

# VISCOTEK 305 TDA USER MANUAL

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# HEALTH AND SAFETY INFORMATION

## Notices, warnings and definitions

The following symbols may be used in various locations within this manual. These signs provide the following information:

Certification IEC 61010-1 for electrical equipment control and laboratory use.

|  |  |
|--|--|
|  | Indicates potentially hazardous situation.                                     |
|  | Electrical shock caution.  |
|  | High temperature caution. Possibility of injury under specific conditions.     |
|  | Laser radiation warning.   |
|  | Certification IEC 61010-1 for electrical equipment control and laboratory use. |

Other information contained in this manual is subject to change without notice. Malvern Instruments assumes no responsibility for errors that may appear in this document. The manual is believed to be accurate at the time of printing. Malvern Instruments shall not be liable for damages resulting from the use of this document.

|  |  |
|--|--|
|  | <b>WARNING!</b><br>This detector is a highly sensitive instrument. Only trained personnel should operate this equipment. Read the manual before using, and follow appropriate safety procedures. Using the equipment in a manner not specified by the manufacturer may compromise the safety protection of the unit.   |
|  | <b>WARNING!</b><br>To avoid the risk of electrical shock, disconnect the power cord before removing top cover. The power cord ends are the primary disconnecting devices. Do not position equipment so that it is difficult to access the cord. Use only power cords that are SVT,18awg – 3 conductor, unshielded, NEMA 5-15P, 10A , 60C, UL listed component, US equivalent #312003-0 |
|  | <b>Caution!</b><br>To avoid possible damage to electrical components, power off the detector before removing cover.  |
|  | <b>WARNING!</b><br>HOT surfaces inside. Wear appropriate protection when handling detector module.   |
|  | <b>WARNING!</b><br>Use correct voltage on fuse module and switch (sw6) (100/240 VAC 50/60 Hz) to avoid damage and possible fire hazard. When fuse replacement is necessary, use only the fuse specified on the chassis rear cover (5.0 Amperes 250V type T).   |
|  | <b>WARNING!</b><br>When connecting power cord to detector, use a properly grounded receptacle.   |
|  | <b>Caution!</b><br>The detector has a flow cell that is pressure rated at 100 psi. Do not connect any tubing or device that might cause the back pressure to exceed the above pressure rating.   |

**Note:**

Indoor use only. The room temperature operation of the instrument is from 5 to 25°C. The maximum operating humidity rating is 20 - 80% non-condensing at 31°C and de-rated to 67% at 25°C at altitudes <2000 M.



The laser diode used in this product has been classified as a Class 1 Laser Product that complies with 21 CFR 1040.10 and 21 CFR 1040.11. Avoid direct exposure to the beam. Serious eye damage or possible blindness could result from direct eye contact with the beam emerging from the instrument. Pay attention to the warning stickers inside the instrument. Contact Malvern Instruments Helpdesk if service is needed.

**Note:**

The detector can use many different types of solvents. If a leak occurs inside the unit, exposure to the solvent is possible. Consult the MSDS for any health and safety precautions.

**WARNING!**

Some solvents can be flammable. Do not operate the detector if the unit is leaking solvent or at temperatures that are above the flash point minus 25°C of the solvent.

**WARNING!**

This product must be connected to a protective earth. The metal parts of the unit are earthed using a protective earth connection.



The Light Scattering Detector assembly contains no user serviceable parts. Contact Malvern Instruments if service is needed.

## Laser safety precautions

During normal operation the operator is shielded from direct exposure to the laser, however users should be aware of the following points in order to maximize safety:

- Laser safety eyewear is not supplied with the system. It is the customer's responsibility to supply the correct laser safety eyewear to anyone who may be exposed to the laser radiation. Reputable distributors of laser safety eyewear can recommend the best product for your specific needs.
- The instrument should be used in a well-lit room. Under these conditions, the human eye pupil has a relatively small opening, limiting the damage potential of laser radiation.

## Power cords and Power safety

The notes in this section indicate best practice and should be adhered to when connecting the Malvern instrument to the power supply.

### Power cord set requirements

Power cord sets must meet the requirements of the country where the product is used. For further information on power cord set requirements, contact a Malvern Instruments representative.

### General requirements

The requirements listed below are applicable to all countries:

- The power cord must be approved by an acceptable accredited agency responsible for evaluation in the country where the power cord set will be installed.
- The power cord set must have a minimum current capacity of 10A (7A in Japan only) and a nominal voltage rating of 125 or 250 volts AC, as required by each country's power system.
- The area of the wire must be a minimum of 0.75mm<sup>2</sup> or 18AWG, and the length of the cord must be less than 3m.
- The power cord must be routed so it is not likely to be walked on or pinched by items placed upon it or against it, or become wet. Particular attention must be paid to the plug, the electrical outlet, and the point where the cord exits the product.

**WARNING!**

Do not operate this product with a damaged power cord set. If the power cord set is damaged in any manner, replace it immediately.

**WARNING!**

Do not use the power cord received with this product on any other products.

## Power safety information

The following notes indicate guidelines to follow when connecting the Malvern

Instruments power supply using single and multiple extension leads/power strips, connection via AC Adapters and use of Uninterruptible Power Supplies (UPS).

**WARNING!**

To prevent electric shock, plug the instrument or accessory into correctly earthed electrical outlets.

The power cord supplied is equipped with a grounding connection to ensure grounding integrity is maintained.

### Advice on use of Extension leads/power strips

Follow this advice when using single or multiple socket extension leads. These are also called trailing sockets.

- Ensure the lead is connected to a wall power outlet and not to another extension lead. The extension lead must be designed for grounding plugs and plugged into a grounded wall outlet.
- Ensure that the total ampere rating of the products being plugged into the extension lead does not exceed the ampere rating of the extension lead.
- Use caution when plugging a power cord into a multiple socket extension lead. Some extension leads may allow a plug to be inserted incorrectly.
- Incorrect insertion of the power plug could result in permanent damage to the instrument or accessory, as well as risk of electric shock and/or fire. Ensure that the ground connection (prong/pin) of the power cord plug is inserted into the mating ground contact of the extension lead

### Advice on use of AC adapters

**WARNING!** Do not use adapter plugs that bypass the grounding feature, or remove the grounding feature from the plug or adapter.

- Place the AC adapter in a ventilated area, such as a desk top or on the floor.
- The AC adapter may become hot during normal operation of the instrument or accessory. Use care when handling the adapter during or immediately after operation.
- Use only the Malvern-provided AC adapter approved for use with the instrument and/or accessory. Use of another AC adapter may cause a fire or explosion.

### Advice on use of Uninterruptible Power Supplies (UPS)

To help protect the instrument and/or accessory from sudden, transient increases and decreases in electrical power, use a surge suppressor, line conditioner or UPS.

## Sample handling warnings

- Always handle all substances in accordance with the COSHH (Control Of Substances Hazardous to Health) regulations (UK) or any local regulations concerning sample handling safety.
- Before using any substance, check the Material Safety Data Sheets for safe handling information.

- Use the instrument in a well-ventilated room, or preferably within a fume cupboard, if the fumes from the sample or dispersant are toxic or noxious.
- Wear personal protective equipment as recommended by the Material Safety Data Sheets if toxic or hazardous samples are being handled, particularly during sample preparation and measurement.
- Wear protective gloves when handling hazardous materials, or those that cause skin infections or irritations.
- Do not smoke during measurement procedures, particularly where inflammable samples are used or stored.
- Do not eat or drink during measurement procedures, particularly where hazardous samples are used or stored.
- Take care when handling glass (e.g. sample vials and pipets). Hazardous materials may enter a wound caused by broken glass.
- Always test a new sample or mobile phase for chemical compatibility before use.
- Always label samples for analysis using industry standard labeling, particularly if they are handled by a number of staff or stored for long periods. Clearly mark any operator hazard and associated safety precautions that are required for the handling of dangerous materials.
- Keep a record of all hazardous substances used in the system for protection of service and maintenance personnel.
- Always adopt responsible procedures for the disposal of waste samples. Most local laws forbid the disposal of many chemicals in such a manner as to allow their entry into the water system. The user is advised to seek local advice as to the means available for disposal of chemical wastes in the area of use. Refer to the Materials Safety Data Sheets.
- The surfaces of the system may be permanently damaged if samples are spilt on them. If a spillage does occur, disconnect the system from the power supply before scrupulously cleaning up the spillage.

## Disposing of the instrument and accessories

When the need eventually arises to dispose of the system, this should be done in a responsible manner. Follow these guidelines:

- Refer to any local regulations on disposal of equipment; in Europe refer to the information below.
- Seek advice from the local Malvern Instruments representative for details.
- Decontaminate the instrument if hazardous materials have been used in it.

## Disposal of Electrical & Electronic Equipment

The following is applicable in the European Union and other European countries with separate collection systems.

This symbol on the product or on its packaging indicates that when the last user wishes to discard this product it must not be treated as general waste. Instead it shall be handed over to the appropriate facility for the recovery and recycling of electrical and electronic equipment.

By not discarding this product along with other household-type waste, the volume of waste sent to incinerators or landfills will be reduced and natural resources will be conserved.

For more detailed information about recycling of this product, please contact your local city office, your waste disposal service, or your Malvern Instruments representative.



# DESCRIPTION OF INSTRUMENT

## Overview

This section describes the hardware within the TDA 305. The complete system is comprised of a number of detectors plumbed in series within the instrument. Each of the detectors is considered in turn.

A separate data acquisition device is not necessary for the Model 305 TDA. The data acquisition device (DAD) has been integrated into the detector, available through the serial interface located on the back panel. The DAD system is more than a simple chromatography board, which only converts analogue voltage signals from GPC detectors into digital values for processing by a computer. It is designed for unattended data acquisition, leaving the computer free to run other applications. The DAD collects and stores analogue data and controls linked accessories

## Flow Paths

Figure 1 shows a plumbing diagram for the whole instrument.

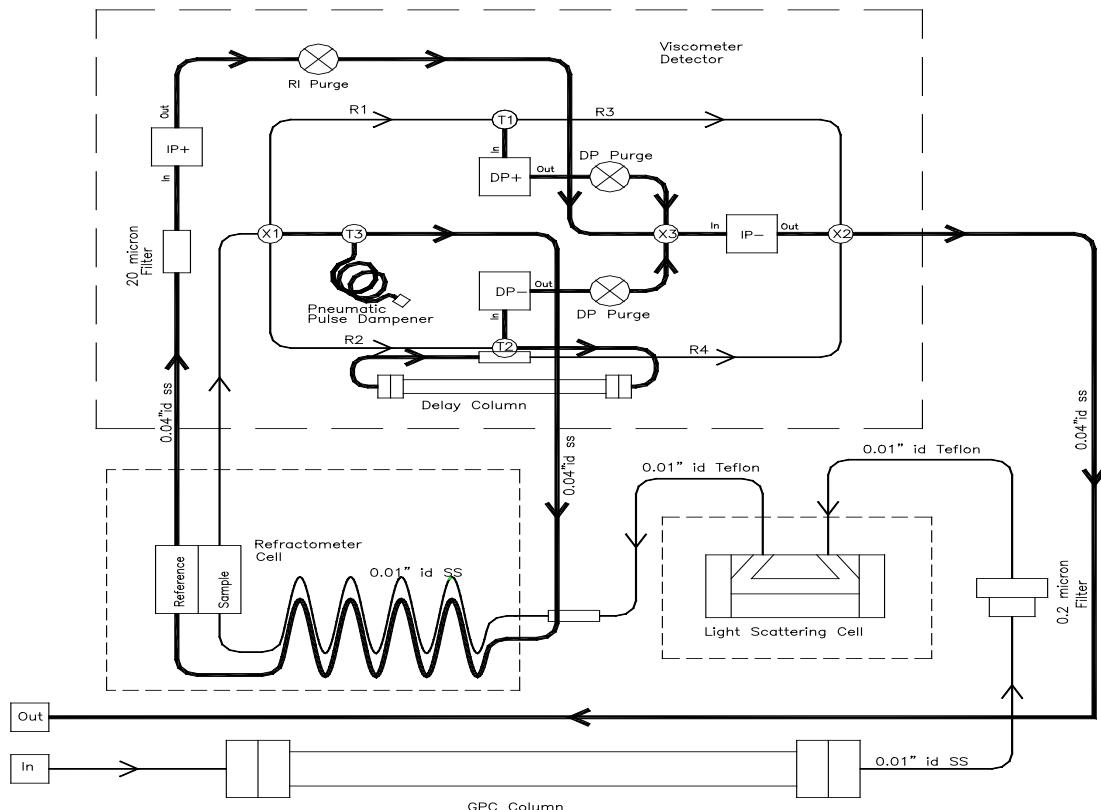


Figure 1: Viscometer, RI and LS Plumbing Diagram

### Refractometer plumbing and flow path

Figure 1 includes a schematic of the flow through the refractometer. Note that the diameter of the tubing is not the same throughout the refractometer. The tubing from the inlet through to the sample side of the cell is 0.01 "id; all other tubing is 0.04" id, from the bottom of the reference cell and out to the RI Purge solenoid. Although the pressure drop also depends on

length, the strong dependence on bore radius or diameter means that the wide bore tubes shown have small resistance compared to the narrow bore tubes. Only with this relationship clearly in mind can one understand the flow patterns described below.

The reference cell of the refractometer is filled with mobile phase solvent by opening the RI purge valve. Solvent flow is then diverted from the Viscometer to the Reference side of the RI flow cell. Solvent then passes through a 20 micron filter, the positive Inlet Pressure transducer cavity and Cross 3. It then flows through the negative Inlet Pressure transducer cavity, Cross 2 and finally exits to waste via the Outlet bulkhead port fitting.

## Viscometer plumbing and flow path

Figure 1 includes a schematic of the flow through the viscometer. Poiseulle's Law of flow through tubes requires that the pressure drop across any tube is inversely proportional to the diameter raised to the fourth power.

$$[A.1-6] \quad P \propto \frac{8LQ}{\pi R^4} \quad \text{where } L = \text{length}, Q = \text{flowrate}, \text{ and } R = \text{radius}$$

Note that some of the tubing shown in figure 1 is small bore (shown with fine line width) and other tubing is wide bore (shown with thick line width). The wide bore tubing is 0.040" diameter and the fine bore tubing is 0.010" diameter. Although the pressure drop also depends on length, the strong dependence on bore radius or diameter means that the wide bore tubes shown have negligible resistance compared to the narrow bore tubes. Only with this relationship clearly in mind can one understand the flow patterns described below.

The viscometer differential pressure transducers, having positive and negative cavities, are purged using electronic solenoids from the front panel of the instrument. These purge procedures must be carried out independently to properly redirect the flow through the transducer cavities and remove air bubbles and purge the cavities with new mobile phase solvent. Normal flow is directed into the viscometer and split at a tee connecting capillaries  $R_1$  and  $R_2$ . When the DP purge ports are closed, the flow is directed only through the four capillary bridge  $R_1 - R_4$ . The  $IP^+$  cavity is in series before the bridge and is purged by opening the Refractometer Purge valve. The  $IP^-$  cavity is in series after the bridge and is continuously purged by the viscometer waste flow.

## Light scattering plumbing and flow path

Figure 1 includes the flow path through the light scattering detector. Flow enters the light scattering cell from the post-column filter or the UV cell and then exits to the refractometer detector of the 305TDA. A laser beam is focused on the end of the cell and scattered light is measured at 90 degrees and at 7 degrees.

## Front Panel

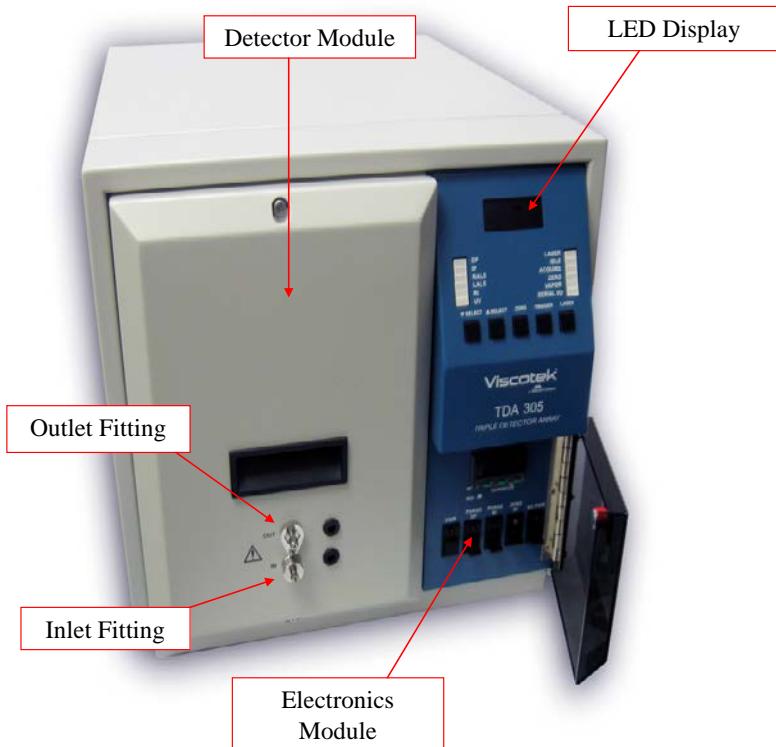


Figure 2: Front Panel of the Model 305 TDA

The LED display shows the voltage of the detector signals (figure 2), which are indicated as DP, IP, RALS, LALS, RI or UV by the appropriate light on the left hand side of the electronics module. The values shown on the meter are in units of millivolts, which matches the analogue output voltages available on the back panel. For the DP and IP signals, the electronics automatically scale the millivolt readings to Pa and KPa. The LS reading corresponds to mV from the photodiode amplifier of the light scattering detector and the refractometer signal corresponds to the mV output of the refractometer. Full scale signal ranges are established in the software package for each channel between 500mV, 1000mV, and 2500mV FS.

- In the centre of the electronics module is a set of five push-buttons: ↓SELECT, ↑SELECT, ZERO, TRIGGER, and lastly LASER:
- The SELECT buttons allow the user to select which of the detector signals is displayed on the LED display meter. You can cycle up or down through the display using the appropriate button.
- The ZERO button is an electronic zeroing adjustment control for offsetting each detector. It is only recommended to use this with the IP transducer signal. The IP signal is read by the computer as an absolute pressure, **so it is critical that the IP signal be zeroed only when the flow is off**. Use the SELECT button to highlight the IP signal and press the zero button to electronically zero the detector.
- The TRIGGER button can be used to start an acquisition with a manual injector. Remember to connect a contact closure signal cable output to the VAC terminal strip if using an auto-sampler.
- The LASER button controls power to light scattering detector laser diode. The laser turns on automatically when data collection is initiated by the software. It is recommended that the laser is switched on for 30 min before acquisition to allow it to stabilise. Pressing the LASER button will deactivate and reactivate the laser.

On the right is another bank of lights, which indicate operation status: LASER, IDLE, ACQUIRE, ZERO, VAPOR and SERIAL I/O:

- The LASER light indicates whether or not the power to the laser diode is on. It is recommended that this switch be left ON at all times since a warm-up period is required for the LASER source to become stable. NOTE: The laser operates in a constant optical power output mode. This is not a pulsed laser unit.

- When the IDLE indicator light is illuminated, the instrument is ready to communicate to the host computer. This light also serves as an indicator light for the self diagnostic routine during the power up routine. At unit power up, this LED is off until self diagnosis is completed. Should the unit fail self diagnosis, the LED will not be properly set.

**Note:** A failure may be indicated by either none or several of the LEDs being lit.

- When the ACQUIRE indicator light is illuminated, the instrument is in the process of acquiring data. This LED will automatically turn off after each runtime cycle.
- The ZERO indicator light is only illuminated when IP is selected and the ZERO button is pushed. As stated above, this should not be performed with other detectors
- The VAPOR light will only illuminate when the vapor sensor is activated by a leak in the detector module. This will also shut off the solvent delivery system if a shut off cable is attached to the terminal strip on the back of the detector. This is a contact closure sent to the pump if a leak occurs. **The connector is polarity sensitive! Pin #1 must be connected to the pump stop and pin #2 connects to pump ground.**
- When the SERIAL I/O light is on serial data is being transmitted to the computer.

Below the logo on the lower portion of the electronics module is a dark plastic cover that encloses several switches and a push-button control panel used for operating the Model 305 TDA. They include: MAIN POWER button, RI PURGE and DP PURGE, RI ZERO, as well as a DC POWER switch.

- TEMPERATURE – This is the temperature controller for the oven compartment. Programming is dependent upon the push button control panel fitted:

#### 3 button control panel

To program, press and hold the far left hand button. The temperature and the setting will begin to flash. Set the temperature with the two right hand buttons. One button will take the temperature set point up; the other will take it down. Once you have the correct set point, release the left hand button. You will see a small LED dot in the upper left corner of the display begin to flash if you have increased the set point above the current temperature. Once equilibrated, the LED dot will go on and off indicating the temperature is cycling.

#### 4 button control panel

The temperature set point is held in Set Point 1 (SP1). Press MENU once and the display will show 'SP1' then press ENTER. The current temperature setting will be displayed with the least significant digit flashing. Press UP or DOWN to change the setting. Holding the button down for three seconds will cause the next higher digit to start flashing and increment or decrement. To save the value, press ENTER then press DOWN to reset the controller.

- The PWR push-button turns the AC power On/Off. This switch will disable all electronic components including the heater circuit. This switch must always be ON during data acquisition.
- The PURGE RI switch is used to control an electronic valve that is used to purge the reference side of the RI cell with fresh solvent. Normal pump flow-rate is required for a period of approximately 5 minutes (or until a stable reading is achieved) to adequately purge the RI. Additionally, the positive signal side of the Inlet Pressure transducer is purged simultaneously. This switch must be in the OFF position for data acquisition.
- The PURGE DP switch is used to control a pair of electronic valves that are used to purge both cavities of the Differential Pressure transducer. Again, normal flow is required for approximately 5 minutes (or until a stable reading is achieved) to adequately purge the viscometer.
- The ZERO RI switch is used to adjust the optical alignment of the Refractometer signal during the initial setup and upon solvent change-over. To make adjustments hold the switch either up or push down depending upon the algebraic sign of the signal displayed. **Note Once the zero has been obtained, do not change or re-zero the signal while running samples**
- The DC PWR switch controls the electronics within the TDA apart from the oven. This is effectively used to turn the instrument electronics on and off. It should be used when removing the detector module or when there is a requirement to re-boot the electronics. Use of this switch does not affect temperature control.

Located to the left hand side of the electronics module is the Oven Compartment that contains space for columns and the Model 305 TDA detector module. This compartment may be removed from its housing, after switching off the DC PWR, by loosening the

two panel screws located at the center top and bottom of the compartment front panel. A handle is provided in the front and rear of the drawer as an aid in removal.



**Note:** No locking mechanism is provided for sliding the detector drawer out of the oven chamber. **Also, HOT surfaces can be encountered upon removal.**

On the outside and to the bottom of the oven compartment are the sample inlet and outlet ports. These are used to connect the Model 305 TDA to the GPC/SEC system.

## Back Panel

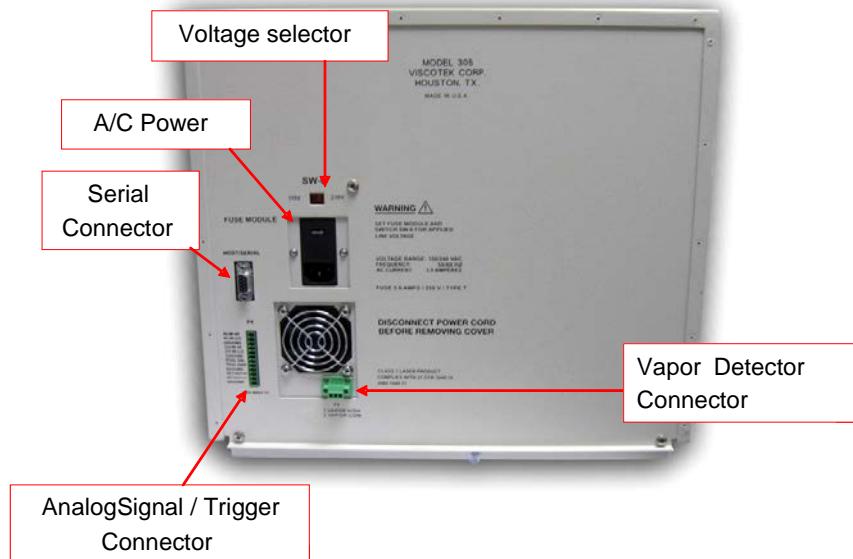


Figure 3: Back Panel of the Model 305 TDA

The back panel, shown in figure 3, contains the fused A/C power module. This module contains the receptacle, a fuse holder and a voltage selector switch. The voltage is selectable for either 110 or 220 VAC. The main power on/off push-button is wired directly to the AC power supply.

The back panel also contains the analogue signal input/output connector and the auto-sampler trigger input. This includes inputs for an external UV detector, and a RI detector as well as an output for one detector signal. Connections to the barrier strip may be made by using a small flat-head screwdriver supplied with the instrument. The maximum level of signal inputs is 2.5 VDC. **The device may be damaged by voltages exceeding 12VDC**

A nine-pin serial connection port is located on the rear of the instrument. This communication socket allows the instrument to be connected directly to a PC through the standard RS232 serial port. The nine-pin connector cable may be oriented in one direction only.

The back panel also contains the vapor detector connector. A two wire connector can be connected here and to the back of the solvent delivery unit to the pump stop or shut down terminal. When the vapor sensor is activated by a leak, the detector will send out a contact closure signal to the pump to shut off flow to the system. **The connector is polarity sensitive! Pin #1 must connect to the pump stop and pin #2 must connect to pump ground.**

## Components

Figure 4 shows a picture of the inside of the detector module containing the light scattering and RI detectors and the UV cell.

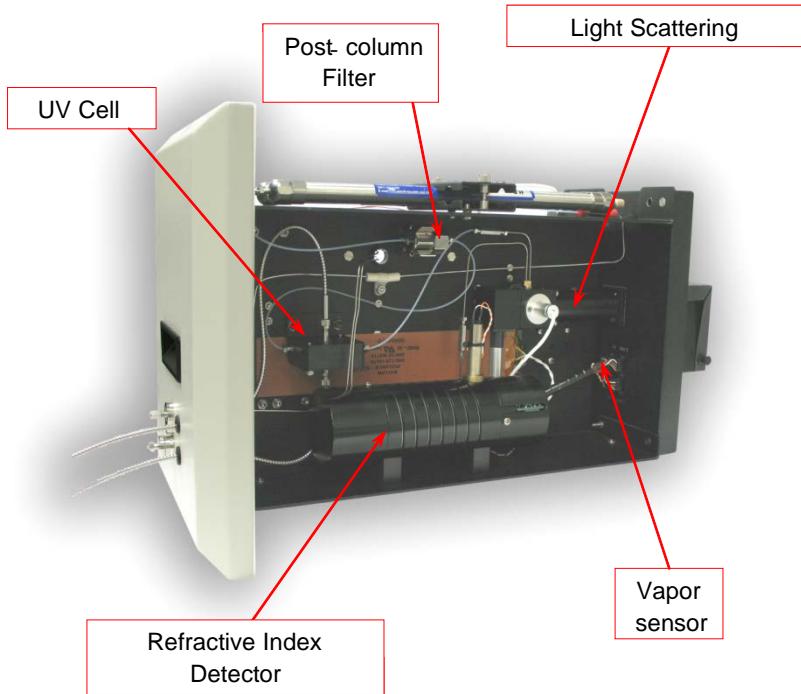


Figure 4: Vapour sensor, Light Scattering and Refractive index detectors

## Refractometer (RI)

The RI detector is a conventional dual cell, deflection design shown in figure 5. Diffuse light from the source is directed onto the mask behind the dual cell, passes through the mask, cell, slit arrangement, and is then reflected by the adjustable concave mirror. The concave mirror focuses and aims the beam back through the cell onto the mirrored prism. The prism splits the beam into two component beams, each component hitting a separate photodiode detector. The photodiodes generate an electric current proportional to the light received. These electric currents are then subtracted from each other and the amplified difference forms the RI signal output.

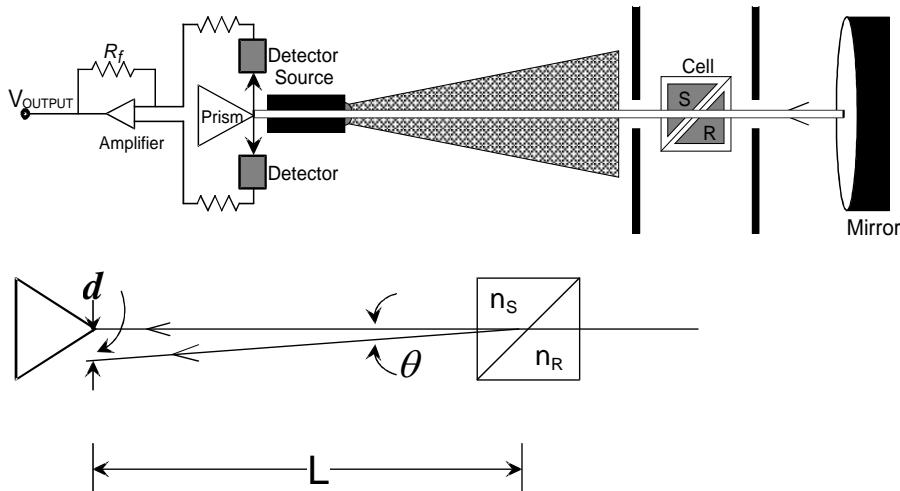


Figure 5: Optical Schematic of RI detector

When both sides of the cell (sample and reference) contain liquid of identical refractive indices, no deflection of the beam is observed. This would be the expected condition at chromatographic baseline, for example. The concave mirror is then aligned so

that near zero signal is output. When the sample elutes into the sample side of the dual cell, the light beam is refracted (deflected) according to Snell's Law of Refraction:

$$[A.3-1] \quad \frac{n_s}{n_r} = \frac{\sin \theta_r}{\sin \theta_s}$$

where  $\theta$  is the angle of incidence with respect to the normal of the plane separating the two liquids, and r and s refer to the reference and sample liquids, respectively.

The RI signal output can be shown to be proportional to the difference in refractive index of the sample and reference liquids.

$$[A.3-2] \quad \frac{n_s}{n_r} = \frac{\sin 45}{\sin(45 - \theta)} \approx \frac{\sin 45}{\sin 45 - \sin \theta}$$

(For small angular deflections of  $\theta$ )

Here it is assumed that the RI cell is a 45° cell, which is the usual case. We then rearrange to:

$$[A.3-3] \quad \frac{n_s - n_r}{n_s} = 1.414 \sin \theta$$

However,  $\sin \theta \approx \frac{d}{L}$  and  $V_{\text{output}} \propto d = L \sin \theta$ .

Inserting and rearranging, we find

$$[A.3-4] \quad \frac{n_s - n_r}{n_s} = k' V_{\text{output}} \quad \text{where } k' \text{ is an instrument constant.}$$

In the usual application, the reference liquid is the solvent for the sample, so that we may equally state that the signal is proportional to the sample concentration and the refractive index increment ( $dn/dc$ ) of the sample.

$$[A.3-5] \quad V_{\text{output}} = k C \frac{dn}{dc}$$

The constant  $k$  is an instrument sensitivity constant (calibration constant) for the refractometer. Because the refractometer is not designed to be an absolute refractometer, but a detector, the only way to determine the instrument constant is by calibration with a standard solution of known concentration and  $dn/dc$  value. This procedure is accomplished through the use of the Viscotek OmniSEC software only when using the universal or triple detection modules. Once the instrument constant is determined by calibration, it should remain constant for an indefinite period of time unless the solvent is changed, some optical re-alignment is made, or the cell is discolored due to residue accumulating on its surface.

The sample equilibration tubing is wrapped in a helical groove on the outside diameter of the RI cylinder block, figure 4. The RI detector is fully contained within the RI block assembly, as noted previously. It consists of the aluminium block and its two end caps, one covering the source/detector end and the other cap covering the cell/mirror end (the front end)

The reference cell can be purged under normal flow conditions by switching ON the RI purge valve. Continue solvent flow for several minutes or until a stable RI signal is obtained.

The flow path during purge condition can be followed from the flow diagram in figure 1. Flow is always first through the sample cell, then through a Tee to the top of the reference cell, through the purge valve, then out to the viscometer.

## RI Specification

|   |                    |
|---|--------------------|
| <b>Cell volume (<math>\mu\text{L}</math>)</b> | 12                 |
| <b>Maximum backpressure on cell</b>           | 30 kPa (5 psi)     |
| <b>Maximum Signal (V)</b>                     | 10 (nom.)          |
| <b>Baseline Noise (mV)</b>                    | 1.0 or less        |
| <b>Baseline Noise (RIU)</b>                   | $2 \times 10^{-8}$ |
| <b>Baseline Drift (mV/hr)</b>                 | 5.0                |

## Viscometer

The viscometer detector utilizes the original Viscotek-patented technology of the four-capillary bridge to minimize the influence of flow rate fluctuations and maximize the sensitivity of detection.

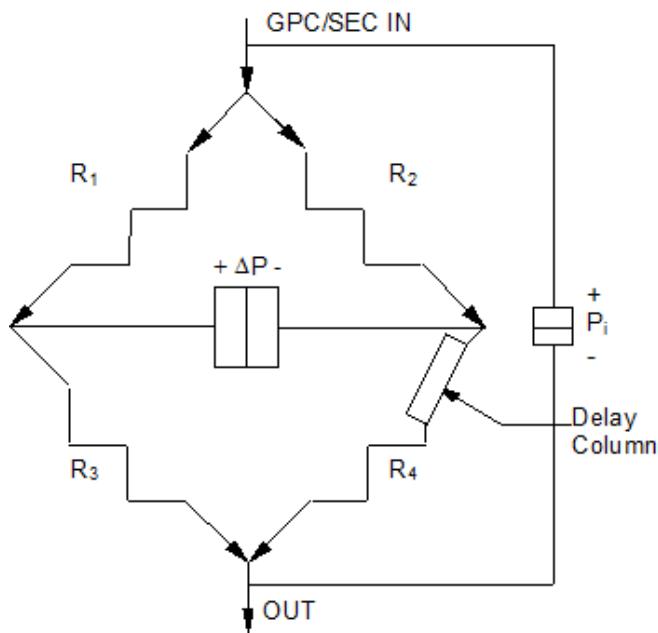


Figure 6: Four-Capillary Bridge

Consider that the four capillaries  $R_1 - R_4$  have equal flow resistances, figure 6. Then when pure solvent of viscosity  $\eta_0$  is flowing in all four capillaries, the differential pressure  $DP = P_2(+) - P_2(-)$  will be zero, or have some near zero baseline value. Then as the polymer solution elutes through the bridge, the viscosity in capillaries  $R_1$ ,  $R_2$ , and  $R_3$  will increase to  $\eta$ , while the pure solvent remains flowing through capillary  $R_4$  due to the delay volume column. Now it is found that  $DP$  is no longer zero, but increases in approximate proportion to the specific viscosity of the solution. The exact equation is shown below.

$$[A.4-1] \quad \eta_{sp} = \frac{4DP}{IP - 2DP}$$

where  $DP$  = Pressure across middle of bridge =  $P_2(+) - P_2(-)$

$IP$  = Inlet pressure through bridge top to bottom =  $P_i(+) - P_i(-)$

$$\eta_{sp} = \text{Specific viscosity of the solution} = \frac{\eta - \eta_0}{\eta_0}$$

$DP$  is typically measured in Pa;  $IP$  is typically three orders of magnitude larger than  $DP$  and is measured in kPa. Thus, Equation [A.4-1] can be approximated as

$$[A.4-2] \quad \eta_{sp} \approx \frac{4DP}{IP}$$

The inlet pressure is more or less constant, being primarily a function of the flow rate of the solvent and its viscosity. The pressure  $DP$  provides the "viscometer chromatogram," as it is nearly proportional to the change in viscosity, *i.e.*, specific viscosity. Note that the  $IP$  will sometimes show a slight change as the sample elutes. This is essentially a "single capillary viscometer" response to the sample. The full power of the bridge to cancel flow rate pulsation and noise is seen in the  $DP$  signal.

### Delay columns

It is clear that the delay column is an important component of the bridge, as it provides the reference solvent to the reference capillary  $R_4$  during the period of elution of the polymer. If the delay volume provided by the delay column is well-matched to the elution profile (chromatogram), the polymer solution will not break through into capillary  $R_4$  until well after the sample has eluted and  $DP$  has returned to baseline, figure 7.

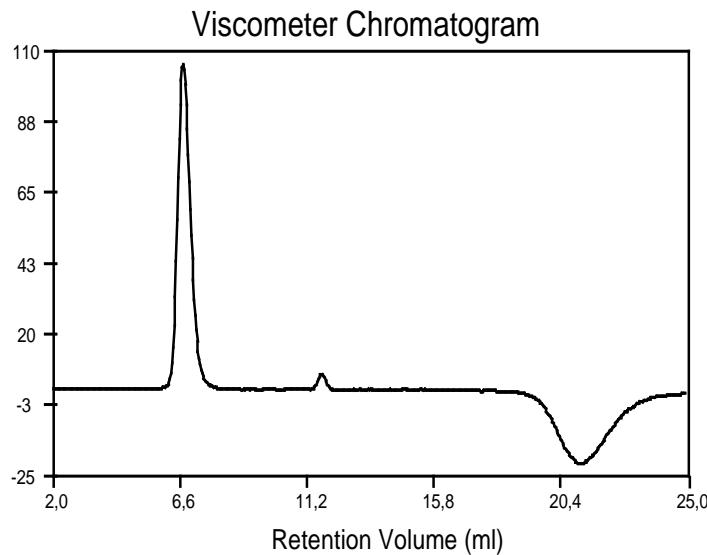


Figure 7: DP Chromatogram Showing Ideal Delay Volume

However, if the delay volume is too small, the breakthrough will occur too early, interfering with the  $DP$  chromatogram, as shown in figure 8, below.

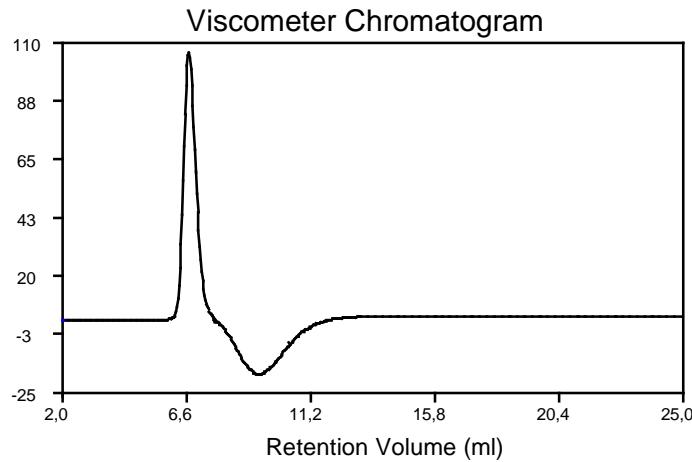


Figure 8: DP Chromatogram Showing Delay Volume Too Small

The third possibility is that the delay volume is too large, a situation that will result in overlap of the breakthrough negative DP peak with the chromatogram of a subsequent injection. This may always be prevented by delaying the start of the next injection, but at a loss in sample throughput.

So it is important that the delay volume be matched to the chromatographic columns being used for the separation. Malvern provides three columns for this purpose: small (CLM9001 for FIPA applications only), medium (CLM9002 for one to two analytical columns) and large (CLM9003 for three to four analytical columns). They are easily changed out whenever the SEC columns are increased or decreased.

### Capillary bridge

Figure 8A shows the viscometer side of the detector module. The four capillaries R<sub>1</sub> - R<sub>4</sub> are wrapped together in a harness and suspended in the center panel module. The inlet tubing is 0.01" id. Most other tubing connections to the bridge (to transducers, purge ports, viscometer exit tubing) are 0.04" id.

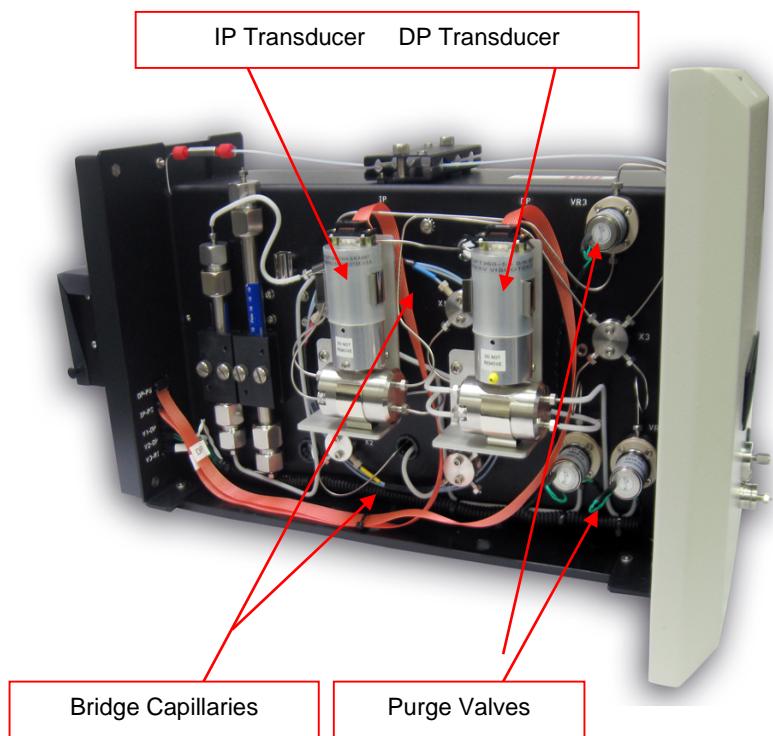


Figure 8A: Viscometer Detector Components

### Pressure transducers

The DP and IP transducers are based on a piezoresistive sensor element. All wetted surfaces of these transducers are made of 316L stainless steel for corrosion resistance. Both pressure transducers are mounted in the detector module's control temperature oven. Connections via 0.04" id ss tubing go to the transducers and to the purge valves. The IP transducer is positioned in series with the RI reference purge and is thus purged with the RI.

### Purge solenoids

The transducers must be purged completely of air bubbles in order to obtain an optimum baseline. This is accomplished by using the purge switches located on the front panel while flow is on and allowing solvent to drain to waste for no more than 15 minutes.

When the DP purge valves are open, the solvent flow is diverted through the DP(+) and DP(-) transducer cavities through the purge valves in preference to flowing through the bridge, due to the high resistance of the bridge. The flow splits more or less equally between the DP(+) and the DP(-) purge ports. One may conclude that air bubbles are purged from the transducers when the

reading on DP becomes steady for several minutes while purging. **Note:** In order to purge the DP transducer, GPC/SEC flow must be turned on.

It is also advisable to purge the transducers after changing mobile phase solvents to make sure that no air is in the system and that all previous solvent is removed.

### Delay column

The delay column is mounted in the Viscometer using the mounting clamp(s) provided. It is connected on its inlet end in the bridge using 0.04" id SS tubing. On its outlet end it is connected to R4 using a length of 0.04" id tubing and a 1/16" union.

Any air bubbles in the delay column will affect the DP signal stability the same as if they were in the DP transducer. Therefore, it is equally essential that the delay column be purged of any air. Normally, the continuing flow of mobile phase will keep the column free of air bubbles. However, if some air is introduced into the system, it will take the equivalent of a chromatographic run to purge them out. During this time, the viscometer signal may be noisy.

### Viscometer signal output

The transducer signals from both the IP and DP transducers are amplified, and scaled. No adjustments of amplification or scale are available to the user. Output on the back panel is fixed according to the following scale:

| Transducer | Output sensitivity | Maximum Output |
|------------|--------------------|----------------|
| DP         | 1.0 mV/Pa          | 2500 Pa        |
| IP         | 10 mV/kPa          | 100 kPa        |

**Note:** The front panel display will over-range at 1999mV with a full scale voltage setting of 2.5V. The instrument will over-range at 500mV and 1000mV, as well if those full scale gain settings are chosen.

The IP signal is an absolute value and therefore *must* be precisely zeroed *with the flow off*. Routine checking of the IP zero is a necessary practice. To zero the IP signal, turn the GPC flow off, push the select button until the IP channel is selected. Push the zero button.

### Viscometer specifications

|                                      |                  |
|--------------------------------------|------------------|
| Capillary Dimensions                 | 0.01" id x 24" L |
| Bridge Balance                       | 1 %              |
| Maximum Flow Rate (H <sub>2</sub> O) | 1.5 mL/min       |
| Maximum Flow Rate (THF)              | 3 mL/min         |
| DP Noise Maximum*                    | 1.0 mV (1.0Pa)   |
| DP Noise Typical*                    |                  |
| DP Drift Typical**                   | 1.5 mV/hr        |

## Light scattering detector

The Light Scattering detector is composed of a cell block and Laser diode mount. Figure 9 is an expanded view of the cell block. It is contained inside the heated oven compartment. It contains both the 90° and the 7° detectors housed in separate optical blocks.

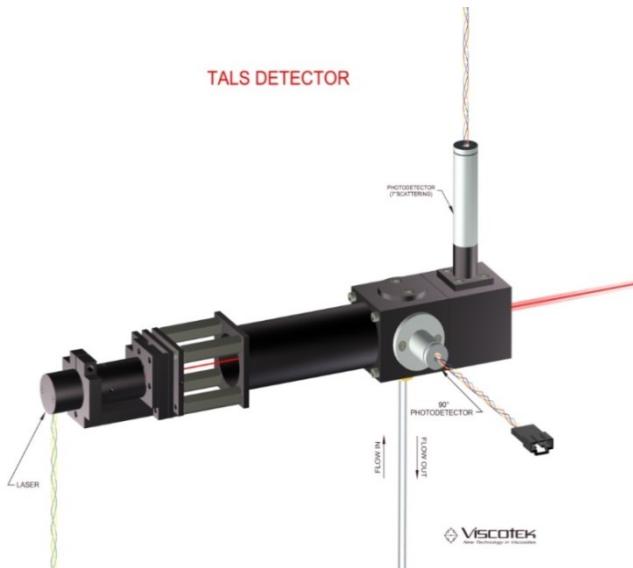


Figure 9: Light scattering detector assembly

## Light scattering detector specifications

|                        |   |
|------------------------|---|
| <b>Measuring Angle</b> | 90° and 7°                                |
| <b>Light Source</b>    | Constant optical power output laser diode |
| <b>Wavelength</b>      | 670 nm                                    |
| <b>Output Power</b>    | 3 mW                                      |
| <b>Detectors</b>       | Photodiode/Amplifier                      |
| <b>Cell Volume</b>     | 18 µL                                     |

## Vapor sensor

Refer to figure 4 for the location of the vapor sensor in the Model 305 TDA. The sensor is mounted in the rear of the 305 TDA module. The 4-lead sensor plugs into a socket for easy replacement. Note that the socket has a notch cut for the sensor key alignment. Hence, the socket's notch should be aligned with the sensor's orientation tab. Should a solvent leak occur in the 305 TDA module, the chemical sensor's output signal will increase and exceed a set threshold level. This will cause the vapor bar LED on the front panel to flash and it turn activate a solid-state relay. This relay will provide a contact closure to shut down the external solvent pump.

The vapor sensor can be adjusted more or less sensitive depending on the solvent used. A level detector circuit (U21) monitors the chemical sensors output signal. The detection level is determined by potentiometer VR5 on the CPU board. The factory setting for VR5 is 4.0 VDC. Clockwise rotation of VR5 increases the sensitivity of the detector. The voltage setting of VR5 can be measured across C1 on the CPU board, which is located directly below VR5. It should be noted that the chemical sensor has a built in heater, which requires a warm-up period of approximately 5 minutes. During the initial power-up state, the vapor detector output signal will be higher than its operating steady state level. Should a false alarm be detected, turn VR5 counter-clockwise to decrease the sensitivity of the detector.

# THEORY

## GPC/SEC separation

The principle of GPC/SEC is the separation of molecules based on their hydrodynamic radius ( $R_h$ ) or volume ( $V_h$ ), not molecular weight. The separation process takes place in a column which is packed with porous, microparticulate material of typically 5 to 20  $\mu\text{m}$  such as styrenedivinylbenzene gel, silica gel etc.

Because of their size, the larger molecules are excluded from some of the pores in the packing material and therefore elute faster through the column than the smaller molecules. In effect, the molecules are separated by size, with the largest eluting first and the smallest last.

The GPC/SEC process starts with a continuous flow of the mobile phase through the system by means of a solvent delivery device, most commonly an isocratic pump. An in-line solvent degasser is employed to eliminate any dissolved gases in the line so that signal instability and noise are reduced. The sample is injected into the system either manually or by an autosampler.

The sample solution is then carried through the column(s) where the size separation process takes place. When the sample elutes from the column(s) it passes through a detector or series of detectors and the output is analyzed by a GPC/SEC software package on the computer.

The extent of the data analysis depends on the type and number of detectors used in the experiment. Depending on the choice of detectors, various types of calibrations and/or calculations are employed to compute parameters like molecular weight (MW), molecular weight distribution (MWD), intrinsic viscosity ( $\eta$ ) or molecular density, hydrodynamic radius ( $R_h$ ), and radius of gyration ( $R_g$ ). It is also possible to obtain additional information on macromolecular structure, conformation, aggregation, branching and copolymer/conjugate composition.

## Molecular weight

The primary goal of GPC as a technique is normally the measurement of molecular weight. While a single molecule may have a defined molecular weight, a population of molecules will include a range of molecular weights and these must be described in some way.

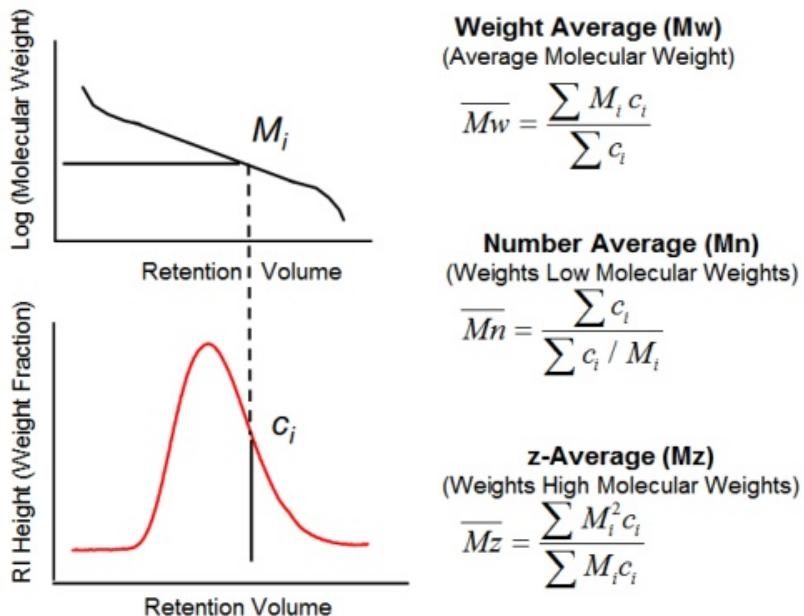


Figure 10: Molecular weight moments and distribution

The most common way of doing this is by using a molecular weight distribution that describes the molecular weight of the whole sample by displaying the fraction of sample that has a given molecular weight. The molecular weight distribution is therefore a graphical representation of the range of sample molecular weight within the sample. The mean molecular weight of the sample will be somewhere within this distribution and the width of the distribution is termed 'polydispersity'. The narrower the distribution, the smaller the molecular weight range, and the lower the polydispersity.

In order to describe the whole distribution numerically, three values are used from the distribution in order to describe it. These are the molecular weight moments  $M_n$ ,  $M_w$ , and  $M_z$  (number-, weight- and Z-average molecular weight). These are calculated according to the equations shown in figure 10, where  $M_i$  is the molecular weight at the  $i^{\text{th}}$  data point, and  $C_i$  is the concentration (or weight-fraction) at the  $i^{\text{th}}$  data point. Dividing  $M_w$  by  $M_n$  gives a value of the polydispersity.

The molecular weight distribution can therefore be derived from any experiment where the molecular weight at each point on the chromatogram is known. This is possible for conventional and universal calibration as well as triple detection but the accuracy of the molecular weight will depend on the technique used, as described in the following sections.

The molecular weights of different samples can therefore be described in this way. While proteins are usually monodisperse in molecular weight with low polydispersity and similar values for the molecular weight moments, polymers are usually characterised by broader distributions and higher polydispersities.

## Conventional Calibration

Conventional Calibration is a widely used GPC technique that employs a single concentration detector, typically a Refractive Index (RI) Detector or UV/VIS to obtain a relative molecular weight.

We have seen from the separation theory that in GPC/SEC molecules are separated according to their hydrodynamic volume and then characterized by a detector or series of detectors as they elute from the column. In Conventional Calibration, a relative molecular weight (MW) and molecular weight distribution (MWD) are determined from the measured retention volume (RV) by means of a calibration curve (log MW vs. RV), which must be set up with the aid of a number of standards of known molecular weight.

The process of conventional calibration is as follows:

- Inject a series of narrow polymer or protein standards of known molecular weight.
- Measure the retention volume (RV) of the resulting peak apex.
- Construct a calibration curve of Log(MW) vs. RV

**Note:** Retention Volume is measured instead of Time to eliminate dependence on flow rate, and you must select standards that cover the entire Molecular Weight range of the sample to be analyzed.

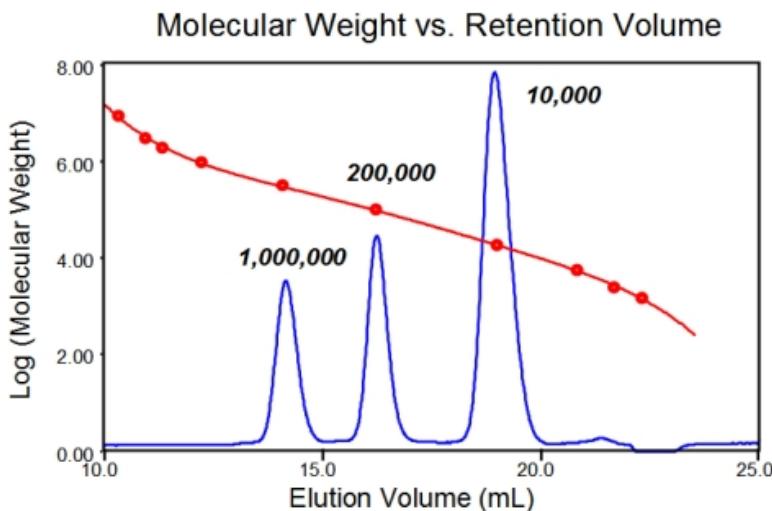


Figure 11: Conventional calibration chromatogram and molecular weight curve

Having gathered this calibration data, we now create a calibration curve to describe the relationship between retention volume and molecular weight; this is usually done by fitting the data to a polynomial:

$$\text{Log } M = A_0 + A_1 V + A_2 V^2 + \dots + A_n V^n$$

Now when the unknown sample is analyzed, it is a simple process to estimate its molecular weight. The measured values are then used to calculate the molecular weight distribution and moments as described in the molecular weight section.

## Universal Calibration

In Conventional Calibration we discovered an inherent problem occurs when we analyze a sample which is chemically different than the standards used to calibrate the column. Unfortunately this is frequently the case, so to improve on Conventional Calibration we use an additional detector, the four-capillary differential viscometer detector, and a technique called Universal Calibration.

As previously mentioned in Conventional Calibration and the separation theory, the principle behind GPC/SEC is that macromolecules are separated based on their hydrodynamic radius or volume. If this is the case, to accurately calibrate a column we need to form a relationship between the hydrodynamic volume and the retention volume (Universal Calibration), as opposed to the molecular weight and retention volume (Conventional Calibration). The viscometer detector makes this possible because it directly measures intrinsic viscosity ( $\text{IV}$ ), which is inversely proportional to the molecular density of the sample.

The product of molecular mass and intrinsic viscosity provides the hydrodynamic volume:

$$\text{MW} \cdot \text{IV} = 5/2 \cdot N_A \cdot V_h$$

Because a viscometer detector coupled with a concentration detector (RI or UV/VIS) can give us a direct measurement of intrinsic viscosity ( $\text{IV}$ ), we can now create a calibration curve of  $\text{Log}(M_w \cdot \text{IV})$  vs. retention volume (RV).

The procedure is as follows:

- Inject a series of narrow standards of known molecular weight.
- Measure the retention volume of the resulting RI peak apex.
- Allow the software to calculate the IV of the peak from the viscometer detector signal.
- Construct a calibration curve of  $\text{Log}(\text{MW} \cdot \text{IV})$  vs. retention volume.

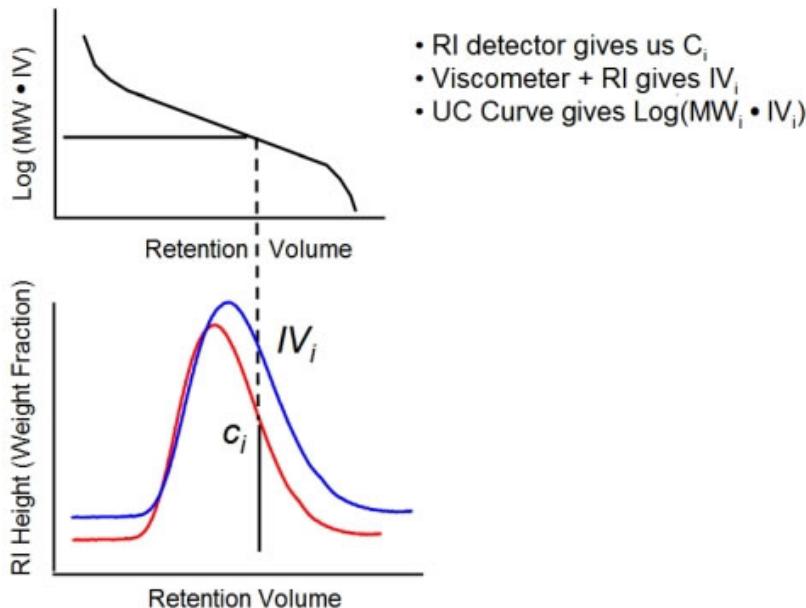


Figure 12: Molecular weight measurement using universal calibration

Now when we analyze our unknown sample, at each data point (RV) we can look up on the calibration curve to find  $Y = \text{Log}(\text{MW}_i \bullet \text{IV}_i)$ . Since we measure the IV<sub>i</sub> from the viscometer we can calculate that  $\text{MW}_i = 10^Y / \text{IV}_i$ .

From here, we can determine the M<sub>n</sub>, M<sub>w</sub> and M<sub>z</sub> averages. In addition to being able to determine true molecular weight distributions (M<sub>n</sub>, M<sub>w</sub> and M<sub>z</sub>), molecular size (R<sub>h</sub> and V<sub>h</sub>) and intrinsic viscosity (IV), the viscometer detector also gives us important structural information on conformation, branching and aggregation. Intrinsic viscosity, which is the inverse density of the polymer coil in solution, represents a direct and sensitive structural parameter and, therefore, is an important parameter in the polymer industry.

The well-known Mark-Houwink relationship:

$$\text{IV} = K \bullet \text{MW}^a$$

can be obtained by means of the double-logarithmic plot of intrinsic viscosity against MW. The Mark-Houwink plot is the central plot used for the analysis of polymer structure. It reflects structural changes in the polymer, such as branching and chain rigidity. The slope, described by the Mark-Houwink exponent "a" can vary between 0 for solid spheres and 2 for rod-shaped structures.

Changes in slope in the Mark-Houwink plot are used to identify when branching occurs.

## Light scattering

Light scattering detection is used to measure the absolute molecular weight of a sample, in that it does not rely on column calibration to relate sample retention volume to molecular weight. Light scattering measures the overall intensity of the scattered light from a molecule which is directly proportional to its molecular weight.

In order to determine molecular weight, the light scattering detector utilizes the Rayleigh equation:

$$\frac{KC}{R_\theta} = \left( \frac{1}{M_w} + 2A_2 C \right) P_\theta$$

where:

$$K = \frac{4\pi^2}{\lambda_o^4 N_A} (n_o \frac{dn}{dc})^2$$

$$P_\theta = 1 + \frac{16\pi^2 n_o^2 R_g^2}{3\lambda_o^2} \sin^2 \left( \frac{\theta}{2} \right)$$

$R_\theta$  = Rayleigh ratio  
 $A_2$  = 2<sup>nd</sup> virial coefficient  
 $\lambda_o$  = laser wavelength  
 $N_A$  = Avogadro's number  
 $n_o$  = Solvent refractive index  
 $dn/dc$  = differential refractive increment  
 $R_g$  = Radius of gyration  
 $\theta$  = Measurement angle

At low concentrations and zero angle, A<sub>2</sub> is very small and P<sub>θ</sub> is close to 1, so the equation is reduced to:

$$R(\theta)|_{\theta \rightarrow 0} \equiv KCM$$

which simply states that R<sub>θ</sub>, the Rayleigh ratio (the ratio of scattered light intensity to incident light intensity), is equal to an optical constant (K) multiplied by the concentration multiplied by the molecular weight.

In order for this to hold true, the intensity of the scattered light must be measured at an angle of 0°. Since such a measurement would require you to look directly into the incident beam, which is not possible, the intensity must be estimated at zero angle from measurements at other angles relative to the incident beam. The TDA has two different light scattering detectors to achieve this:

### Right-angle light scattering (RALS)

Relatively small molecules with a radius of gyration less than approximately 15 nm, scatter light effectively isotropically, or equally in all directions. Such molecules also scatter only a small amount of light making high sensitivity a requirement.

The RALS detector measures the intensity of the light scattered at 90° with respect to the incident beam. At 90°, refraction and flare from material interfaces is minimized which maximizes the sensitivity of the RALS detector. Since scattering in this size range is isotropic it does not matter that we are not measuring away from zero angle as the scattering intensity at all angles will be the same.

The use of the RALS detector therefore maximizes sensitivity for small molecules which scatter only a small amount of light but scatter it isotropically.

## Low-angle light scattering (LALS)

As molecular size increases, the intensity of the light scattered by the molecule starts to vary with angle and this effect becomes increasingly significant with size.

The LALS detector measures the intensity of the scattered light at 7° relative to the incident beam. As can be seen in figure 13, at this angle, the error due to the angular dissymmetry of the scattered light is minimal over the size range studied by GPC. These particular values are true for a sample with  $\text{dn}/\text{dc}$  of 0.185 in an aqueous solution, but the relationship is true for all samples. The assumption is therefore that this error is small enough to be ignored and a measurement at 7° can be treated as a measurement at 0°.

The use of the LALS detector therefore minimizes the angular dependence of the result for all molecular sizes relevant to GPC.

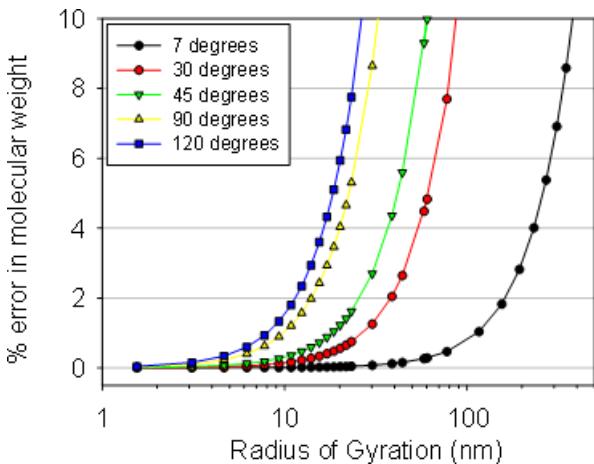


Figure 13: Angular errors in molecular weight measurement

## Triple Detection

Triple detection employs a concentration detector, viscometer and light scattering detector acting in concert, with each detector providing complementary but different information:

- Concentration is measured with an RI or UV, and is necessary for the determination of both molecular weight and intrinsic viscosity.
- The viscometer detector provides a direct measurement of intrinsic viscosity or molecular density, to provide information on structure.
- The light scattering detector provides a direct measurement of absolute molecular weight and eliminates the need for column calibration.

The data from the detectors is combined to provide even more information. As we have seen, the viscometer and light scattering detectors must be used in combination with at least one concentration detector. Combination of the molecular weight and intrinsic viscometer data enables calculation of molecular size. As stated in the universal calibration section:

$$\text{MW} \cdot \text{IV} = 5/2 \cdot N_A \cdot V_h$$

Rearranging this equation leads to:

$$M_w \cdot IV = \frac{10}{3} \cdot \pi \cdot R_h^3 \cdot N_A$$

allowing the calculation of the hydrodynamic size ( $R_h$ ). Since the only limit of this calculation is the sensitivity limit of the detectors, it is possible to measure  $R_h$  down to < 1 nm. This is all done without the need for lengthy column calibration. Only detector calibration is required.

The combination of triple detection therefore allows measurement of concentration, molecular weight, size and intrinsic viscosity from which, information on conformation, branching and aggregation, among others, can all be derived from a single experiment.

## Co-polymer analysis

A common addition to the Triple detector system is the UV/VIS detector which can act as a supplemental concentration detector. This Tetra Detector System yields another level of detail; since we have two concentration detectors, it is possible to determine molecular weights for co-polymers and protein conjugates, in addition to being able to quantify the amounts of the two co-monomers present across the distribution.

This is calculated using a pair of simultaneous equations shown below:

$$\delta RI = ([A] \bullet \frac{dn}{dc} A) + ([B] \bullet \frac{dn}{dc} B)$$

$$\delta UV = ([A] \bullet \frac{dA}{dc} A) + ([B] \bullet \frac{dA}{dc} B)$$

Simply, the signal magnitude at each detector is the sum of the contribution of the two components of the sample. By solving this pair of simultaneous equations, the concentration of each component can be calculated. This is only possible if the dn/dc and the dA/dc for both components is known.

When the composition of the co-polymer is known, the overall dn/dc for the sample can also be calculated as:

$$\frac{dn}{dc}_{\text{complex}} = \left( \frac{1}{1+\delta} \right) \frac{dn}{dc} A + \left( \frac{\delta}{1+\delta} \right) \frac{dn}{dc} B$$

Using the complex dn/dc allows the Rayleigh equation to be solved for the molecular weight of the copolymer ensuring that the correct molecular weight is calculated even when the polymer itself is of a mixed composition.

Furthermore, a UV detector enables us to calculate molecular weight when neither the concentration nor the dn/dc is known. This is often the case when dealing with proteins. With knowledge of the protein's extinction co-efficient it is possible to determine its concentration via the UV detector.

Now that the concentration is known, it's possible to determine the dn/dc from the RI detector, and hence all their parameters are available to determine molecular weight using the light scattering detector.

## Calibration

Multi-detector methods such as universal calibration, triple detection and co-polymer analysis remove the need for column calibration as the sample is measured directly. Nevertheless, it remains necessary to calibrate the system. The purpose of this is to calibrate the detector responses to a sample whose properties are known.

The solution employed by Viscotek systems is to run a single narrow calibration standard with a known concentration, molecular weight and intrinsic viscosity. This allows the detector constants to be calculated which relate the property measured to the magnitude of the signal measured.

The second purpose of the calibration is that it allows delay volume and band broadening calculations to be performed.

The delay volume represents the distance between the detectors that the sample must travel. At a given time, different fractions of the sample will be present in each detector cell. This distance must be calculated and corrected for in order to correctly relate each detector signal to the same sample fraction.

As the sample moves through the chromatography system, it will invariably diffuse out. This is most significant within the detector cells and is a phenomenon known as band broadening. This band broadening affects the shape of the peak and the fraction of the sample that is present in the detector cells at a given moment. In order to accommodate for this, OmniSEC can correct for the band broadening to ensure that the calculated result is as accurate as possible. The peak broadening calculations are critical to good multi-detector GPC/SEC and the calculations used in the OmniSEC software are the result of over 20 years experience of refinement.

Once a narrow standard has been used to calibrate the detector responses and correct for the inter-detector delay volumes and the band broadening, it is common practice to measure a second molecular weight standard. This usually has a broader molecular weight distribution and a higher polydispersity. If the molecular weight of this second standard is correctly measured by the system, then the calibration has been successful and the instrument is performing well enough to move on to unknown sample.

# INSTALLATION

## Installation specifications

|                                |   |
|--------------------------------|---|
| <b>Instrument Model:</b>       | 305 TDA   |
| <b>Dimensions:</b>             | 16" W X 22" D X 15" H   |
| <b>Weight:</b>                 | 33 Kg (73 lbs).   |
| <b>Operating line voltage:</b> | 100 - 240 VAC   |
| <b>Fuse Type:</b>              | 2 – 5.0A 250 V Type T   |
| <b>Line frequency:</b>         | 50/60 Hz  |
| <b>Line current:</b>           | 100 V – 2.0 Amps, 120 V – 2.0 Amps,<br>220 V – 1.0 Amp, 240 V – 1.0 Amp |

## Installation kit for model 305 TDA

| Quantity | Item                            | Quantity | Item  |
|----------|---------------------------------|----------|---|
| 1        | Power Cable Kit                 | 4        | SS Frit - 0.5 micron                              |
| 1        | 12-Pin Phoenix Connector        | 50       | Filter Membranes:                                 |
| 1        | Viscotek Screwdriver            | 25       | 2046 nylon (Aqueous)                              |
| 1        | Allen Head Wrench, 0.050"       | 25       | 2013 PTFE (Organic)                               |
| 1        | 64" SS Tubing, 1/16"x 0.01 "id  | 3        | PS Narrow Calibration Standard)                   |
| 1        | 60" TFE Tubing, 1/16"x 0.01 "id | 3        | PS Broad Calibration Standard                     |
| 1        | 120" TFE Tubing, 1/16"x 0.03"id | 3        | PEO Narrow Calibration Standard                   |
| 10       | Valco Nuts, 1/16"               | 3        | Dextran Broad Calibration Standard                |
| 10       | Valco Ferrules, 1/16"           | 1        | Instrument Manual                                 |
| 3        | Valco Cap, 1/16"                | 2        | 4 M (10 Ft). Analog Signal Cable (1 Pr..Shielded) |
| 2        | Valco Plug, 1/16"               | 1        | 3 M (10 Ft). Trigger Cable (1 Pr.)                |
| 1        | Valco Union, 1/16"x 0.03"id     | 1        | 3 M (10 Ft). Serial Cable                         |
| 1        | RALLS Filter:                   | 1        | 3 M (10 Ft). Diagnostic Cable                     |
| 4        | Teflon O-ring                   |          |   |

## Connection to a SEC system

The Model 305 TDA is designed to be connected directly to the outlet of a properly functioning SEC system by the minimum length possible of 0.01" id SS tubing.

- Check the power module and SW6 on the back and make sure that the voltage set is correct for the AC power source (100/120V or 220V/240V) used. Connect the power cord and turn on the main power switch on. Installation of instrument should provide 1 cm of ventilation space around TDA.
- The detector is normally checked out and shipped with THF solvent. If possible, it is best to use THF for the initial checkout at the site. If another mobile phase is to be used, be certain that it is miscible with THF before proceeding.
- Install the RI Zero motor assembly.
- Connect a waste line to the outlet on the front of the detector.
- Place the waste line in an appropriate waste container.
- Install the detector module into the electronics module. **WARNING!** Front panel DC PWR switch must be "off" before installing detector module.
- If the instrument is a 305 TDA with a LS detector verify that a post column light scattering filter is properly installed below the column mounting platform, figure 14.

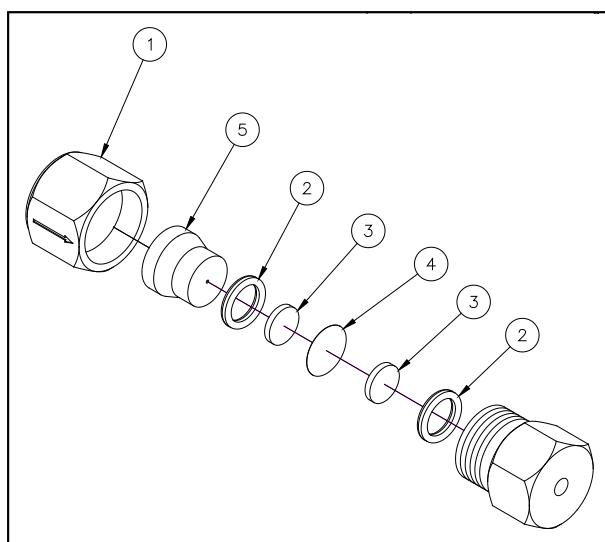


Figure 14: Post-column filter assembly

- Connect the solvent line from the pump to the inlet connector on the front of the detector.
- With the solvent flowing slowly, install the separation columns. Increase the solvent flow to normal flow-rate after at least two column volumes have eluted through the columns.
- Allow the columns to equilibrate for at least 2 to 3 hours.
- Program temperature to working value and allow the detectors to stabilize. This could take a minimum of 4 hours and possibly overnight.
- Purge the Refractometer detector for approximately 5 minutes or until the displayed value becomes stable.
- Purge the Viscometer detector for approximately 5 minutes or until the displayed value becomes stable.
- Turn off the flow; wait several minutes and then zero the Inlet Pressure transducer. Resume normal flow.
- Run a "Quick run" to verify proper detector operation.

- Run a test sample using the PS or PEO narrow calibration standard provided. If using another solvent, use an appropriate narrow standard for that solvent. Use one SEC column only for this test.



**Note:** if the detector is to be moved after installation, either remove the detector module assembly or tighten the module into the electronics and use multiple people as the whole detector heavy (> 30 Kg).

## Installation with other detectors

Before making any plumbing connections with any external detector you should consult with your local Malvern representative or Helpdesk. In general, if an external UV detector is to be used, plumb the detector before the 305 TDA. The same advice is true for connecting the Zetasizer µV DLS detector. Do not connect anything after the 305 that will exceed the backpressure limits. Connect any analog signal cables to the analog signal terminal strip located on the back of the 305 TDA.

### CORROSIVITY OF SOLVENT AND COMPATIBILITY WITH INSTRUMENT

The Model 305 TDA detector array will function with most commonly used solvents with the exception of very strong acids (hydrochloric, sulphuric, etc.). Solvents such as THF, Formic acid, acetic acid, NMP, DMF, DMSO, and water are acceptable for use in the 305 TDA.

It is important that when changing from one type of solvent to another that both solvents be miscible. If they are not, then you will need to use one or more intermediate solvents for change over. Failure to go through a proper intermediate may result in damage to the instrument or unstable baselines for a prolonged period of time.



# OPERATION

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## Purging

### Air bubbles

The presence of air bubbles in any of the detectors will greatly increase the level of baseline noise in the signal. It is imperative that bubbles be kept out of the detectors. There are basically two ways for air bubbles to get into the system:

#### By entry from the outside

This ordinarily happens only when the detector is disconnected and then reconnected. Air may enter by siphoning of solvent from the detector, or from air in the connecting tubing. To minimize this, always immediately plug off the Sample In port when disconnecting the connecting tubing. Then before reconnecting the tubing, be sure to purge the tubing of any air that may have entered.

#### By spontaneous bubble formation in the solvent (out-gassing)

If the solvent is not thoroughly degassed before use, it can occasionally form bubbles spontaneously on the wall of any static cavity. The best way to prevent this from happening is to use only filtered / vacuum degassed mobile phase solvent. For this purpose, an in-line solvent degasser before the pump is most convenient and highly recommended. Air bubble problems seldom occur in systems operating with an in-line degasser. Sparging is not a recommended method for degassing solvent.

Purging is accomplished by operation of the RI and DP Purge valves as previously described.

### Switching mobile phase

The second reason for purging is upon changing mobile phase solvent composition.

Be certain that the new mobile phase is miscible with the old solvent that is in the detector. In most cases, the detector is shipped in THF, which is miscible with virtually all organic solvents and also with water. However, it is not miscible with all aqueous salt solutions. The best practice when going from THF to an aqueous salt solution is to go through an intermediate flush with distilled/deionized water.

To make a change of mobile phase in the columns, disconnect the detector from the system while making the solvent changeover in the columns. Reconnect the detector only after the columns are sufficiently purged and equilibrated with the new mobile phase. The reason for this is that columns frequently discharge residues upon solvent changeover and these residues can sometimes coat the capillary walls of the viscometer bridge and deposit debris on the surface of light walls causing high background and noise.

To replace the columns with another set that is already equilibrated to the new mobile phase solvent:

- Remove the old column set and replace with an in-line filter, if one is not already in place. The in-line filter will prevent particle contamination from the pump entering the detector and possibly plugging up the viscometer capillary tubing.
- Change to the new mobile phase solvent ensuring that the viscometer detector never goes off scale.
- Purge repeatedly until the refractometer and the viscometer baselines are both close to zero and stable.
- Install the new columns and allow time for temperature and flow equilibration.
- Perform a few more purges as necessary to ensure that the baselines are low and stable

For a more detailed explanation see the relevant section in the technical note in the appendix "General Instrument Care". If unsure, contact your local Malvern representative or the helpdesk.

## Zeroing

## IP Zero

The IP zero can be adjusted by selecting the appropriate channel and pushing the zero button on the front panel. When doing this, the instrument must be idle and the flow should be stopped.

**Note:** The IP measurement is treated as an “absolute” measurement by the data system, unlike the RI and DP signals which are baseline corrected. For this reason, the IP must never be zeroed with the flow on.

## LS Zero

It is not ever recommended to zero the light scattering detectors

## RI zero

The RI can be zeroed by first selecting the RI signal with the select button on the front panel, and then by moving the RI zero switch either up or down depending on the algebraic sign of the signal. If the signal is too positive, then the switch is pushed down. Conversely, if the signal is too negative, then the switch is pushed up. Typically, this response is zeroed when the flow rate is on, the detector has been sufficiently purged, and after the temperature has stabilized overnight.

## Viscometer bridge balance

An important operating variable for the viscometer is the balance of the capillary bridge. At the factory, the bridge is balanced to 1% or better. During use, the balance can deteriorate due to partial plugging of one of the capillaries. The balance is determined by measuring the pressures at baseline condition and using the ordinary formula to compute the *apparent* specific viscosity due to the bridge imbalance. As seen earlier:

$$[\text{A.2-1}] \quad \eta_{sp} = \frac{4DP}{IP - 2DP}$$

where  $DP$  = Pressure across middle of bridge  $= P_2(+) - P_2(-)$

$IP$  = Inlet pressure through bridge top to bottom  $= P_i(+) - P_i(-)$

$$\eta_{sp} = \text{Specific viscosity of the solution} = \frac{\eta - \eta_0}{\eta_0}$$

## Check balance procedure

The procedure to check the balance is simple:

Turn the flow on and allow to run a few minutes.

Turn the flow off. Wait 2 minutes. Let pressure go completely to zero.

- Zero the IP signal.

Turn the flow back on again.

- Record the initial jump of the DP signal and its settling value (Pa). An initial jump value greater than 150 Pa indicates air is in the system.
- Record the IP value (kPa). If air in the system has been ruled out as the problem, compute the balance as below.

For example, if the inlet pressure IP is 30 kPa and DP reads 40 mV = 40Pa , the apparent specific viscosity with the flow through the balance is easily determined:

$$\eta_{sp} = \frac{4DP}{IP - 2DP} = \frac{4 \times 0.040}{30 - 2(0.040)} = 0.00535$$

**Guideline:** If the balance gets worse than 3% ( $\eta_{sp} > 0.03$  at baseline), contact your local Malvern representative or the helpdesk.

## Baseline noise

The measurement of baseline noise can be done most rigorously using the actual digital values of the signal to compute the RMS or standard deviation about the mean. There may be some applications where this degree of rigor is necessary, but in most chromatography situations it is adequate to estimate the noise graphically as shown in figure 15 below.

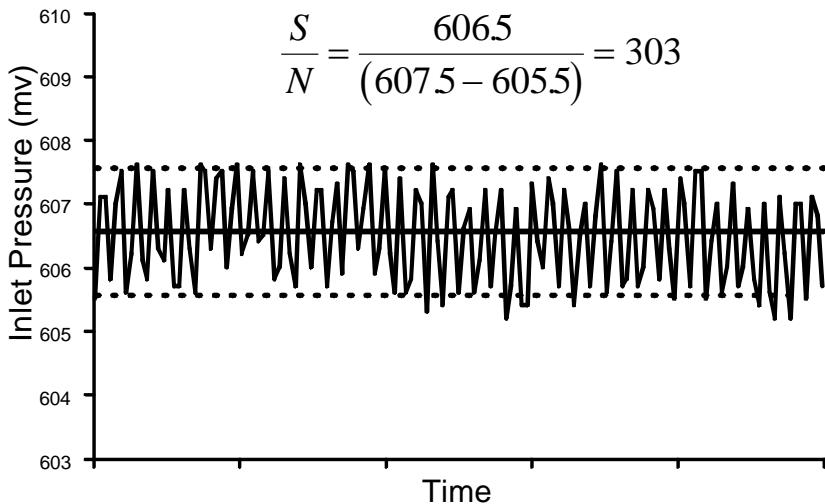


Figure 15: Method of Estimating Baseline Noise

Observe that the noise is estimated not by the peak-to-peak method, which is inappropriate for random noise, but by a visual average of the highest and lowest excursions.

For the signal from the viscometer, the pulsation is usually more rhythmic as it is a direct consequence of pump pulsation. In this case, it is more appropriate to use the peak to peak method as in figure 16:

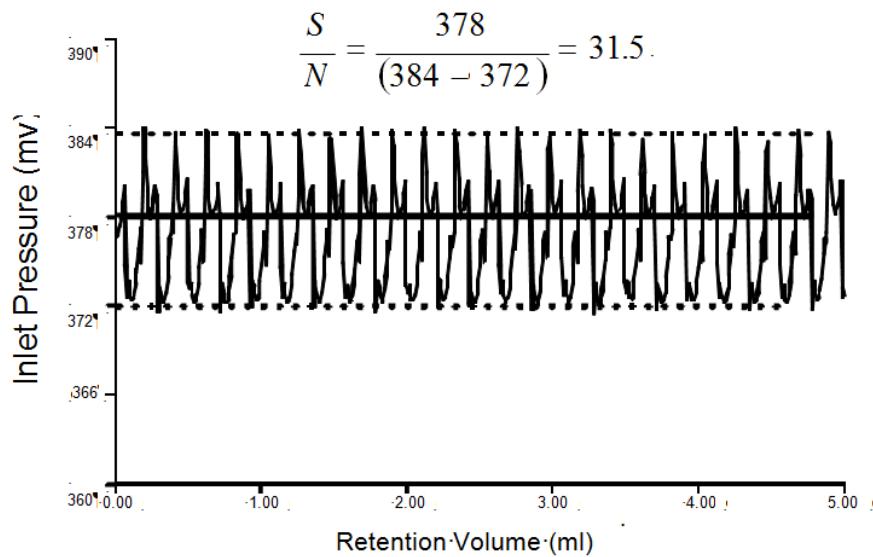


Figure 16: Measurement of Pump Pulsation from the IP Signal/Noise

DP and light scattering baselines should generally contain less than 1 mV noise.

## Light scattering background

From time to time the background scattering should be checked following this procedure: Flush out the detector with pure solvent or monitor the baseline after a reasonable amount of time flushing.

It is most important that the RALS and LALS baselines are stable. Typically the LALS baseline will be slightly higher than the RALS but the actual baseline value for each will vary depending on the solvent used. These values should not change significantly in the same solvent and typically, the LALS baseline should be less than 300 mV in most solvents. Please contact the Malvern Technical Service and Support department for further instructions.

## Standard sample analysis

Periodically it is a good idea to check the performance of the detector by running a polymer standard sample and comparing it to that obtained upon installation. The recommended standard for organic solvent use is one of the narrow MWD polystyrene standards. For aqueous solvent use, one of the narrow MWD polyethylene oxide or polyethylene glycol standards are recommended. Choose a standard somewhere in the molecular weight range of 20K - 100K. Before running the standard, check the baseline performance as described in the previous sections. If the detector baselines are within specifications, run the standard sample and calculate the intrinsic viscosity. Intrinsic viscosities of the standards should follow the Mark-Houwink relationships below.

$$[\eta] = KM^a$$

|                | <i>a</i> | <i>K</i>              |
|----------------|----------|-----------------------|
| <b>PS</b>      | 0.71     | $1.28 \times 10^{-4}$ |
| <b>PEO/PEG</b> | 0.65     | $5.88 \times 10^{-4}$ |

The sensitivity of the RI detector should not change greatly from installation.

The viscometer sensitivity factor can be checked by using a standard material of known intrinsic viscosity. The IV calibration factor should always be within 0.9 to 1.1.

# TROUBLESHOOTING

## Refractometer

### General function

Noise in the refractometer baseline can be characterized as either random, pulsation, or drift type.

### Baseline noise & drift

Random white noise generated by the refractometer is generally the lowest level of the triple detector configuration. Its value should be less than 1.0 mv and often approaches that of the individual bits. The most frequently encountered form of baseline noise in the RI signal is that of drift. The RI is extremely sensitive to compositional changes in the solvent and to thermal variation. Often when mixed solvent systems are used it is necessary to continuously stir the mixture to minimize the drift caused by the continuous change in composition (possibly caused by the more rapid evaporation of one component over another). The Model 305 TDA is temperature controlled to minimize thermal effects encountered in the RI.

Thermal equilibration is normally slow and may require several hours to accomplish. We recommend leaving the oven on for periods of time when the detectors are not being used continuously, but are needed frequently, to minimize start-up time for analysis.

## Viscometer

### DP pulsation

Pulsation noise in the DP baseline is a common problem. In most cases it arises from pump pulsations, but it may possibly come from electronic sources. To eliminate the latter possibility, turn off the pump flow. If the pulsation noise on the DP goes away, electronic sources are clearly eliminated. Assuming it is pump-related, there are three possible causes to consider:

#### Air bubbles in the DP transducer and/or the delay column.

Any air in the DP transducer cavities or in the delay column will amplify normal pump pulsation response to unacceptable levels. A simple purge of the viscometer can alleviate this problem. However, if that does not work, it may be necessary to find out where the air is coming from. Make sure to purge the viscometer prior to doing these tests.

- Shut off flow and remove the pneumatic pulse dampener from its tee, inserting a plug instead. The pulse dampener can be found at the bottom of the viscometer if it has been installed.

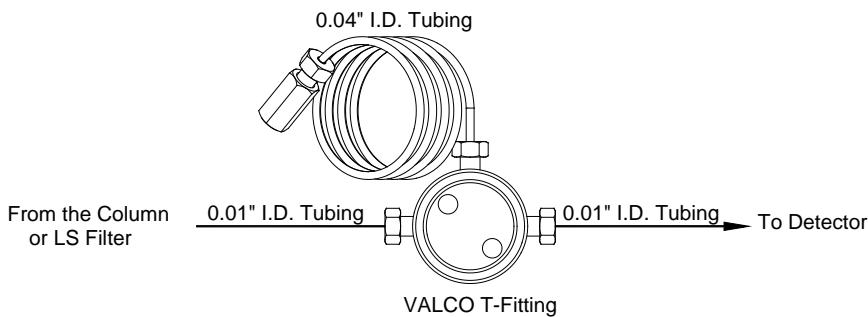


Figure 17: Pneumatic Pulse Dampener

- Restore flow to viscometer and wait until baseline is re-established.
- Open the plug on the pulse dampener tee while observing the DP baseline. If the DP returns to near zero with less than 50 Pa overshoot, there is no air in the DP transducer or in the column. If more than 50 Pa of overshoot is observed, an air bubble

may be present. To eliminate air bubbles, simply open the detector purge ports and allow at least one column volume of solvent to flow through.

- If the baseline still has excessive noise, stop flow, remove and cap the delay column. Use a dead volume union to connect the tubing together. Restore flow and wait for baseline to equilibrate. If noise is gone, then there is air in the delay column. Contact the Malvern Helpdesk for further instructions.

### **Excessive imbalance in the capillary bridge.**

The ability of the bridge to cancel pump pulsations is directly proportional to the degree of balance. Normal balance is better than 1% as calculated in the Viscometer bridge balance section and is unacceptable above 3%. The higher the imbalance, the lower the pump pulsation will need to be. Contact the Malvern Technical Service Department if the balance is out of range.

### **Excessive pump pulsation, as measured by the IP signal/noise in figure 16.**

If this is excessive ( $\text{IP S/N} < 200$ ), check the operation of the pneumatic pulse dampener. Drain the tube with the flow off, recap, and measure IP noise again. Finally check the operation of the pump itself.

### **DP noise**

Random noise in the DP transducer is usually electronic rather than flow related. To confirm that this is the case, simply turn off the pump flow. If the noise continues, it is electronic in nature. Normal random (white) noise as measured in the baseline noise section should be 1.0 mV or less. If this level is exceeded with the flow off, check the DP transducer. To check this as a possible source of noise, stop the flow. If the noise disappears, the transducer is at fault and will need to be replaced. If the noise does not disappear, electronic noise elsewhere in the output board circuit is indicated.

### **DP constant noise**

Constant or rhythmic noise in the DP baseline, such as a sudden decrease in the baseline, or a spike, etc., is usually caused by flow problems such as a faulty pump seal, check valve, or bad column frit, or occasionally by the passage of an air bubble through the system. This can be confirmed by observing the IP baseline overlaid together with the DP baseline. If the IP baseline shows a large change coincident with the change in the DP baseline, some fault in the flow stability is indicated.

## **Light Scattering Detector**

### **General function**

If there is any question as to the instrument function, or if the instrument has been moved around, the response of the instrument should be compared to the results of a known standard. If the signal on either of the light scattering detectors, but more often the LALS, has exceeded the maximum limit, it is likely that the cell has become dirty and will need to be cleaned.

If the light scattering detectors are registering no signal, it may be that the laser is faulty and needs replacing.

### **Baseline noise**

If there is any question about the source of baseline noise, the columns should be removed, the LS filter changed, and the detector should be flushed with pure clean solvent (THF is preferred). Once the fresh solvent has thoroughly flushed out the system, the baseline noise should be less than 1 mV peak to peak on average. If the instrument shows more noise than expected, excluding any occasional fluctuations, the cell or the lenses may need cleaning or replacement. Please contact the Malvern Helpdesk for further instructions.

Any other equipment repair should be done by trained Malvern service personnel. If the equipment is to be returned to depot for service, decontamination procedures must be performed prior to shipment and decontamination documents must be included in shipment. Contact the Malvern Helpdesk for these documents, RMA numbers and for further instructions regarding decontamination.

If during normal operation a spill occurs, use appropriate decontamination procedures. Do not use any cleaning agents that could react with spilled material. If there are questions about the use of cleaning agents on the equipment, contact Malvern helpdesk for further instructions.

# APPENDICES

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Malvern has a wide range of technical and application notes describing the common applications and best practices for using our systems. The three technical notes on the following pages are reproductions from our library that should be useful to help a new user get started. These are:

**Appendix I: General Instrument Care of the Viscotek TDMax GPC/SEC system**

**Appendix II: How to calibrate Viscotek GPC/SEC detectors for synthetic and natural polymer measurements**

**Appendix III: How to calibrate Viscotek GPC/SEC detectors for protein measurements**



# Appendix I:

## General Instrument Care of the Viscotek TDAmax GPC/SEC system

### Introduction

The Viscotek GPC/SEC systems (Fig.1) combine the separating power of chromatography with the analytical power of light scattering detection and viscometry for size and structural characterization of molecules in solution.



Figure 1: The TDAmax system.

Viscotek GPC/SEC systems are used to analyze a wide range of molecules, ranging from polymers to proteins. This versatility means that the systems can be run in many different conditions and solvent setups.

This technical note details information about maintaining Viscotek GPC/SEC systems and aims to help you:

- Maximize your system's reliability.
- Prevent damage to the instrument.
- Reduce unnecessary downtime.
- Recognize rising issues.
- Minimize avoidable service visits.

### Organic Systems

Systems running in organic solvents are generally simple to maintain. The main danger of damage to the instrument occurs during changeover between two organic solvents. This must be performed according to the protocol outlined in Section 4 "**Solvent compatibility and changeover**" which can be found on pages 5 - 7 of this technical note.

Our recommended guidelines for Viscotek GPC/SEC systems running in organic solvents are set out in Table 1.

### Aqueous Systems

The nature of running systems in aqueous buffers means that they are at risk of microbial contamination. If a microbial culture is allowed to grow in a system, it can disrupt the chromatography and can also damage the detectors. Whilst minor contamination can be eliminated by flushing the system with cleaning agents, major contamination will require a service visit.

Recirculating aqueous buffers or stopping the flow without flushing the system with a preservative buffer is not recommended. This action will significantly increase the likelihood of microbial contamination and quickly render the instrument unusable.

Table 2 sets out our recommended guidelines for Viscotek GPC/SEC systems running in aqueous buffers.

|            | Recommended Action  | Reason  | Refer to section...                   |
|------------|---|---|---------------------------------------|
| Instrument | <b>Running mode:</b> When in use, systems should be accelerated from their standby flow rate and allowed to equilibrate for one hour before injection of standards and samples. | Allows baselines to stabilize.  |                                       |
|            | <b>Standby mode:</b> When not in use, leave the system recirculating in pure solvent at a flow rate of 0.1 ml/min.  | Preserves the pump and prevents build up of preservatives or salts within the system.                 | <b>System storage</b>                 |
|            | <b>Shutdown mode:</b> If the system is not going to be used for an extended period then contact your local Malvern office for advice.   |   |                                       |
|            | Use an in-line degasser during GPC/SEC measurements.  | Removes dissolved gases in solvent, which may affect chromatography.                                  |                                       |
|            | Columns must be run and stored according to manufacturer guidelines.  | Prolongs the life of the column.  | <b>Column Exchange</b>                |
| Solvent    | Ensure that there is sufficient solvent in the reservoir for the run duration and stand-by mode.  | Prevents the system drying out.<br>Prevents damage to the pumps.                                      |                                       |
|            | Clean or periodically replace solvent aspirators.   | Ensures good solvent delivery.  |                                       |
|            | Use HPLC grade solvents.  | Minimizes contamination of detectors.   | <b>Solvent and sample preparation</b> |
|            | Filter solvent using a 0.2 µm PTFE or nylon membrane.   | Removes particulates that may cause blockages within the detectors, tubing and cause noisy baselines. |                                       |
|            | Regularly empty the waste and drain bottles.  | Prevent spillages of harmful chemicals.   |                                       |
| Sample     | Follow recommended protocol when changing the system between two different solvents.  | Prevents damage of instrument detectors.  | <b>Special Care Instructions</b>      |
|            | Ensure that samples are fully dissolved.  | Prevents blockage, preserves column and improves data quality.  | <b>Solvent and sample preparation</b> |
|            | Filter samples using a 0.45 or 0.20 µm Teflon (PTFE) membrane.  | Removes undissolved material.<br>Prevents blocking of column.<br>Maintains stable, clean baselines.   |                                       |

**Special Care Instructions: Changing solvents**



If more than one organic solvent is required, the changeover must be performed according to the protocol outlined in this technical note. Refer to the "Solvent compatibility and changeover" section. Also check solvent miscibility guidelines.

**Table 1:** Recommended guidelines for Viscotek GPC/SEC systems running in organic solvents.

|            | Recommended Action  | Reason  | Refer to section...                   |
|------------|---|---|---------------------------------------|
| Instrument | <b>Running mode:</b> When in use, systems should be accelerated from their standby flow rate and allowed to equilibrate for one hour before injection of standards and samples. | Allows baselines to stabilize.  |                                       |
|            | <b>Standby mode:</b> When not in use, leave the system flowing in a preservative buffer at a flow rate of 0.1 ml/min.   | Prevents build up of salts or microbial contaminants within the system.                             | <b>Special Care Instructions</b>      |
|            | <b>Shutdown mode:</b> If the system is not going to be used for an extended period of time then follow the recommended protocol for shutdown.                                   | Preserves the pump and prevents build up contaminants within the system.                            | <b>System storage</b>                 |
|            | Use an in-line degasser during GPC/SEC measurements.  | Removes dissolved gases in solvent.   |                                       |
|            | Columns must be run and stored according to manufacturer guidelines.  | Prolongs the life of the column.  | <b>Column Exchange</b>                |
|            | Change post-column filter weekly or after every sample set.   | Prevents contamination of light scattering detectors. Reduces noise from shedding columns.          | <b>Post-column filter replacement</b> |
| Solvent    | Ensure that there is sufficient solvent in the reservoir for the run duration and stand-by mode.  | Prevents the system drying out.<br>Prevents damage to the pumps.                                    |                                       |
|            | Clean or periodically replace solvent aspirators, empty autosampler drain and waste bottles.  | Ensures good solvent delivery and prevents microbial contamination.                                 |                                       |
|            | Use high quality water for buffer preparation.  | Minimizes contamination of detectors.   |                                       |
|            | Filter solvent using a 0.2 µm nylon membrane.   | Removes particulates that may cause blockages and noisy baselines.                                  | <b>Solvent and sample preparation</b> |
|            | Replace buffer solutions weekly.  | Prevents microbial contamination.   |                                       |
| Sample     | Ensure that samples are fully dissolved.  | Prevents blockage, preserves column and improves data quality.                                      |                                       |
|            | Filter samples using a 0.45 or 0.20 µm nylon or cellulose membrane.   | Removes undissolved material.<br>Prevents blocking of column.<br>Maintains stable, clean baselines. | <b>Solvent and sample preparation</b> |

**Special Care Instructions: Avoiding microbial contamination**



The use of additives such as sodium azide (0.02% w/v), alcohols such as methanol or ethanol (10% v/v), or organics such as acetone or acetonitrile can significantly reduce the risk of microbial contamination. Refer to the "Preventive Cleaning" and "Cleaning a system" sections in this Technical Note.

**Table 2:** Recommended guidelines for Viscotek GPC/SEC systems running in aqueous solvents.

# Solvent and sample preparation

## Solvent preparation

Careful preparation of organic and aqueous solvents can significantly reduce the risk of particulate and microbial contamination.

We recommend that only HPLC grade solvents are used in organic systems. For aqueous systems, HPLC grade bottled water or ultra-pure water from a purification system must be used. It is essential that both organic and aqueous solvents are filtered through a 0.2 µm PTFE or nylon filter.

For aqueous running solvents, we recommend:

- The use of additives such as sodium azide (0.02% w/v), alcohols (methanol or ethanol, 10% v/v) or acetone and acetonitrile. However, these should only be used if they have been shown to be compatible with the separation method and/or analyte.
- That glassware is cleaned with detergent and rinsed with neat alcohol, and left to dry before use.
- That aqueous solvents are kept away from direct sunlight and stored at 4°C, if not immediately used.

## Sample preparation

The most important point to consider with respect to sample preparation is that GPC/SEC is a solution-based technique.

Consequently, good chromatography relies on the sample being fully dissolved. Samples should therefore be given sufficient time to fully dissolve.

Sample solutions must be clear, not cloudy or turbid. We recommend that 0.45µm or 0.2µm filters are used prior to sample injection. Typical filters are PTFE (Teflon) for samples in organic solvents and nylon or cellulose for samples in aqueous buffers.

## Routine maintenance

From time to time, routine maintenance will be required in order to keep the system in the best condition. This should be planned to avoid analysis delays and sufficient time should be set aside.

## Column exchange

Columns should be used and run according to the manufacturer's instructions. To exchange columns, the flow rate should be reduced to 0.1 ml/min or stopped. The columns can then be safely removed from the flow path.

In the TDA, the columns are kept inside the column oven. To access the columns, the DC power should be turned off, the screws on the front of the system loosened and the detector module carefully removed from the chassis.

If new columns require the solvent in them to be changed, the columns should be purged to waste for at least two column volumes before connecting them to the detector. Follow the column manufacturer's instructions regarding the solvent change. It is likely that some material will elute from the columns due to the new solvent. This should always be prevented from entering the detector as it may be a source of contamination.

The new columns are inserted into the flow path carefully to avoid allowing any air into the system. It is worth watching the connections for a couple of minutes to check for leaks. The detector module is then replaced, the screws tightened, and the DC power switched on. When the flow is accelerated, care should be taken to make sure the detectors are stable and do not exceed the measurement range. If any air has been introduced, the detectors signals will be moving randomly. They should be purged and allowed to return to baseline. It may take a few column volumes for the new columns to equilibrate.

Solvent exchange and storage of the column should be performed according to the column manufacturer's instructions.

## Post-column filter

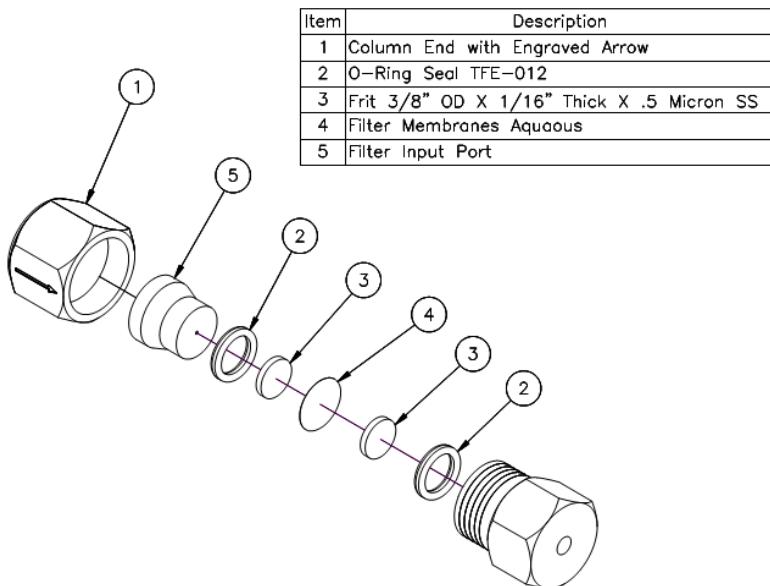
The Viscotek TDA systems incorporate a post-column filter, which is used to collect any particles shed by the column. Although undetected by most conventional detectors, such as UV and RI, these shed particles will scatter significant amounts of light and thus light scattering signals and baselines will be affected.

Replacement of the filter membrane is recommended:

- If the system is running with an increased pressure.

- If a different buffer is to be used.
- Prior to system cleaning.
- After system cleaning.

The post column filter is situated in line between the columns and the first detector. To replace it, the system flow should be reduced to 0.1 ml/min or stopped. The DC power to the TDA should be turned off and the module removed from the chassis. The filter housing is then removed from the flow path.



**Figure 2: Diagram of the components of the post-column filter.**

Within the housing of the post column filter are two frits within two O-rings, sandwiching a membrane filter (Figure 2). It is generally recommended to replace the filter, frits and o-rings all together. (If the supply of frits is limited, they can be re-used but their orientation must be maintained to prevent previously collected material escaping into the detectors). Typical filters are PTFE for organic solvents and nylon or cellulose for aqueous buffers and 0.2µm pore size are used.

Having replaced the filter, the inlet of the filter housing is connected to the flow path. Mobile phase should be allowed to pass right through the housing to ensure that no air enters the system before connecting the filter outlet it to the detectors and replacing the module.

## Preventive Cleaning

Systems running a single organic solvent do not usually require regular cleaning, unless contaminants are introduced via sample injection. Aqueous systems require flushing with a cleaning solution about once a month. Cleaning and flushing a system can take up to a few days to achieve maximum benefits and can take place over a weekend, for example. This helps prevent the build up of most contaminants, but will also alleviate symptoms of minor contamination such as elevated baselines and noise or pump backpressure increases.

## Cleaning an aqueous system

The cleaning process must be treated in the same way as a solvent changeover. Therefore, before carrying out this procedure, please ensure that Section 4: '**Solvent compatibility and changeover**' has been read and fully understood.

### To clean your system:

- Remove columns and store according to manufacturer's instructions.
- Reconnect the flow path using a union.
- Replace the post-column filter.

- Replace the running buffer reservoir with one of the cleaning solution specified below and allow it to be flushed through the system overnight at a rate of 0.1 ml/min.
  - (a) *Sodium Azide solution (0.02% w/v)*. A very effective bactericide.
  - (b) *Methanol: water (10% v/v)*. A useful cleaning buffer, since most aqueous buffers are miscible with this mixture without precipitation of buffer salts. It is however, very important to check miscibility with the type and concentration of buffer beforehand as precipitation can lead to blockages and leaks.
  - (c) *Acetone: water (10:90 v/v)*.
- After flushing overnight, purge both of the detectors several times and perform at least 8 washes of the autosampler. Flushing the system with a few hundred milliliters of cleaning solution should remove any contamination in the majority of cases. Progress can be monitored by collecting data and watching the baseline signals of the detectors. After a reasonable period of cleaning, the detector signals should stabilize (green trace in Fig. 3).
- Prepare fresh running buffer and carry out solvent change; remembering to keep the viscosity detector signals on scale by reducing the flow rate. Or simply exchange solvents at a flow rate of 0.1 ml/min, overnight.
- Purge the RI detector several times, wash the autosampler needle and replace the post- column filter. During this process, the solvent filter and pump filter may also be replaced although this is not always necessary.

## System storage

If the system will not be used for an extended period of time - e.g. over a holiday period - then the following protocol for system shutdown should be followed. This will prevent salt deposition, particulate and microbial contamination, as well as preserving solvent.

### Aqueous Systems

Using pure water, follow Steps 1-5 in the protocol outlined in '**Cleaning an aqueous system**'. Also ensure that the detectors are purged with at least 20 mL of clean solvent and that 8 washes of the autosampler needle are carried out. Repeat these steps with a methanol: water solution (10% v/v). Stop the pump and stopper the inlet and outlet ports located at the front of the TDA chassis. Then switch off the detectors and instrument.

To restart the system, use the protocol outlined in '**Solvent compatibility and changeover**'. The system may require cleaning.

### Organic Systems

Generally organic systems can be left recirculating at 0.1 ml/min in pure solvent - e.g. THF, DMSO - for up to one month in the same solvent. Beyond this time, the risk of solvent oxidation increases. It is recommended that the user change the solvent and continue recycling until such time that the instrument is used for analysis.

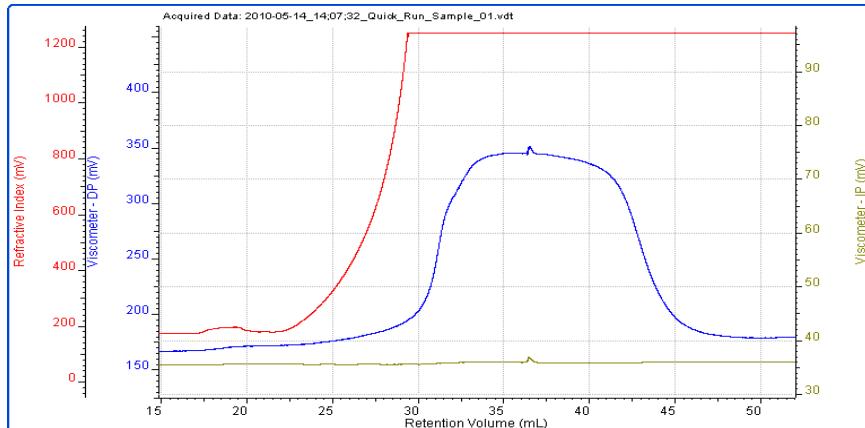
Please contact the local Malvern office for advice about shutdown procedures for organic systems.

## Solvent compatibility and changeover

It may be necessary to use a different solvent dependent on application or during cleaning. Changing solvents requires care and patience to ensure that the changeover is complete and the system is not damaged by the process.

During solvent changeover, the viscometer bridge and pressure transducers are at **significant risk of damage**. The nature of the viscometer setup means that when the new solvent reaches the viscometer, a significant signal response occurs. This signal will persist for as long as the delay column takes to fill with the new solvent. Once this has happened, the detector will return to its baseline level. This can be seen in the blue trace in figure 3. When the new mobile phase reaches the detector at 30 ml the signal increases from 170mV to 350mV. This returns to 170mV when the delay column has filled with the new mobile phase at 42.5ml. The magnitude of the signal shift is dependent on the relative viscosities of the two different solvents.

During solvent changeover, the flow rate **must** be reduced so that the viscometer signals, both IP (inlet pressure) and DP (differential pressure) remain **on scale** at all times. (We recommend that the gain is set to 2.5V in the '*Configure...*' dialogue so that the full detector scale can be seen.) The simplest way to guarantee a successful solvent changeover is to set the flow rate to 0.1 ml/min and allow the solvent exchange to occur overnight.



**Figure 3: Chromatogram showing detector signals from refractive index (red), viscometer DP (blue) and viscometer IP (dark yellow).**

Once the viscometer bridge has rebalanced, the flow rate can be accelerated to the running mode flow rate; but, always ensuring that the viscometer signal stays on scale. The refractive index and viscosity detectors should be purged repeatedly to ensure they too are flushed out.

This should be considered every time the solvent is changed, whether for instrument cleaning or to run the chromatography in a different mobile phase.

## Solvent compatibility

Viscotek systems are compatible with most solvents and buffers.

Aqueous solvents are solutions prepared in pure water and do not contain any organic solvents or alcohols. Aqueous solvents are largely interchangeable since their viscosities are not significantly different.

If a system running in an aqueous solvent is changed for another solvent containing an organic component then the main concerns are salt precipitation at the buffer interface and damage to the viscometer. To minimize salt precipitation, we recommend that the system is flushed with >50mL of pure water prior to exchange with the new solvent. To prevent damage to the viscometer, follow the recommended protocols outlined in the next subsection '**Solvent changeover: Aqueous to Aqueous**' and Steps 2 and 3 in **Organic to Aqueous**'.

Organic solvent exchange is more complicated due to the vastly different viscosities of organic solvents. Generally, it is recommended to use one system for a given organic solvent. If this is not possible, the exchange of the solvents this should be carried out as outlined in the next subsection '**Solvent changeover: Organic to Organic**'.

It is strongly recommend that users check the miscibility of the solvents being interchanged. Common GPC/SEC organic solvents are summarized in Table 3. These solvents are all miscible and may be interchanged according to the described protocol.

| Common solvents       | Running temperature (°C) | Common solvents            | Running temperature (°C) |
|-----------------------|--------------------------|----------------------------|--------------------------|
| Tetrahydrofuran (THF) | 35                       | N-methyl-pyrollidone (NMP) | 50-60                    |
| Dichloromethane (DCM) | 30                       | Dimethylsulphoxide (DMSO)  | 50-60                    |
| Acetone               | 35                       | Dimethylformamide (DMF)    | 60                       |
| Chloroform            | 35                       | Dimethylacetamide (DMAc)   | 60                       |
| Toluene               | 35                       |                            |                          |

**Table 3:** Commonly used organic solvents and their typical running temperatures. All of these solvents are miscible.

## Solvent changeover

### Aqueous to Aqueous

- Remove analytical columns and replace with a union.
- Replace post-column filter.
- Change the solvent reservoir to pure, filtered water and draw 30mL through the degasser.
- Pump at least 50mL of pure, filtered water – no salt or buffer - through the system at flow rate between 0.1 and 1 mLmin<sup>-1</sup>. Keep viscometer signals on scale.
- Purge the RI and DP with 20mL of pure water.
- Change the solvent reservoir to the desired solvent and draw 30mL through the degasser.
- Pump the new solvent through the system between 0.1 - 1.0 mLmin<sup>-1</sup>. Keep viscometer signals on scale.
- Purge the RI and DP with at least 20mL of new solvent.
- Increase the flow rate to the normal running mode and allow the detector baselines to stabilize before injecting standards or samples.

### Aqueous to Organic

- Follow the first **five** steps detailed above in the previous subsection '**Aqueous to Aqueous**'.
- Change the solvent reservoir to the desired organic solvent e.g. 10% methanol solution, acetone, acetonitrile etc.
- Draw 30mL through the degasser.
- Pump the new solvent through the system at flow rates between 0.1 – 1.0 mLmin<sup>-1</sup>, keeping the viscometer signals on scale. This can be done overnight at a flow rate of 0.1 mLmin<sup>-1</sup>.
- Purge both the RI and DP with at least 20mL of the new solvent.
- Increase the flow rate and allow the detector baselines to stabilize before injecting standards or samples.

### Organic to Organic

- Remove analytical columns and replace with a union.
- Replace post-column filter.
- Change the solvent reservoir to acetone and draw 30mL through the degasser.
- Pump at least 120mL acetone through the system at flow rate between 0.1 and 1 mLmin<sup>-1</sup>. Keep viscometer signals on scale. This can be done overnight at a flow rate of 0.1 mLmin<sup>-1</sup>.
- Purge both the RI and DP with at least 20mL acetone.
- Change the solvent reservoir to the desired solvent and draw 30mL through the degasser.
- Pump the new solvent through the system at flow rates between 0.1 – 1.0 mLmin<sup>-1</sup>, keeping the viscometer signals on scale. This can be done overnight at a flow rate of 0.1 mLmin<sup>-1</sup>.
- Purge the RI and DP with at least 20mL of the new solvent.
- Increase the flow rate to the normal running mode and allow the detector baselines to stabilize before injecting standards or samples.

### Organic to Aqueous

It is strongly recommended not to switch one system from running organic to aqueous solvents or vice versa. Although possible, this can lead to extended downtime and more frequent issues than when a system is kept solely in organic or aqueous solvents.

The only exception to this may be during the installation where an instrument has been shipped in THF and is to be run in aqueous mobile phase. If a system is to be switched from THF to water, then the protocol outlined in '**Solvent changeover: Organic to Organic**' must be followed.

## Common symptoms of rising issues

There are a number of symptoms which indicate when some routine maintenance might be required.

### Increasing pump pressure

The most common symptom a system may display is an increase in pump pressure. Over time, one of the filter elements within the system may get blocked.

Within a GPCmax, there is a filter before the injection valve. If the pump shows a pressure above ~ 0.4 MPa /60 psi with just a wide bore waste line attached to the GPCmax outlet at a flow rate of 1 mL/min, then this post- pump filter should be replaced.

The post-column filter will become blocked with particles shed from the column and this will be noticeable in the GPCmax pump pressure. To test this, disconnect and reconnect the filter housing to the flow path. If the pump pressure is above 1 MPa, the filter needs to be replaced. Blockage of the filter can take anywhere from days to months depending on the quality of the column and can also be affected by poor sample or mobile phase quality. If the pressure has increased, the filter should be replaced as described below.

Other issues that may cause an increase in pump pressure can include debris build up in the pump heads and also degraded columns. If none of these improves the situation, it is best to contact a Malvern representative.

### Light Scattering Detector Noise

Another common symptom is an increased level of noise in the light scattering detectors. This is caused by particles of debris passing through the system. Since they are generally larger than the molecules of interest in the sample, they cause spikes in the light scattering detectors and the low angle detector (LALS) is always more sensitive to this than the right angle detector (RAL).

To determine whether or not a system requires cleaning, turn off any digital filtering and de-spiking functions in the software and then monitor the light scattering signal. Software de-spiking and digital filtering algorithms are useful for improving data but can sometimes mask possible issues that, if visible, would indicate a need for system cleaning.

Using a fresh and filtered buffer, collect data for a period of 30 minutes and monitor the baselines of the detector signals.

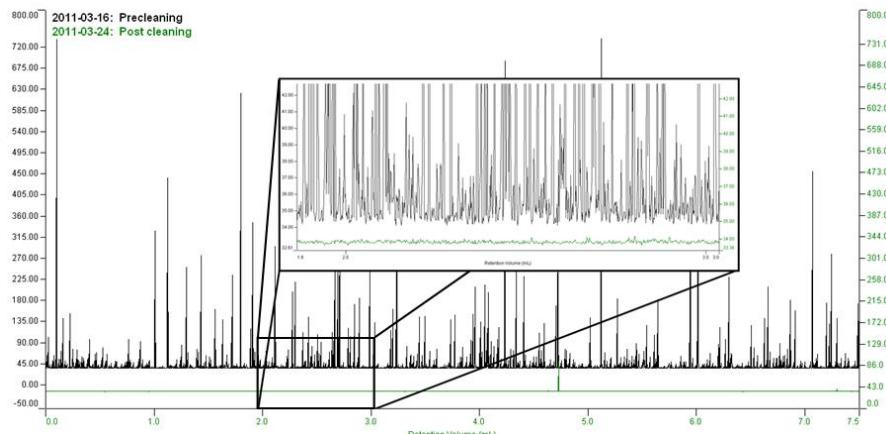


Figure 4: Comparison of RALS signals pre- and post- cleaning. Prior to cleaning (black) the RALS signal contains many spikes from dirt in the system. Post cleaning (green) the signal is much more stable.

Figure 4 shows a comparison of the RALS signal for a PBS run buffer pre- and post-cleaning. As evident in this figure, the pre-cleaning signal, in the absence of any de-spiking or digital filtering, shows a large degree of signal spiking across the entire run. If the baseline light scattering signals resemble this, then the system needs some level of cleaning. After cleaning, the signal spiking is infrequent and the results are consistent with those typical of newly installed systems.

## Elevated light scattering baselines

As well as becoming noisy, the light scattering detector baselines may themselves increase. For instance, a new system would be expected to have RALS and LALS signals beneath 50 mV when running only solvent and depending on the solvent in use. If the light scattering cell becomes contaminated or dirty, these, and particularly the LALS detector baseline may increase. This will eventually reduce the dynamic range of the instrument and if they increase above the upper limit of the detector, will make it unusable. The light scattering cell itself may then require cleaning.

If this happens it is best to contact your local Malvern representative for advice.

## Noise in other detector signals

It is less common for the other detectors to become noisy however it can occur.

Noise or drift in the viscosity detector or the refractive index detector could be due to an air bubble trapped in the system, dripping from the waste line, incomplete solvent exchange or a poorly mixed solvent.

To rectify these, ensure the waste line is not dripping, perform a series of purges of both the RI and the DP, place the mobile phase reservoir on a magnetic stirrer and allow an extended period of equilibration.

## Poor chromatography

If the detector baselines are changing or becoming noisy after sample injection, this may be due to the sample itself. If the peak is not a nice shape and shows significant tailing, most notably in the light scattering detectors, or the descending edge of the peak is noisier than the ascending edge, it is likely that the sample is interacting with the column and is not being separated solely by size, which is a pre-requisite for GPC/SEC. In this situation different mobile phase compositions should be used to try to optimize the chromatography to obtain the best results. It may also be worth using a different type of post-column filter as the interaction may be here.

Poor analytical conditions can also lead to partial deposition of sample material within the detector system. This can affect the light scattering cell, most notably by permanently increased scattering background (the baseline). This can also affect the viscometer delay column. Deposits within the delay column lead to a permanent negative bias of the viscometer DP signal.

If the detector signal returns to its usual baseline after an extended run time, the detector itself is not heavily affected. The most likely consequence of sample/column interaction is poor data and a requirement to clean out the system more frequently.

# Appendix II:

## How to calibrate Viscotek GPC/SEC detectors for synthetic and natural polymer measurements

### Calibration Procedures

Calibrating a Viscotek multi-detector system generates the instrument constants and performs any required offset and band-broadening calculations. For systems making measurements of synthetic polymers such as polystyrene or natural polymers such as polysaccharides, the calibration process is essentially identical. For these systems, calibration is performed using a sample of known molecular weight and with a narrow polydispersity. The calibration is then checked against a second sample with a wider polydispersity before moving on to sample analysis.

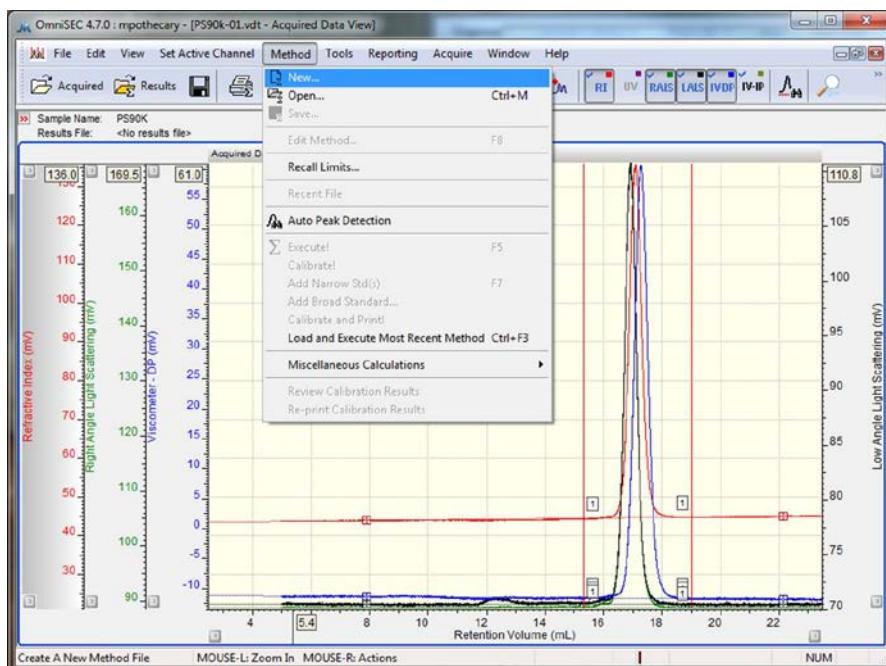


Figure 1: Set limits and baselines around all the peaks in the standard sample and select 'New' in the 'Method' menu.

Carefully creating a method takes just a few minutes and following this protocol will ensure that calculated results are as accurate and reliable as possible. Once the method is created, it takes just seconds to analyze the subsequent samples.

Open the data file to be used as the calibration standard in OmniSEC. If the system is running in organic solvents, this is usually a polystyrene or polymethyl methacrylate. The molecular mass has to match analytical conditions (i.e. column separation range). Furthermore, the molecules have to be isotropic scatterers so their hydrodynamic radius should be  $\leq 10$  nm. Typically a molecular mass of 90 kDa to 100 kDa will be good. If the system is running aqueous buffers this is usually a PEO or a Pullulan. The concentration of the standard sample should have been known and input when the sample was run. The accuracy of this initial concentration is crucial to calculating accurate molecular weights at later stages. Check that all detectors show good signal quality, and then add all of the detectors to the display by selecting the detector buttons (if using an OmniSEC version < 4.7, hold Ctrl while selecting the detectors). Set the baseline and integration limits around the entire sample using the right-click menu, and then select the 'Method – New' option from the main menu (figure 1).

## Setting baselines and limits

Setting baselines and limits can be done by right-clicking on the 'Acquired Data View' and selecting the appropriate options. A quicker way to set limits and baselines is with the shortcut keys. Holding down the 'shift' key and left clicking in the Acquired Data View' will drop a limit at the elution volume corresponding to the current mouse pointer x-position on all of the detector chromatograms. A second left-click at a different location will drop the second limit. Holding down the 'shift' key and right clicking will place baselines on all of the detector chromatograms. If the 'control' key is held down, instead of 'shift', then the limits or baselines will only be placed on the active channel. To remove limits and baselines, select the appropriate option from the right-click menu.

## Calibration procedure

In the Create New Method window, select the 'Blank' option, and then select in the 'Multi-Detectors-Homopolymers' option. The next screen will show the detectors to be calibrated. Select the detectors as appropriate for the system (the concentration detectors is usually the refractive index (RI)) and then click 'Next'. Enter the known parameters for the standard in the 'Standard' window. These should be provided with the standard and those for a PS90k are shown in figure 2. Note that the 2<sup>nd</sup> virial coefficient is not required, however, if the value is known, it can be entered in the appropriate box. If polydispersity is not written on the standard, it can be calculated as  $P_d = M_w/M_n$ . If the UV detector is used, the dA/dc value is equivalent to the extinction coefficient presented as  $A_\lambda = X \text{ ml mg}^{-1} \text{ cm}^{-1}$  (The value at 1% w/v can be used as long as this is consistent with the sample tab).

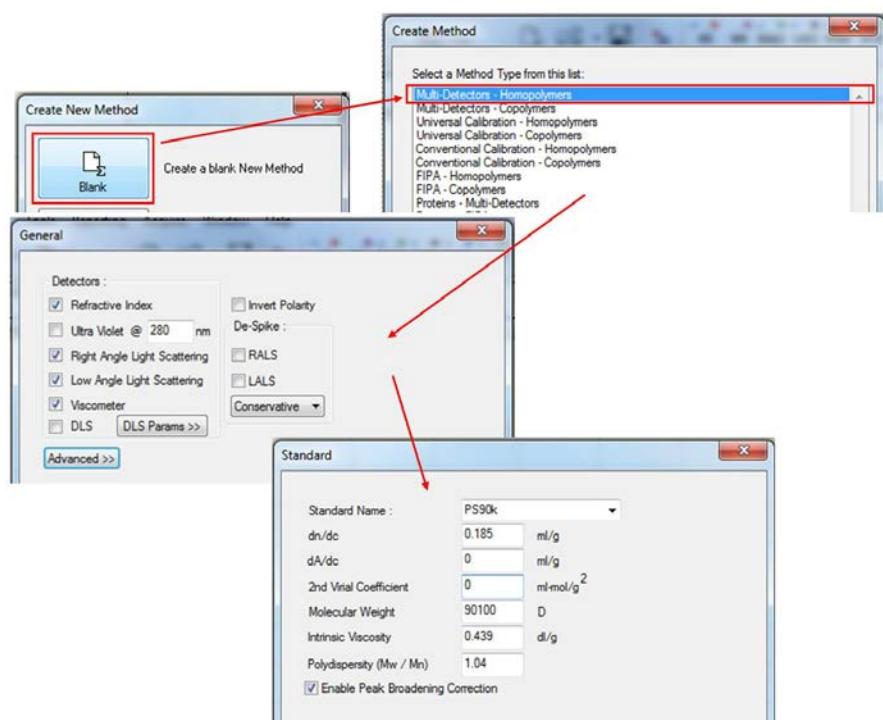


Figure 2: Follow the method wizard through by filling in the appropriate options and clicking 'Next' at each step. The values for a PS90k in THF are shown as an example.

After entering the standard properties, click 'Next', and then fill in the appropriate values in the 'Sample' window (figure 3). If the measured sample is different from the standard, only the refractive index of the solvent needs to be input. (If the dn/dc of the sample is known, it can also be input, in which case, the 'calculate concentration from detector' box must be checked.)

To complete the initial calibration keep the 'peak detection' algorithm set to Manual and then save the calibration with an appropriate name. After saving, the software will ask 'Calibrate the method now?' Click 'Yes' and the calibration results will be displayed.

(If limits have not already been set, a different message will appear – click ‘OK’, set limits and baselines as described earlier and then select ‘Calibrate!’ from the ‘Method’ menu.)

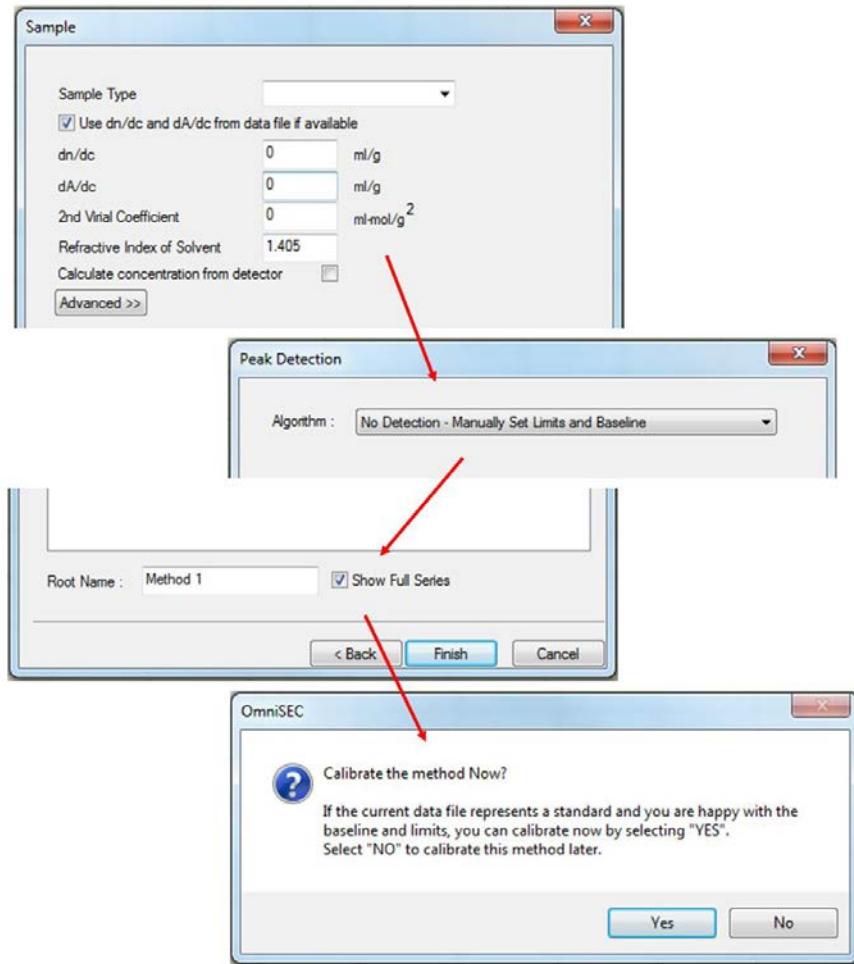
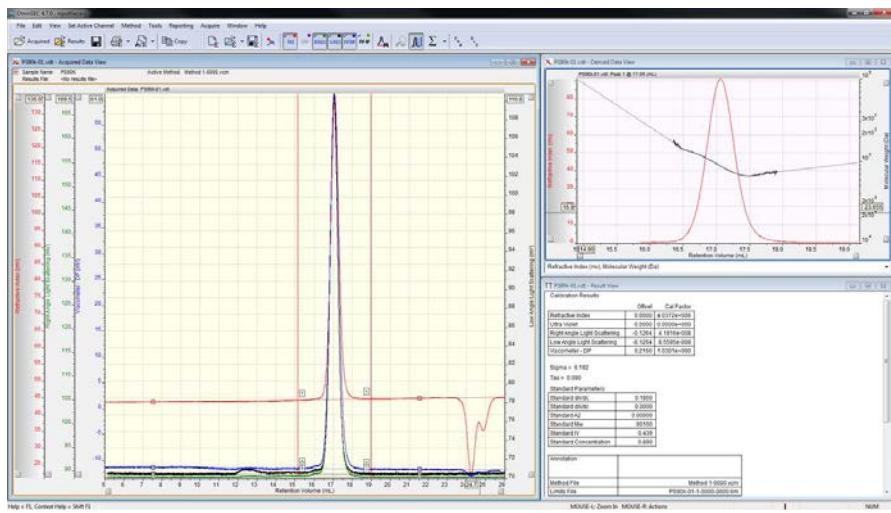


Figure 3: Continue to follow the method wizard through to the end and calibrate the system.

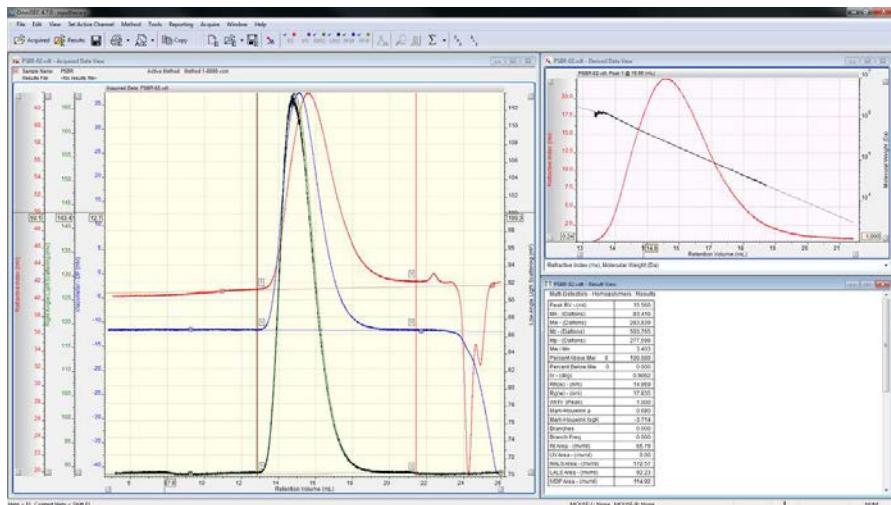
The peaks are aligned and the detector calibration constants reported in the ‘Result View’ (figure 4).



**Figure 4:** After calibration, the calibration results including instrument constants and offsets will be displayed.

The calibration is now effectively complete; however good practice says that it is worth checking the calibration against a different sample whose molecular weight is also known. Correctly measuring the molecular weight of a second sample will give a high level of confidence that the calibration has been successful and the subsequent measurements of unknown samples will give accurate answers.

Open the second standard file or go to it using the 'Close & Next' option in the file menu (or press F12). For organic solvents, this is typically a second polystyrene such as PS280k or a polymethyl methacrylate. For aqueous systems, this is often a dextran such as D70k. Place limits and baselines around the peak as described earlier and then execute using the 'Execute!' button ( $\Sigma$ ) or by pressing F5. The calculated molecular weight should be close to the value stated on the sample. Under suitable chromatographic conditions, the molecular weight trace decreases steadily and monotonously along the chromatogram showing an excellent distribution of the sample as in figure 5. A lack of steadiness in the molecular mass vs. retention volume plot usually indicates an error during calibration such as incorrectly set baselines. A non-monotonous (where the molecular weight increases with retention volume at some point) plot usually indicates unsuitable chromatographic conditions.



**Figure 5:** Typical analysis result for a broad PS280K standard.

The calibration is now complete and the method can be used to analyze other samples in exactly the same way as the second standard. It is best practice to run a standard and calibrate at the beginning of each set of measurements. If calibration every time is not required, then at the very least, a reference standard should be run and analyzed with the existing method to ensure it is working properly.

**Note:** This calibration process is exactly the same for synthetic polymer samples in organic solvents and for natural polymer samples in aqueous buffers. Only the values used in the standard and sample tabs change depending on the specific standards used.

## Background information

It is important to understand "what" is being calibrated during this process. The calibration procedures within the Viscotek OmniSEC software perform two functions.

### Calibration constants -

All detectors generate a voltage output, and in order for the output signal to be correlated with a physical property change, the relationship between the absolute change in the physical property and the change in the detector voltage output must be established. For many GPC detectors, such as refractive index (RI) & ultraviolet spectroscopy (UV) detectors, the relationship between changes in the physical property being measured and the voltage output is linear, with the calibration using a standard of known properties producing a calibration constant. Determination of the calibration constants is the 1<sup>st</sup> function performed during the OmniSEC calibration procedure.

### Offset Volume and band broadening

In multi-detector GPC systems, the signals from multiple detectors are coupled to give the maximum amount of data. However, in order to couple these signals, the elution peaks must be aligned and corrected for band broadening, such that the same elution "slice" is being used in the calculations. The 2<sup>nd</sup> function performed during the OmniSEC calibration is therefore to calculate the offset volume between the GPC detectors and correct for inter-detector band broadening.

There are a variety of GPC detector calibration standards available, either from Malvern or 3<sup>rd</sup> party vendors. When selecting an appropriate calibration standard, there are three things to consider.

- The standard must be suitable for use under the conditions that are to be employed when measuring unknown samples. For example, if the experimental objective is characterization of small molecules and a column set is used with exclusion limit of 50 kDa, a 100 kDa standard is not suitable for calibration. The standard should also be an isotropic scatterer for the conditions applied. This criterium is 'passed' for a hydrodynamic radius of no more than 10 nm. Typically standards of up to 100 kDa mass fulfill this requirement. At the same time, the standard should have a sufficiently high molecular mass in order to produce significant light scattering and viscometer signals for the calibration process.
- All of the injected standard's mass must pass or elute from the column. The voltage response from the concentration detectors is proportional to the sample concentration, and the calibration constant is calculated from the entire standard peak area whose concentration is known if 100% of the sample mass elutes. If the entire injected sample does not elute from the column, an error will be introduced into the calibration constant.
- The calibration standard should have a polydispersity of no more than 1.1. As noted above, the response from the concentration detectors is proportional to the sample concentration. The response from the light scattering (LS) detector however, is a function of both the concentration and the molecular weight. If the molecular weight varies too much across the elution peak, as would be expected for samples with  $M_w/M_n$  significantly greater than 1.1, improper alignment of the LS and RI/UV peaks can lead to error in the off-set volume calibration.



# Appendix III:

## How to calibrate Viscotek GPC/SEC detectors for protein measurements

### Calibration Procedures

Bovine serum albumin (BSA) is a cheap and readily available protein which separates well under most chromatographic conditions. It is therefore an excellent choice for a standard. However, it has a high propensity to form oligomers and these are normally resolved by the chromatography. The instructions below describe how to calibrate a Viscotek SEC system with a sample such as BSA.

Carefully creating a method takes just a few minutes and following this protocol will ensure that calculated results are as accurate and reliable as possible. Once the method is created, it takes just seconds to analyze the subsequent samples.

Open the data file to be used as the standard in OmniSEC. The concentration of the standard sample should have been known and input when the sample was run. The accuracy of this initial concentration is very important. Check that all detectors show good signal quality, and then add all of the detectors to the display by selecting the detector buttons (if using OmniSEC version 4.6, hold Ctrl while selecting the detectors). Set the baseline and integration limits around the entire protein sample using the right-click menu, and then select the 'Method – New' option from the main menu (figure 1).

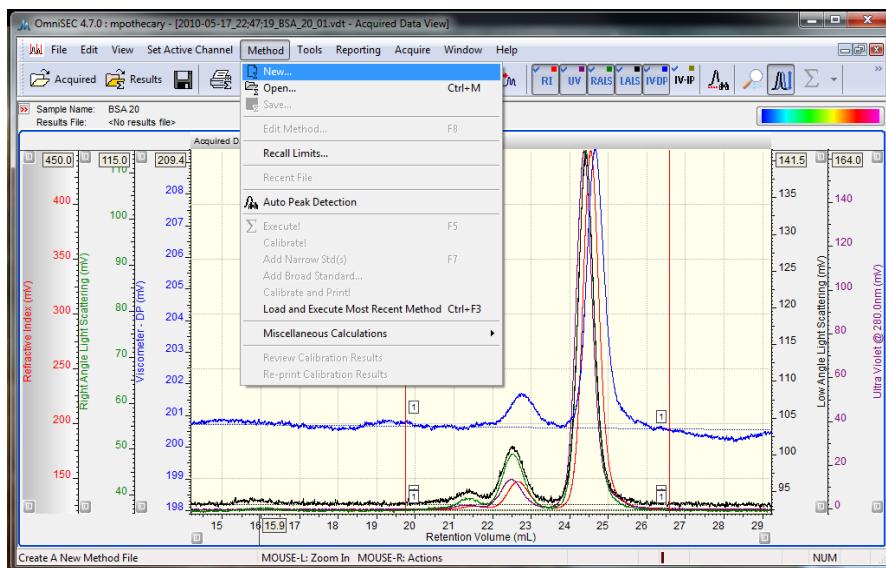


Figure 1: Set limits and baselines around all the peaks in the standard protein sample and select 'New' in the 'Method' menu.

### Setting baselines and limits

Setting baselines and limits can be done by right-clicking on the 'Acquired Data View' and selecting the appropriate options. A quicker way to set limits and baselines is with the shortcut keys. Holding down the 'shift' key and left clicking in the Acquired Data View' will drop a limit at that elution volume on all of the detector chromatograms. A second left-click at a different location will drop the second limit. Holding down the 'shift' key and right clicking will place baselines on all of the detector chromatograms. If the 'control' key is held down, instead of 'shift', then the limits or baselines will only be placed on the active channel. To remove limits and baselines, select the appropriate option from the right-click menu.

## Calibration Procedure

In the 'Create New Method' window, select the 'Blank' option, and then select in the 'Multi-Detectors-Homopolymers' option. The next screen will show the detectors to be calibrated. Select the concentration detector, either refractive index (RI) or ultraviolet (UV), and then click 'Next'. Enter the known parameters for the protein standard in the 'Standard' window. Those for BSA are shown in figure 2. Note, that the 2<sup>nd</sup> virial coefficient and intrinsic viscosity values are not required. However, if the values are known, they can be entered here. If the UV detector is used, the dA/dc value is equivalent to the extinction coefficient presented as  $A_{280} = X \text{ ml mg}^{-1} \text{ cm}^{-1}$  (The value at 1% can be used as long as this is consistent with the sample tab). The molecular weight value that should be entered here is that of the monomeric species (figure 2).

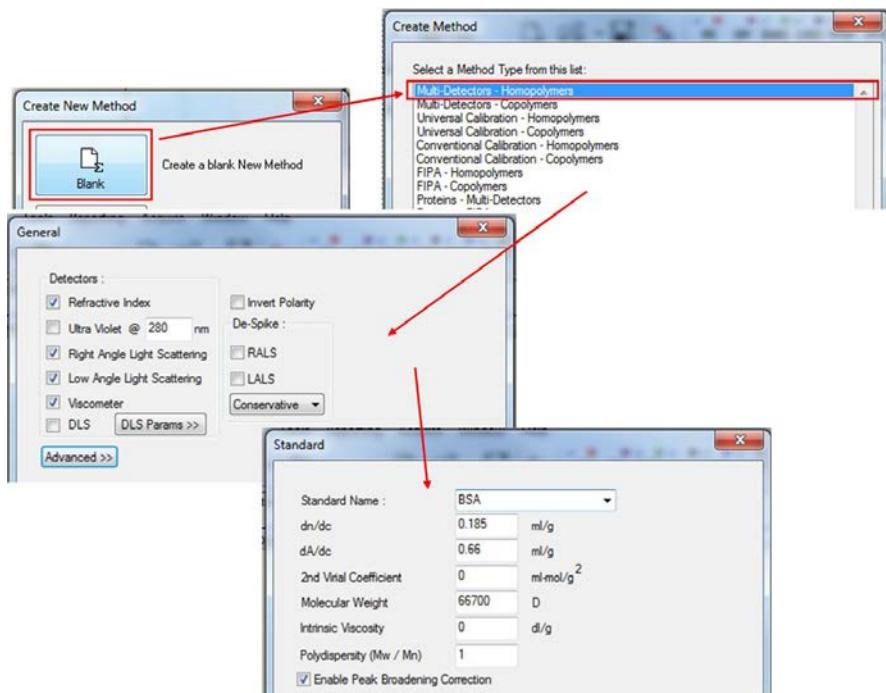
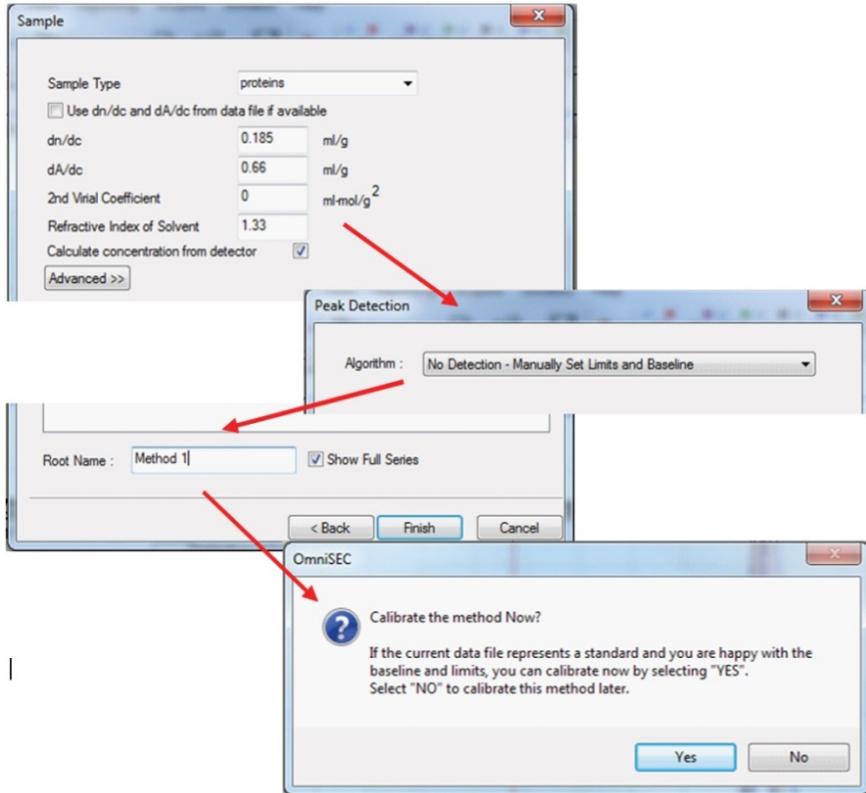


Figure 2: Follow the method wizard by filling in the above options and clicking 'Next' at each step.  
Protein dn/dc is typically constant at 0.185 ml/g.

After entering the standard properties, click 'Next', and then fill in the appropriate values in the 'Sample' window (figure 3).

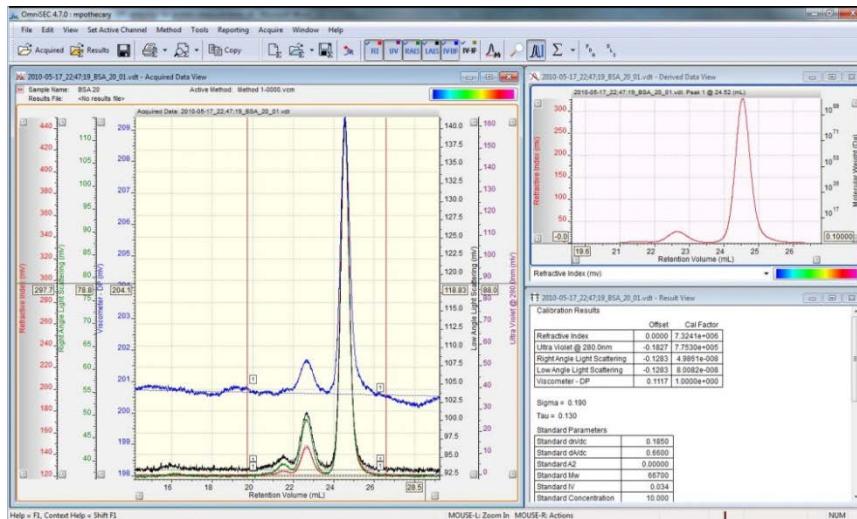


**Figure 3: Continue to follow the measurement wizard through to the end and calibrate the system.**

In the sample window, the box labeled 'Calculate concentration from detector' must be checked. To complete the initial calibration keep the 'peak detection' algorithm set to Manual and then save the calibration with an appropriate name. After saving, the software will ask 'Calibrate the method now?' Click 'Yes' and the calibration results will be displayed.

(If limits have not already been set, a different message will appear – click 'OK', set limits and baselines as described earlier and then select 'Calibrate!' from the 'Method' menu.)

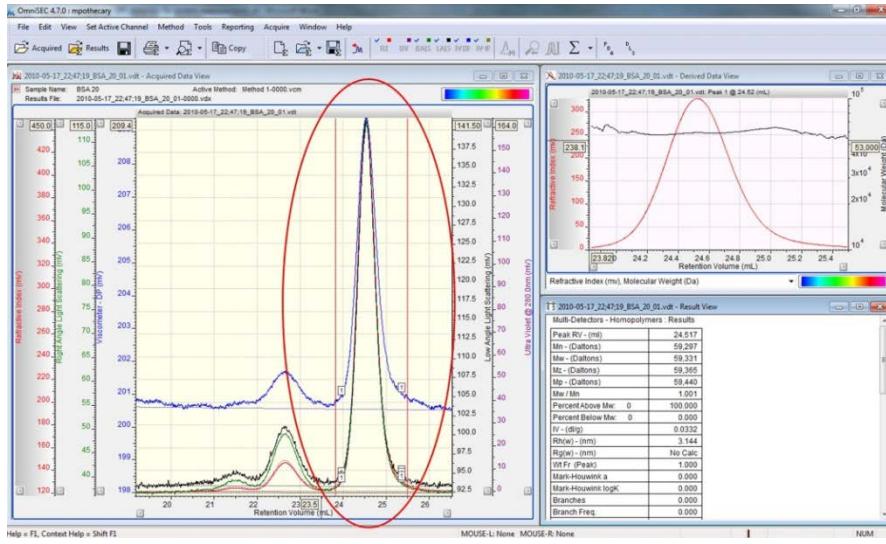
The peaks are aligned and the detector calibration constants reported in the 'Result View' (figure 4).



**Figure 4: After calibration, the calibration results including instrument constants and offsets will be displayed.**

For a single component standard, such as low polydispersity polystyrene, this would complete the detector calibration. However for a multi-component standard, such as BSA, a second stage is required to correct for the fact that the sample has formed some oligomers.

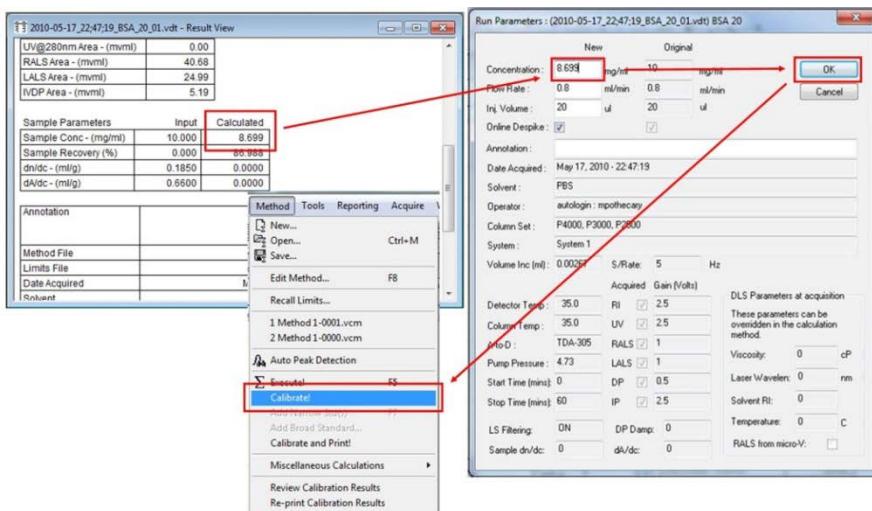
For the next stage, right click the chromatogram and select the 'Clear LIMITS on ALL Channels' or the 'Delete All Limits' option. Zoom in on the monomer peak and set the integration limits around only the monomer peak using the 'Add limits on all Channels' option in the right click menu (figure 5).



**Figure 5:** Delete the original limits then zoom in on the monomer peak and set new limits around just this peak.

Note that the calculated values in the results window represent the sample within the selected limits. As evident in figure 5, the molecular weight results for this 66 kDa monomer are incorrect. This is a consequence of using the monomer molecular weight to represent the weight average molecular weight for the entire sample in the original calibration.

Scroll down in the 'Results View' to the calculated Sample Concentration, press F3 to open the 'Run Parameters' window (or use the File-Run Parameters), and then enter the calculated Sample Concentration in the New Concentration field. Press OK, then recalibrate by selecting the Method-Calibrate option from the Main Menu tool bar.



**Figure 6:** Scroll down in the 'Result View' to the calculated concentration. Copy this value into the new concentration box in the run parameters dialogue (F3). Click ok and re-calibrate.

Once the recalibration is completed, it is always a good idea to review the calibration results. Figure 7 shows the calibration results for the example presented here. The Cal Factors shown are indicative of typical values for detector calibration constants, as are the Sigma and Tau values. Sigma, indicative of band broadening, should be around 0.2, and Tau, indicative of tailing, should be smaller than Sigma.

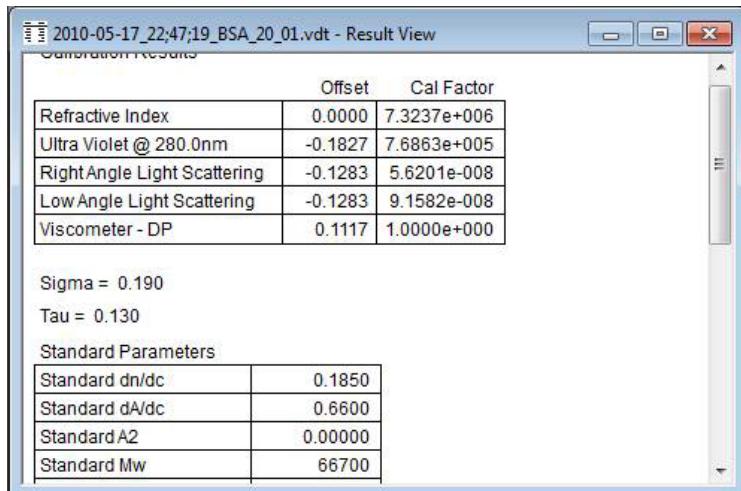


Figure 7: Typical calibration results after the second stage calibration.

As a final check of the calibration results, it is instructive to treat the standard measurement results as an unknown, to see if the calibration yields the expected results. Also, the dimer molecular weight should be accurately calculated by the method.

In the Acquired Data View, add limits around the dimer peak and if the result is not automatically re-calculated, click 'execute' or press F5. The results should now show two columns representing the two peaks. The monomer peak should have a molecular weight very close to that which was input in the standard options (in the case of BSA, 66 kDa) and the dimer peak should have a molecular weight of twice the monomer (133 kDa). Depending on the BSA sample and the resolution, a third peak may be visible representing the trimer and this peak should have a molecular weight of three times the monomer (201 kDa).

The molecular weight traces in the Derived Data View should be plateaus indicating that each peak is monodisperse (to view all peaks together, right click in the 'Derived Data View' and select 'All Peaks Mode' or 'Peak by Peak Mode'). This is the equivalent of the Mw/Mn value in the results table. This value should be close to 1 and is typically less than 1.05. For a mixture of protein oligomers, this is the type of result that one would expect, with each peak showing a constant molecular weight.

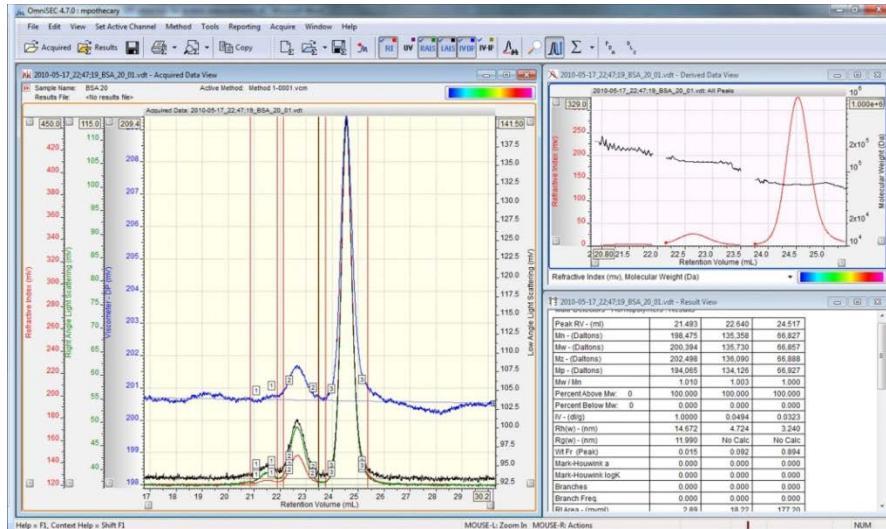
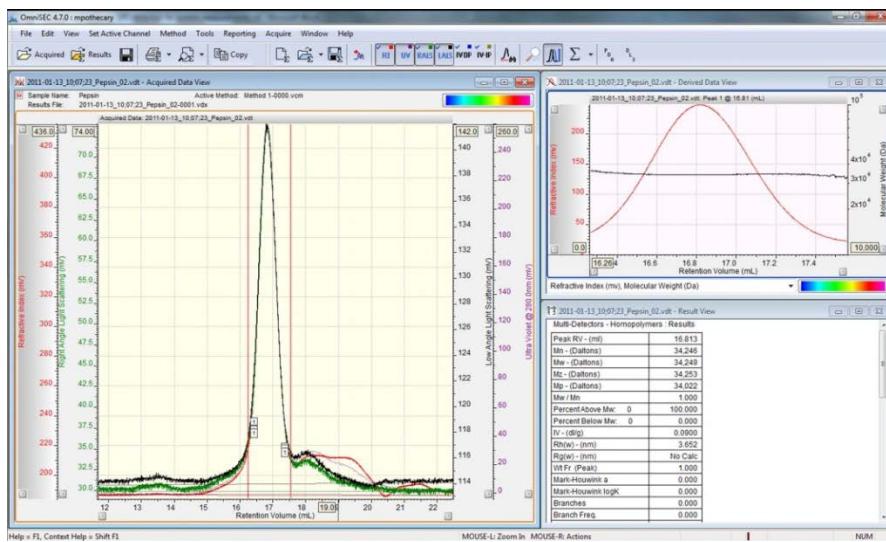


Figure 8: After the second calibration, the molecular weights of all of the peaks should be correctly calculated.

The calibration is now complete and the method can be used to analyze other protein samples. It is best practice to run a standard and calibrate at the beginning of each set of measurements. If calibration every time is not required, then at the very least, a standard should be run and analyzed with the existing method to ensure it is working properly.

To analyze the next sample in the sequence, press F12 to move to the next file or open the sample of choice using the 'File – Open' menu. The file to be analyzed must be in the same folder as the standard. Set limits and baselines around the peak and click 'Execute' or press F5. The sample will be immediately analyzed using the method created.



**Figure 9: Set limits and baselines around the peak and click 'Execute' or press F5 to run the method.**

## Background information

It is important to understand "what" is being calibrated during this process. The calibration procedures within the Viscotek OmniSEC software perform two functions.

### Calibration constants

All detectors generate a voltage output, and in order for the output signal to be correlated with a physical property change, the mathematical relationship between the absolute change in the physical property and the change in the detector voltage output must be established. For many GPC detectors, such as refractive index (RI) & ultraviolet spectroscopy (UV) detectors, the relationship between changes in the physical property being measured and the voltage output is linear, with the calibration using a standard of "known" properties producing a calibration constant. Determination of the calibration constants is the 1<sup>st</sup> function performed during the OmniSEC calibration procedure.

### Offset Volume and band broadening

In multi-detector GPC systems, the signals from multiple detectors are coupled to give the maximum amount of data. However, in order to couple these signals, the elution peaks must be aligned and corrected for band broadening, such that the same elution "slice" is being used in the calculations. The 2<sup>nd</sup> function performed during the OmniSEC calibration is therefore to calculate the offset volume between the GPC detectors and correct for inter-detector band broadening.

There are a variety of GPC detector calibration standards available, either from Viscotek or 3<sup>rd</sup> party vendors. When selecting an appropriate calibration standard, there are three things to consider.

- The standard must be suitable for use under the conditions that are to be employed when measuring unknown samples. For example, if the experimental objective is general protein characterization using a Superdex 200 column with an upper molecular weight limit of 600 kDa, a low molecular weight aqueous standard would be appropriate.

- All of the injected standard's mass must pass or elute from the column. The voltage response from the concentration detectors is proportional to the sample concentration, and the calibration constant is calculated from the entire standard peak area whose concentration is known if 100% of the sample mass elutes. If the entire injected sample does not elute from the column, an error will be introduced into the calibration constant.
- The calibration standard should have a polydispersity of no more than 1.1. As noted above, the response from the concentration detectors is proportional to the sample concentration. The response from the light scattering (LS) detector however, is a function of both the concentration and the molecular weight. If the molecular weight varies too much across the elution peak, as would be expected for samples with  $M_w/M_n$  significantly greater than 1.1, improper alignment of the LS and RI/UV peaks can lead to error in the off-set volume calibration.

When it comes to protein measurements, the requirements for calibration of the GPC detectors are no different. The caveat is that there is currently no protein standard that satisfies the low polydispersity requirement, as proteins tend to form oligomeric structures in solution. While one could select a low molecular weight synthetic polymer standard, identifying one that is compatible with the chromatography and has  $M_w/M_n$  of close to 1 is difficult. An alternative approach is to use a two step calibration technique which facilitates calibration of the GPC detectors using a readily available standard protein that can be resolved into its separate oligomer populations.

Using this technique, the concentration detectors are calibrated first by treating the entire sample as a single entity. In the second step the concentration of the monomer peak is calculated and then the monomer alone is used to calibrate the light scattering detectors.

