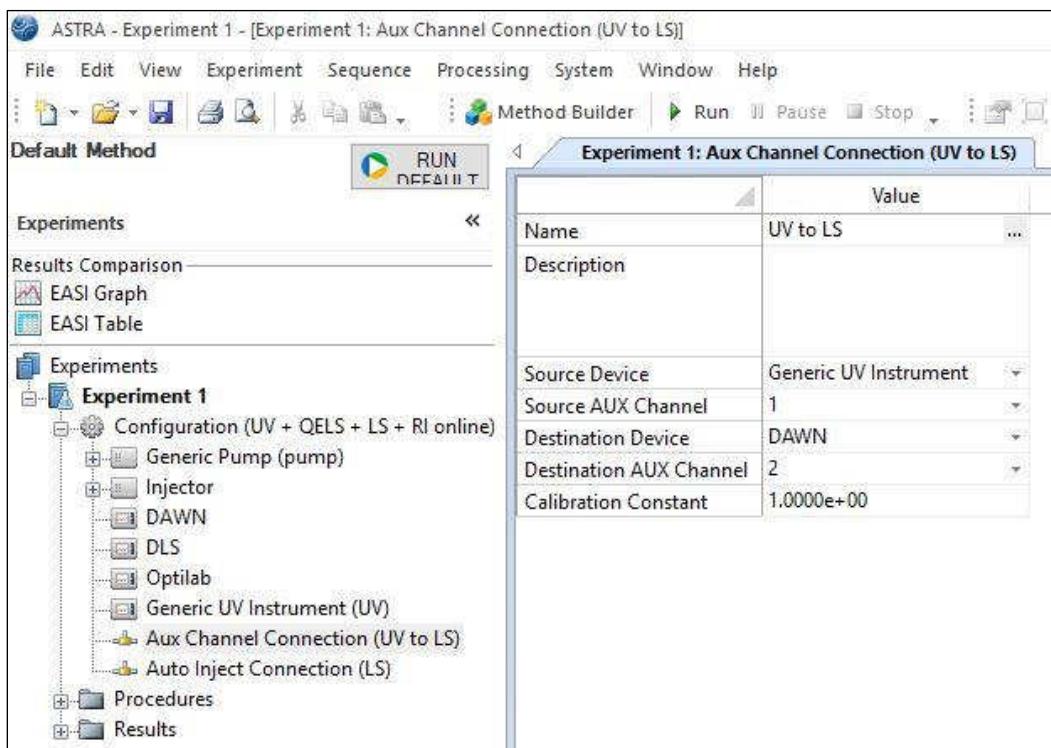


Figure 145. Screenshot of the ASTRA software showing an example experiment method which has the “[Aux Channel Connection \(UV to LS\)](#)” profile expanded under the “[Configuration](#)” sub-menu.

5. Select “OK” to save changes and exit this window.
6. This completes the ASTRA set-up necessary for the UV detector.

Generic RI Detector (if present):

1. Select the appropriate ASTRA method and instrument configuration. Refer to the *ASTRA User’s Guide* for assistance with selecting the appropriate ASTRA method and configuration for your particular detector configuration.
2. Once the correct ASTRA method and instrument configuration has been selected, expand the “Configuration” tree and double-click on “[Generic RI Instrument \(RI\)](#)” to expand this instrument profile. Enter the wavelength of your detector, the only relevant entry in this window, (refer to Figure 146).

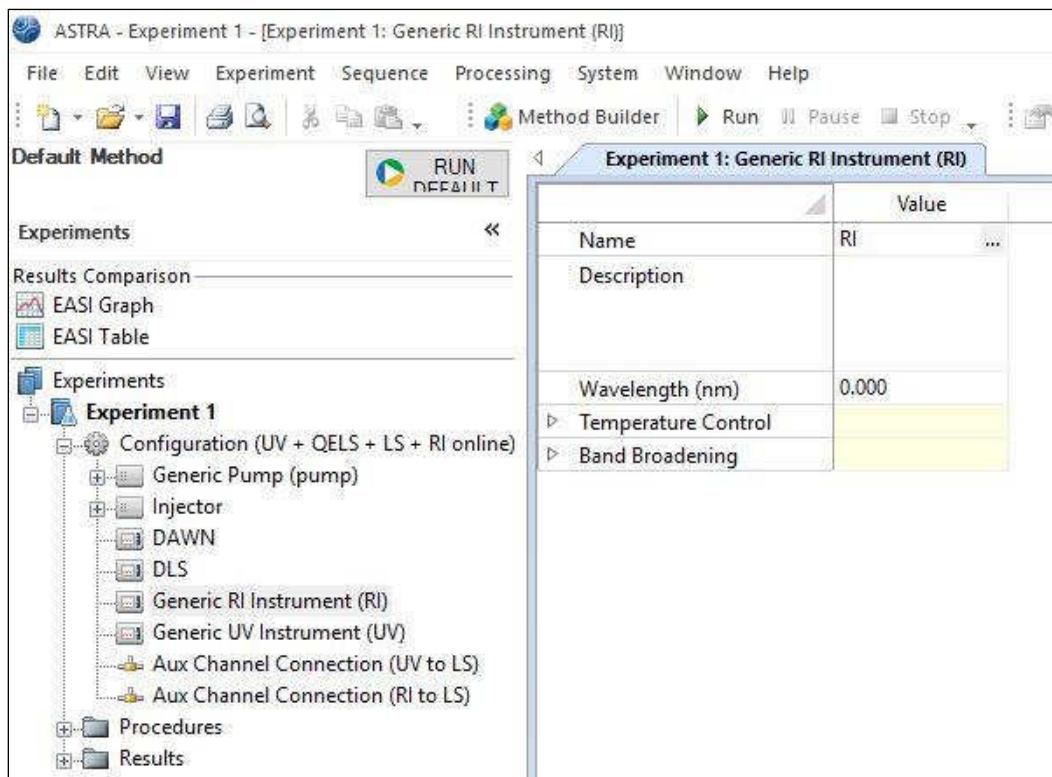


Figure 146. Screenshot of the ASTRA software showing an example experiment method which has the “[Generic RI Instrument \(RI\)](#)” profile expanded under the “Configuration” sub-menu.

3. Select “OK” to save these changes and close the window.
4. Double-click on the “[AUX Channel Connection \(RI to LS\)](#)” to expand this profile as shown in Figure 147.
5. In this window it is possible to define the following items:
 - **Source Device** – the instrument that sends analog data over this connection, typically this is left as is.
 - **Destination Device** – specify the instrument that receives analog data over this connection, typically this is left as is.
 - **AUX Channel** - The input AUX channel corresponds directly to the AUX port that is connected from the RI detector analog output to the back panel of your Wyatt instrument via the RJ-12 cable (refer to Table 11).

- **Calibration Constant** - Unlike with the UV detector, in this case, this value is the calibration constant used to convert from volts to refractive index units, entered in units of RIU/V (refractive index units per volt). The Agilent RID allows attenuation settings between 488 nRIU to 1,000,000 nRIU at discrete values for either 100mV or 1V full scale. A good starting value to use for this constant is 488 nRIU with a 1V full scale output, which results in a 4.88e-4 RIU/V value that should be entered into ASTRA. If sensitivity needs to be decreased this value can be increased accordingly, refer to the Agilent RID detector hardware manual for more information on how to configure this constant.

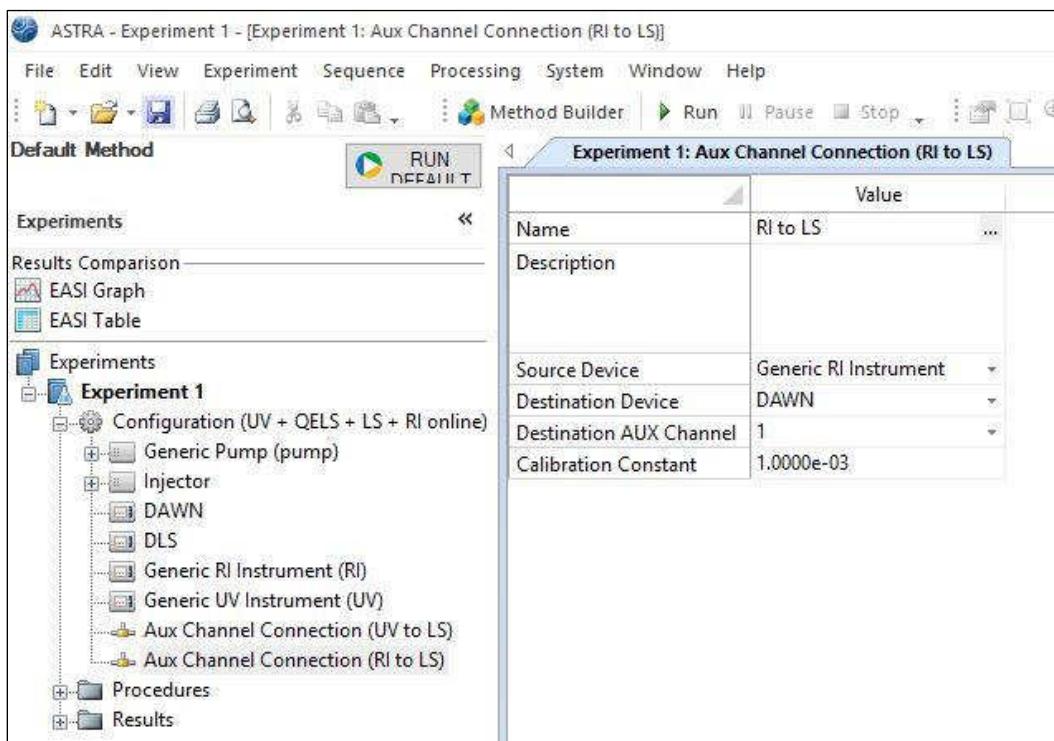


Figure 147. Screenshot of the ASTRA software showing an example experiment method which has the “Aux Channel Connection (RI to LS)” profile expanded under the “Configuration” sub-menu.

6. Select “OK” to save changes and exit this window.
7. This completes the ASTRA set-up necessary for the RI detector.

If you have any questions, please email support@wyatt.com or call Wyatt’s support team at 805-681-9009, option 4 (within the US and Canada).

If you are one of our international customers, feel free to contact your local representative directly. You can find contact information for our global offices at www.wyatt.com/Distributors.

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