

HPLC & UHPLC

Principles of fraction collection using the Vanquish HPLC and UHPLC systems

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Introduction

The technique of fraction collection is continuously evolving and becoming more refined. The approach involves the combination of automated sample introduction, precise liquid chromatographic instrumentation, integrated software control, as well as the fraction collector (FC) apparatus itself. Fraction collection is the process of capturing the eluting fluid from a liquid chromatographic separation into vessels. One measures the presence and concentration of desired analytes in these vessels using different detection methods, ranging from UV detectors to mass spectrometers. These detectors, in combination with the chromatographic data system (CDS), are used to determine the fraction window, based either on detector signal or retention time.

This document focuses on the Thermo Scientific™ Vanquish™ Fraction Collector (VFC) operating at analytical scale fraction collection (0.05–10 mL/min), the Thermo Scientific™ Chromeleon™ CDS software settings, and general factors to consider for optimal translation of chromatographic resolution to fraction purity, and the optimized LC instrumentation.

Fraction collection instrumentation

The ultimate goal of fractionation is to isolate compounds of interest or to segregate impurities while preserving the fraction integrity for the next steps in the analytical workflow. To achieve this, the separation power of the column is matched to the frequency of discrete fractions to preserve analyte resolution. Furthermore, fractions should be preserved under stable storage conditions to protect precious samples against degradation by temperature and light, or contamination by dust.

As already mentioned, one of the most important aspects of fraction collection is that the resolution achieved by the column must be maintained by the detector as well as in the fractions. This 1:1:1 ratio of chromatographic resolution to detection response to fractionation accuracy is only an ideal situation and is rarely achieved in practice. For example, capillary flow dispersion, eddy dispersion and mechanical obstacles can all impact this ratio. Another impediment in fraction collection is scaling up (e.g., nano, analytical, semi-preparatory, preparatory, and industrial). This process is non-linear, and each application range possesses its own characteristics. This technical note focuses on the analytical application range only.

The fluidic path for the system is shown in Figure 1. Like all HPLC approaches, mobile phase passes through an autosampler, a column, and then a detector. What is unique about this technique is that a delay capillary connects the detector to the FC via a fraction collection valve, which, as discussed below, plays an important role in the properties of the FC. Finally, a waste capillary from the fraction collection valve is used for discarding unnecessary eluent.

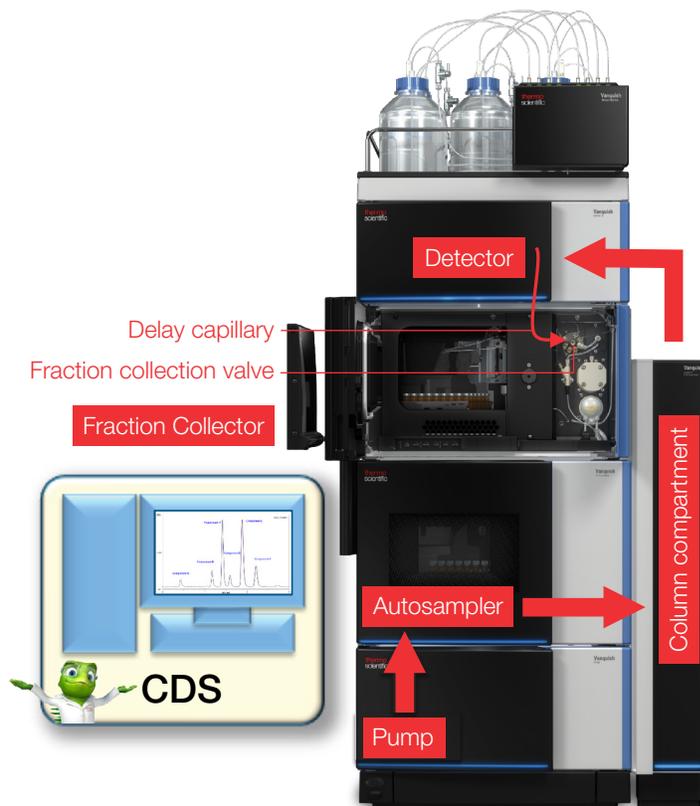


Figure 1. Typical configuration for fraction collection. The fluidic pathway is indicated and the CDS connects the entire setup communicating with the LC system to trigger the collection of fractions by the FC.

Thermo Fisher Scientific offers several FC options, either integrated or non-integrated into Vanquish LC systems. The Vanquish Fraction Collector, which is integrated into the Vanquish LC stack (Figure 1), is the focus of this Technical Note. The non-integrated versions are discussed in Appendix B.

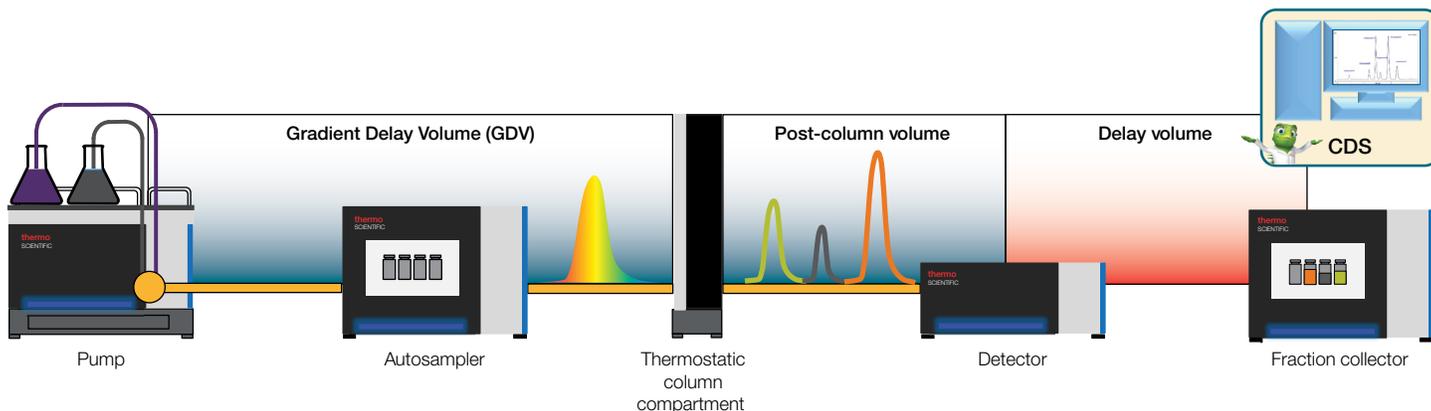


Figure 2. The delay volume is the total volume between the detector and the collection vessel. The value is important for precise fractionation control by the CDS.

The importance of delay volume

The goal of any FC is to ensure that the elution profile is accurately captured in the sample fractions, and this depends upon a signal coming from the detector. This, in turn, can be impacted by the delay volume between detector and the collection vessels (Figure 2). This must be optimized to ensure the ultimate fraction collection performance.

Several factors contribute to the delay volume, the most important of which include the delay capillary, fraction collection valve, needle capillary, and fraction collection needle (Figure 3).

The delay capillary: This connects the detector flow cell outlet and the inlet to the fraction collection valve “sample inlet” port. A short connection with small inner diameter capillary is ideal to keep the delay volume as low as possible. However, the effects of flow rate on the generated back pressure needs to be taken into consideration.

The fraction collection valve: This component is responsible for redirecting the flow between a waste position and a collection position. Its volume will contribute to the delay. Such delay is not only the result of the internal valve volume, but also results from the valve switching time, the magnitude of which depends upon the flow rate. A fast-switching valve is mandatory to minimize delay time. The fraction collection valve of the VFC also includes functions to flush and wash to actively improve the resolution despite unavoidable delay volume in fluidic connections. This will be discussed in detail in the *Flush and wash functions* section.

The needle capillary and fraction collection needle: This capillary connects the fraction collection valve outlet port to the fraction collection needle, which then directs the flow into the collection vessel.

The nature of the droplet

Besides the delay volume contribution of the fluidic connection, the surface tension of the mobile phase will generate a unique volume. The higher the organic content the lower the surface tension, and therefore the smaller the resulting droplet volume.¹

A major advantage of the VFC is that it uses a specially designed collection needle with very small droplet size formation to allow the lowest minimum collection volume.

The needle arm movement

The needle arm movement, combined with sample rack carousel radial movement, delivers the fluidics to an XY-plane position, allowing the eluting solution to be distributed among vessels.

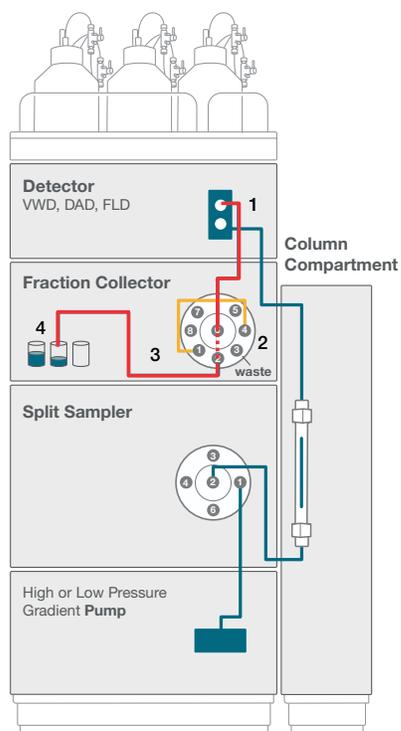


Figure 3. Delay volume in the LC instrument. The delay capillary (1), internal volume of the fraction collection valve (2), needle capillary (3), and fraction collection needle (4) combine to create the delay volume resulting from the fluidic connection.

Fluid can even be placed precisely into the vessels themselves by an additional Z-axis movement of the needle. Ideally, the collection needle moves quickly to its desired location. Increasing the time/distance when moving from one collection vessel to the next results in increased delay time and its corresponding delay volume, the magnitude of which will be affected by the flow rate.

The Role of the CDS

The CDS can impact the delay volume:

1. It controls the collection needle movement as well as the fraction collection valve switching based on whether detector signal or time window is selected.
2. It is responsible for implementing the collection mode. It can use either peak parameters or timed parameters to trigger the fraction collection valve switching times as well as the collection needle movement. Factors such as the delay volume, peak detection settings, and timed collection settings have a direct influence on the delay time (and corresponding volume) of the chromatogram to elution profile in the resulting fractions.

Optimization of the VFC to minimize the delay volume

Factors contributing to the total delay volume depend upon which fraction collector fluidic components are being used. Fluidic optimization with respect to flow rate ranges, while keeping maximum allowed system back pressure in mind, guarantees optimized results for a broad flow range.

The delay capillary and needle capillary must be optimized for the required flow rate range; this is a balance between the generated back pressure and minimum dispersion resulting from minimized delay volume. Table 1 contains recommendations for analytical flow rate ranges and for completeness, includes different flush buffer loops. These can be changed depending on the operation flow range. Although not relevant for delay volume, they do impact the recovery rate, carry-over, and other important parameters (see *Flush and wash functions* section).

The delay volume contributes significantly to the dispersion between the detector and the fractions themselves. Correct determination and implementation are essential for powerful fractionation. Inappropriate delay volume values and correlating fraction collection valve switch times can potentially result in distributing the fractions incorrectly, and not practically achieving the virtual simulated fractionation.

Delay volume determination

The delay volume can be calculated either manually from the volumes of the installed fluidic parts or, more conveniently, determined using the Automated Delay Volume Determination (DVD) function.

Table 1. Recommended capillaries for given application flow rate ranges

	Flow rate [mL/min]					
	Up to 0.5	0.5–1	1–2	2–5	5–10	P/Ns
Delay capillary for time-based fractionation, 0.1 × 350 mm, MP35N, Viper*	■	■	■			6042.2340
Delay capillary for time-based fractionation, 0.18 × 350 mm, MP35N, Viper*				■	■	6042.2337
Delay capillary for peak-based fractionation, 0.18 × 1200 mm, MP35N, Viper	■					6706.1100
Delay capillary for peak-based fractionation, 0.25 × 1500 mm, MP35N, Viper		■				6706.1110
Delay capillary for peak-based fractionation, 0.5 × 800 mm, PEEK, Viper			■			6706.1120
Delay capillary for peak-based fractionation, 1 × 1000 mm, PEEK				■	■	6706.1130
Needle capillary, 0.18 × 415 mm, PEEK-shielded MP35N, Viper*	■	■	■	■		6706.1010
Needle capillary, 0.25 × 415 mm, PEEK-shielded MP35N, Viper					■	6706.1020
Flush buffer loop, 50 µL, PEEK, Viper*	■	■	■	■		6706.1070
Flush buffer loop, 100 µL, PEEK, Viper					■	6706.1080

*included by default in module ship kit

The Automated DVD function of the VFC measures the delay volume specific to the current fluidic configuration. It is also immediately included as a parameter in Chromeleon CDS to ensure optimum fractionation settings for future instrument methods.

Automated DVD is conducted as part of the qualification process and can be measured for any Vanquish LC system with a specific optical detector configuration. A qualification wizard guides the user step-by-step through the process to ensure correct delay volume measurement.

Step 1: Install a specific DVD capillary (Thermo Scientific™ Viper™ Fingertight Fitting System, P/N 6040.2330) to connect the Vanquish Autosampler outlet directly with the flow cell detector inlet (Figure 4A).

Step 2: Place an empty vial into the position specified by the system.

Step 3: Purge and flush the system with water at 1 mL/min.

Step 4: The system automatically injects an air bubble into the fluidic stream.

The time taken for the air bubble to be detected, first by the optical detector and then by the integrated air bubble sensor in the VFC, is used to calculate the total delay volume.

The prerequisite for correct results is to select the installed delay capillary from the Delay Capillary ID × L in the drop-down menu.

If the required capillary is not listed, the value “custom” can be selected to enter the corresponding Delay Capillary ID and Delay Capillary L in the Chromeleon CDS configuration. After subsequently executing the DVD Operational Qualification (OQ), the resulting delay volume is automatically updated with the experimentally determined delay capillary volume.

The resulting delay volume is automatically populated in the module properties and can be accessed via the Vanquish Fraction Collector ePanel within the Chromeleon CDS under the 'More Options' dialog. (Figure 4B).

More operational details for Automated DVD and about supported instrument configuration can be found in the operating manual of the Vanquish Fraction Collector.²

The method-specific delay volume and delay time are auto-set when setting up a new method with the Instrument Method Wizard of Chromeleon CDS, and thus enable high resolution during the fractionation process.

Although the delay volume is determined precisely by empirical measurement and the valve switches exactly, some liquid from the first fraction will always remain in the capillary and collection needle after the valve switched at the end of the fractionation. Besides the delay volume determination, it is necessary to further optimize fraction collector performance—for example, for achieving a high recovery and minimizing carry-over—to guide the left-over liquid into the correct vessel and to minimize cross contamination by choosing an appropriate cleaning procedure. These flush and

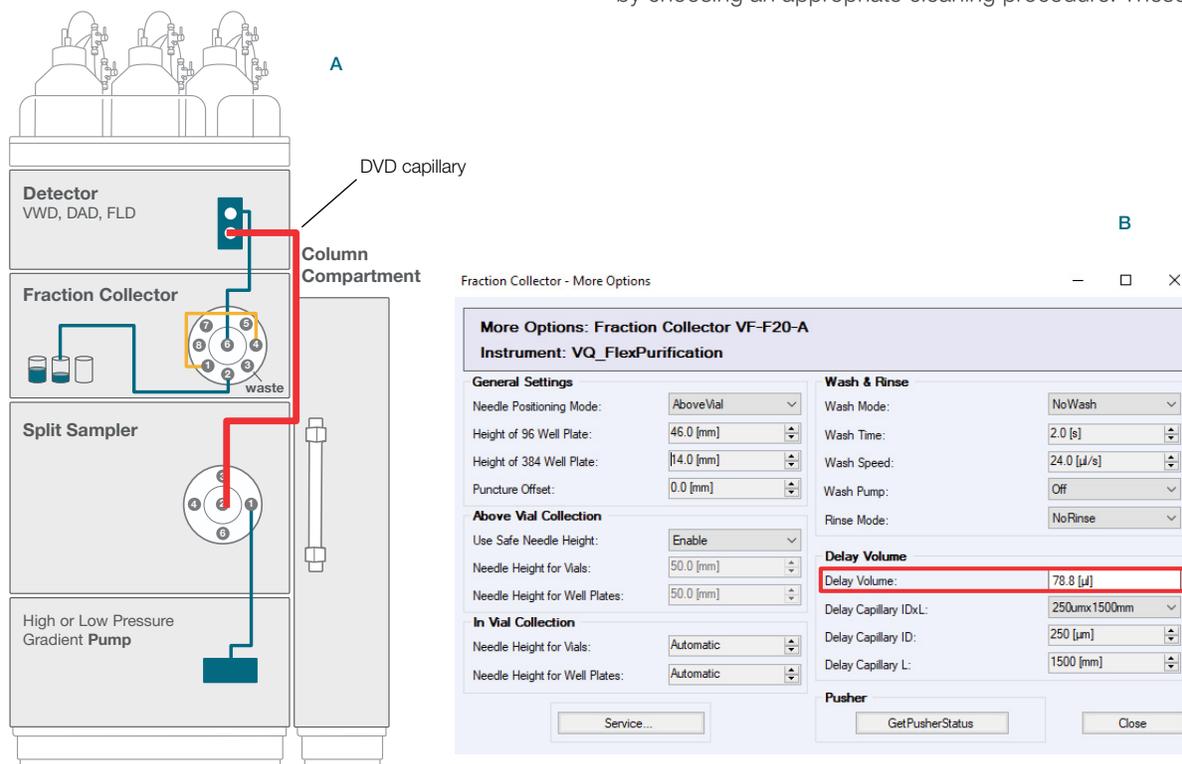


Figure 4. Delay volume determination. Empirically determined delay volume by the automated tools requires installation of a DVD capillary (A). The total delay volume due to the tubing is then displayed on the 'More Options' dialog of the ePanel (B).

cleaning procedures are discussed in the next section.

Flush, rinse, and wash functions

The VFC has several flush, rinse, and wash functions to optimize performance.

In the IDLE status, the fraction collection valve is in the WASTE position and no fractions are collected (Figure 5A). For collecting fractions, the fraction collection valve switches to the COLLECT position (Figure 5B). When the flush function is enabled, the CDS at the end of the fraction collection switches the fraction collection valve from the COLLECT position (Figure 5B) to the FLUSH position (Figure 5C). The needle will stay at the current sample container position. In the FLUSH position, the flush buffer loop filled with flush solvent is switched into the flow path between the detector and the needle, allowing the remaining fraction in the needle capillary and needle to be dispensed into the current tube (Figure 5C) or moved to the next sample container. Thus, the recovery of the main fraction is increased and carry-over of one fraction into the next fraction is minimized (Figure 5D).

Note: The flush solvent used for the flush function should be compatible with the eluent used for eluting fractions. One can use, for example, mobile phase A. If this eluent is not strong

enough to remove compounds present in the needle capillary, needle or flush buffer loop, a separate flush solvent can be selected. For more operational details on flush loop solvent please refer to [2].

The VFC has several different wash options. One can perform both an external needle wash or internal needle rinse or both. This selection of the wash and rinse steps before the collection starts (*Before Collection*), after the collection period ends (*End of Collection*), or both before injection and after the fraction collection run (*Both*) is defined in the Chromeleon CDS Instrument Method Wizard. The option of having no wash is also possible and applies to the external needle wash as well as internal needle rinse.

To remove residual sample from the external surface of the needle between runs, the VFC offers the option to move the needle into the wash port and wash the collection needle externally with an adequately strong (dedicated) wash solvent, while the fraction collection valve is in the WASTE position (Figure 5E).

Note: The recommended external needle wash solvent is 10% methanol in water. This can be adjusted depending upon chemical compatibility and contaminant solubility to meet specific

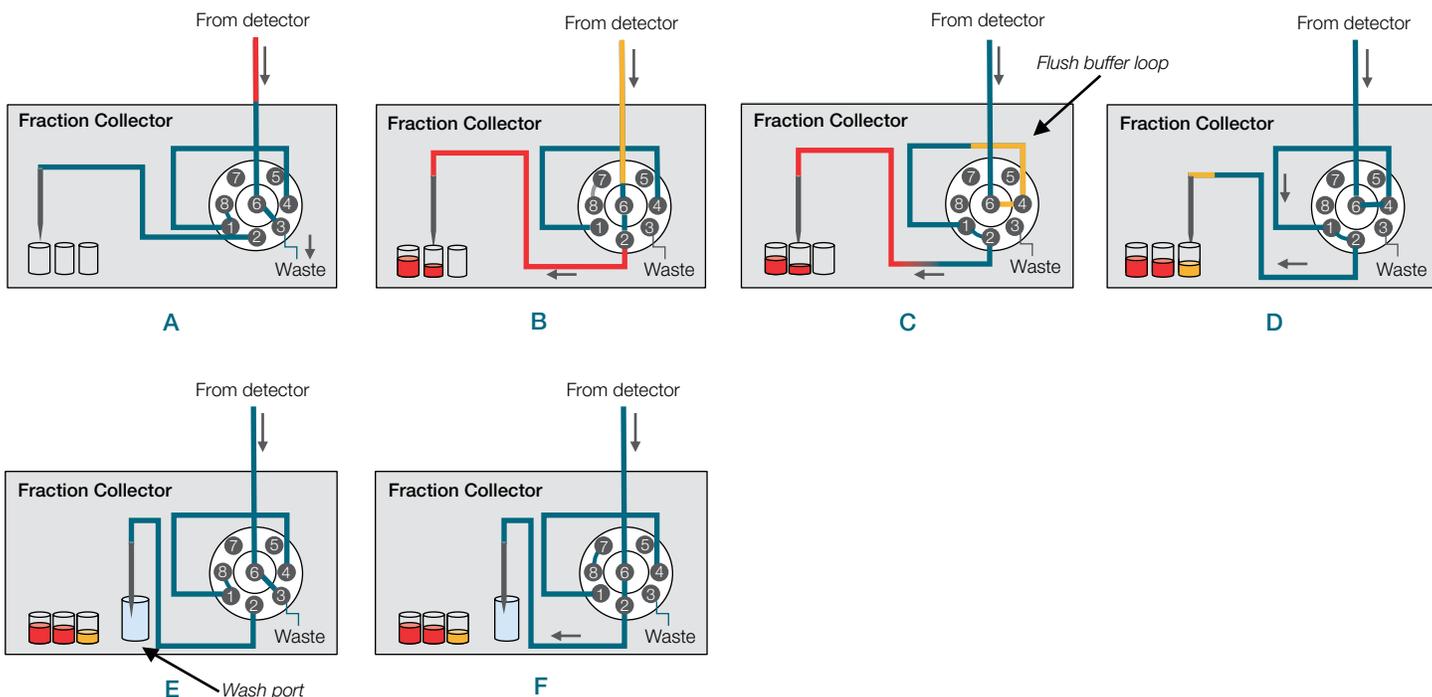


Figure 5. The fraction collection valve of the Vanquish Fraction Collector and its three positions support various functions. In WASTE position (A) the VFC is idle and no fractions are collected. In COLLECT position (B) fractions (red) are collected. The FLUSH position (C) enables high recovery fractionation to separate main fraction (red) from impurities (yellow) (C to D). For the external needle wash (E) the valve is in WASTE position and the needle in the wash port. For the internal needle wash or RINSE mode (F) the valve is in position COLLECT and the needle in the wash port.

external wash needs and reduce carry-over.

Needle wash is defined by the instrument method parameter Wash Mode with two available settings: Wash Speed and

Table 2. Recommended settings for needle wash

Parameter	Setting
Wash Mode	Both
Wash Speed	100.0 $\mu\text{L/s}$
Wash Time	3.0 s

Wash Time (Table 2).

For internal rinse of the collection needle, the needle is moved into the wash port while the fraction collection valve is in

COLLECT mode. With the flow delivery from the LC pump, the mobile phase rinses the inner surface of the needle into the waste to minimize any carry-over and cross-fraction contamination (Figure 5F). The details of the procedure are defined by the instrument method parameter Rinse Mode.

Fraction collection modes

Fraction collection can be achieved either based on a time program or a peak recognition program (Figure 6). The selection of an appropriate collection mode depends on the chromatographic conditions, i.e., resolution of the separated compounds. Recommendations for when to use time-based or peak-based modes are presented in Table 3.

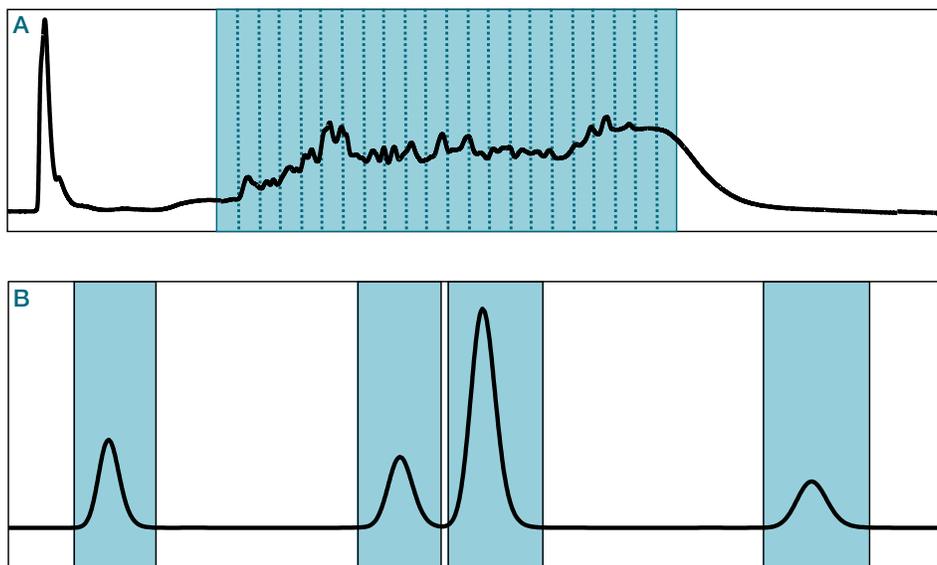


Figure 6. The above chromatograms represent (A) Collect by Time and (B) Collect by Peak.

Table 3. Recommendations for when to use peak-based fractionation and when to use time-base fractionation

Attributes	Time-based	Peak-based
Known stable retention times	●	
Known stable peak shape	●	
Unknown retention times		●
Peak shoulders		●
High resolution		●
Low resolution	●	
Very complex samples	●	

Sample containers and settings

The VFC can handle a wide variety of sample containers including individual vials and well plates. The correct choice will depend upon the experimental design. The VFC features automatic recognition of barcoded sample racks or well plates by a barcode reader to identify the sample container. More details for sample containers can be found in [3]. Various settings for vials and well plates are automatically populated in the CDS program but, as discussed in greater detail below, certain values (e.g., plate height) need to be checked while others (e.g., whether the needle should go into the vial or not) must be selected prior to use of the VFC. This section discusses several important aspects when setting up the VFC for different sample containers and includes how to set the needle height for different well plate formats, and positioning the needle for either above vial or within vial collection.

Well plates

When initially setting up the VFC with 96 well plates (deep or shallow) and 384 well plates, the respective heights of the plates need to be defined in the Chromeleon CDS ePanel in the VFC tab under 'More Options'. The default plate height when the VFC is newly installed in Chromeleon CDS is 50 mm for both 96 well plates and 384 well plates. Since commercially available well plates may show slight tolerances in height (Table 4), height needs to be measured with a ruler and then entered into the fields indicated in Figure 7. Once the plate height is determined the user must decide which needle positioning mode is required.

Table 4. Typical well plate heights

Plate type	Setting
96 deep well plates	44.0–46.0 mm
96 shallow well plates	13.0–15.0 mm
384 well plates	13.0–15.0 mm

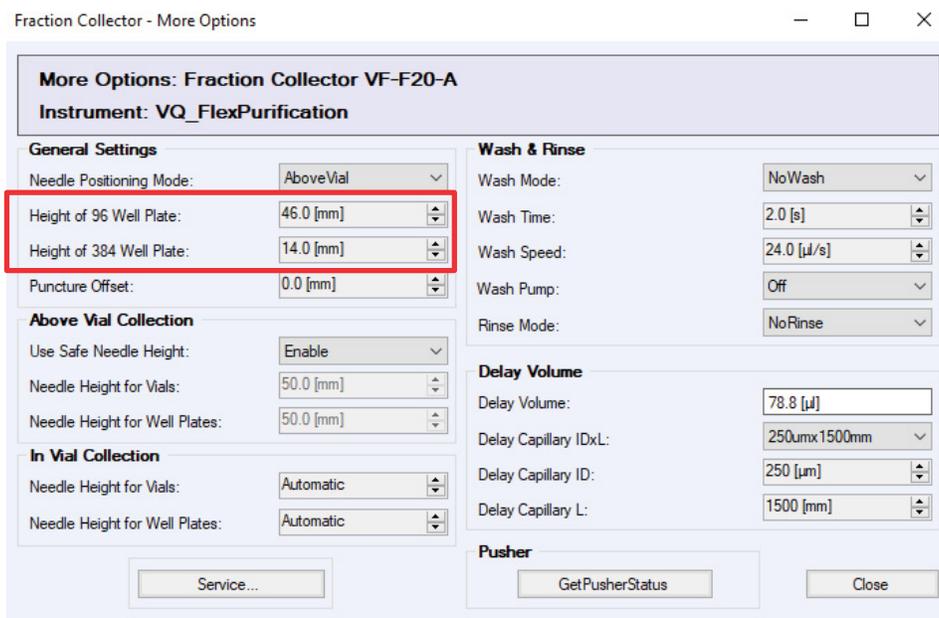


Figure 7. The default settings of well-plate height can be adjusted for exact positioning of the needle compared to the sample container height.

Needle positioning

There are two different needle positioning modes used when collecting fractions: *AboveVial* collection and *InVial* collection. To switch between these modes, a manual change of the collection needle pusher is required and is described in the user manual.²

AboveVial collection: This is the typical selection for general fraction collection and a recommended setting for novice users. *Use Safe Needle Height* is specified in the Instrument Method Wizard for the VFC under the General Settings tab (Figure 8A). The safe needle height is 2 mm above the collection vessel. Deselecting the *Use Safe Needle Height* is used for more advanced needle height positioning where the desired height is above the collection vessel but positioned very close to the top of the vessel. It is recommended to test this prior to collection via the custom needle height positioning procedure explained below.

InVial collection: A typical case in which *InVial* collection is selected is when using capped vials with a septum or well plates with cover (e.g., to prevent sample evaporation) or when it is desirable to fill the collection vial from the bottom. More details for capped vials can be found in [3]. The parameters for the *InVial* needle height are entered in the Instrument Method Wizard under the General Settings for the VFC (Figure 8B). One can separately designate the needle height for vials and well plates.

Selecting “Automatic” will position the needle 2 mm above the bottom of the vial or well plate hence filling the vial or well plate from the bottom. This would typically be done with lower flow rates (~50–300 µL/min) and lower sample concentrations.

In some cases, where the flow rate is above ~300 µL/min, and higher sample concentrations are present, this may result in an undesired effect where the outside of the needle could become contaminated by the collecting liquid. One can collect *InVial* but above the liquid position by entering a value in these fields to a desired height. For example, with a 1.5 mL vial and if the maximum collecting volume is entered as 0.5 mL, one can position the needle *InVial* but above the level of the 0.5 mL volume collected in the vial. Thus, the needle will not be submerged into the collecting liquid. This must be tested to discern the height of the liquid collected and the positioning height of the collection needle (see *Custom needle height* section).

In cases where a capped vessel is used, a puncture offset can be entered to alleviate pressure buildup in the capped vessel when the collection liquid is displacing the air inside the empty vessel. Enter the puncture offset in the Instrument Method Wizard for the VFC under the General Settings tab as seen in Figure 8B.

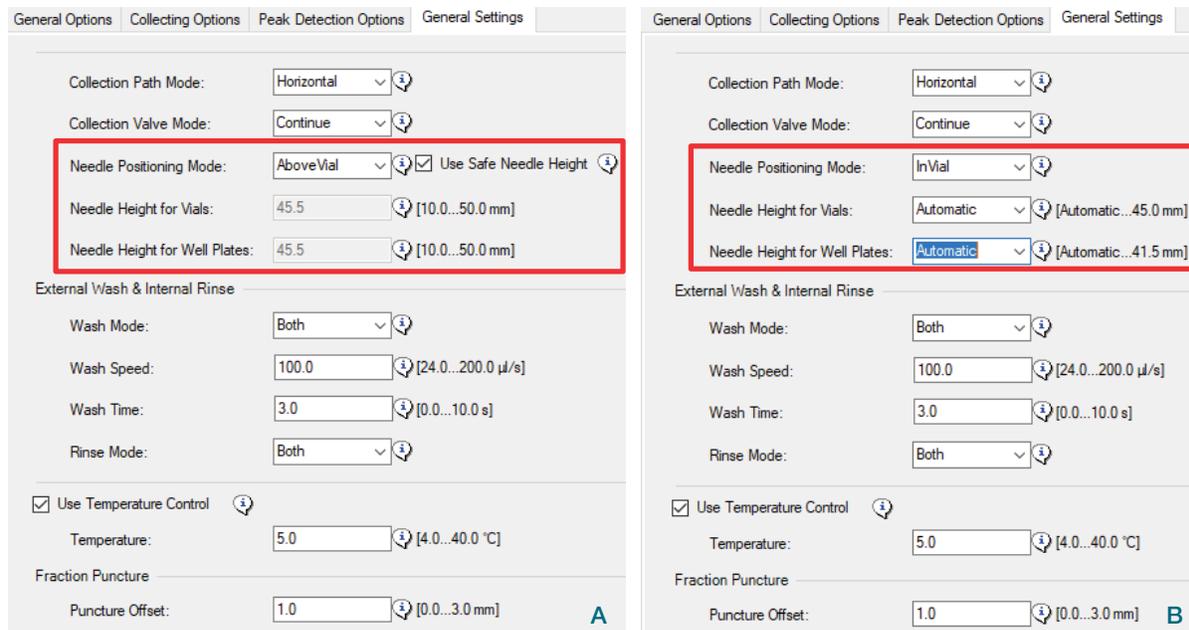


Figure 8. The needle positioning is set to either *AboveVial* (A) or *InVial* (B) in the instrument method wizard.

Custom needle height

For both, *InVial* collection and *AboveVial* collection, the needle height positioning can be customized to give the user flexibility as to where the needle height is to be positioned above or within the vial.

To test the needle height, enter the collection position in the VFC ePanel under 'Move Needle' (Figure 9). Enter the vial position to be tested, press Enter, then click the 'Position' button. The needle will then move to the designated rack and vial position. Then enter the height, press Enter, then click the 'Height' button. This will lower the needle to the designated height. This can be repeated until the desired needle height is met.

Note: Do not enter a different needle position after the needle has been lowered. Instead enter the maximum needle height of 50 mm and then choose the different needle position to avoid damage of the needle while conducting the needle height testing.

Fraction collection software settings

The Chromeleon 7.3.1 CDS is used to perform the fraction collection. Chromeleon CDS provides a step-by-step wizard for method creation. The following provides an explanation of each fractionation parameter but will forego other LC module parameters.

The most important software-related parameters include the following:

Collection channel: Whether it be UV detection or another detection method, setting the channel correctly is essential. For example, if the user collects using the peak-based mode and an unsuitable absorption wavelength is set as the collecting channel, then it is possible to lose much, if not all the desired analytes.

Fraction mode: Select between time-based or peak-based fractionation modes (Table 3).

Delay volume: This is either automatically determined for the VFC (see *Delay Volume Determination* section) or needs to be thoroughly set for other fraction collector models (see Appendix B, Table 8). If set too high, the analytes of interest will be collected in either an earlier-than-desired vessel or drained to waste entirely. If set too low, the analytes will reside in later fractions when correlating the fractions with the source chromatogram.

Automatic fraction pooling: This setting is activated if the user wants to start the fraction collection using the same vial for every sample run. If deactivated, Chromeleon CDS remembers the last collection position from the previous run and then continues with the next collection position.

Fraction collection needle movement mode: The user can select between different movement schemes including *Horizontal*, *SawHorizontal*, *Vertical*, and *SawVertical*. Greater detail can be found in [Appendix A: Chromeleon CDS fraction collection wizard](#).

Note: When the fraction collection arm reaches the end of a row or column, respectively, in *SawVertical* or *SawHorizontal* mode, the needle has a longer distance to travel to the first position in the next row versus in *Vertical* or *Horizontal* mode, respectively. Next to the needle movement mode, the selection of the Collection Valve mode must be considered depending on the application focus. Collection modes are discussed in greater detail in the next section.

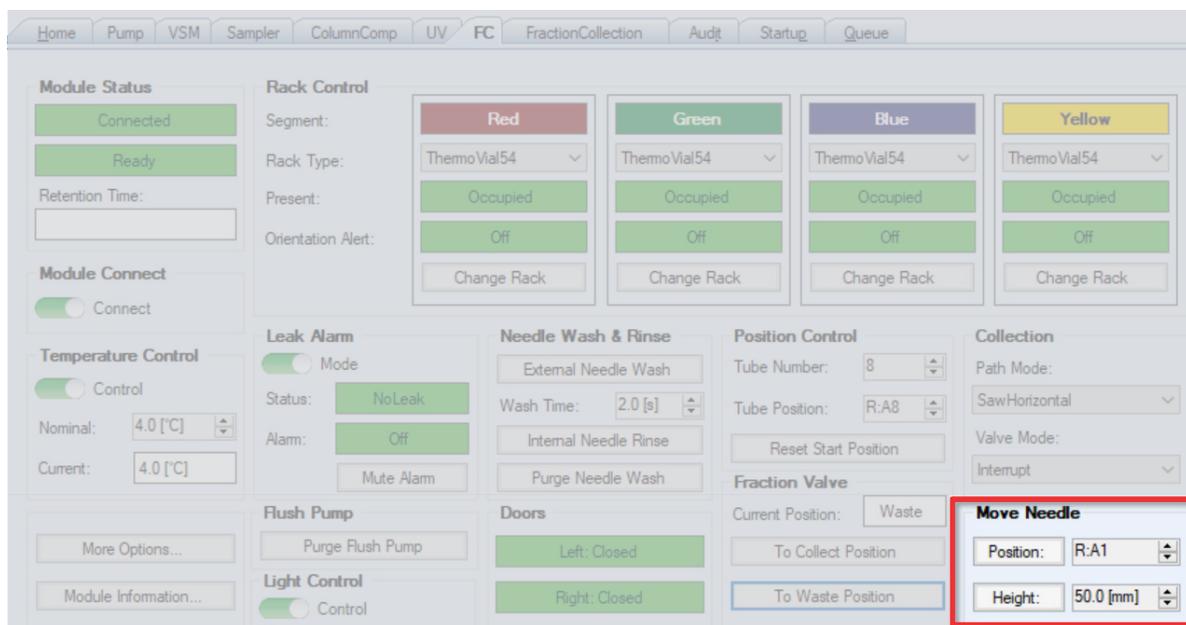


Figure 9. Collection needle height positioning can be customized for flexible positioning of needle. For example, against the liquid level in *InVial* mode or against sample container height in *AboveVial* mode.

Collection valve mode: This can be set in Chromeleon CDS to either *Continue*, with the fraction collection valve remaining in the COLLECT position, or to *Interrupt*, with the fraction collection valve switching to WASTE position. The settings in the software should be considered carefully to avoid unnecessary loss or cross-contamination of fractions, which can have a major impact on the final fraction recovery or carry-over.

It is recommended to set the fraction collection valve to *Continue* at lower flow rates, as the exiting liquid forms and drops slowly. In the Continue mode, no sample is guided to waste, and the loss of sample is minimized. On the contrary, at higher flow rates and in case of NeedleMovement Mode set to *SawVertical* or *SawHorizontal*, sample loss due to spillage or cross-contamination might occur during the needle movement along a long distance. In this case, the choice of *Interrupt* mode, may avoid spillage or cross-contamination but result in material loss to waste and poor recoveries. For example, using *SawHorizontal* mode with the *Interrupt* mode, the fraction collection valve switches to waste when moving from the end of one row to the beginning of the next. Sending the flow to waste during the needle movement is a source of loss, but at higher flow rates and larger peak volumes the impact may be negligible. Therefore, for the best results when using analytical flow, use the combination of *Continue* collection mode with the *Horizontal* movement scheme, to minimize loss.

An advantage of the *Interrupt* mode is that the collection order on the rack is easier to follow and consequently the organizing of sample re-injection easier to set up.

For step-by-step guidance of the fraction collection section of the method editor, please refer to Appendix A.

Performance indicators for fraction collectors

The following section discusses in detail various aspects of the fraction collection process.

Delay

The traditional hardware configuration (Figure 1) places a detector before the fraction collector. Using a UV detector as an example, an analyte enters the flow cell and generates an electronic signal whose intensity is based on the concentration of the sample and absorbing capacity of the analyte. This electronic signal is interpreted by the software to meet conditions of threshold and slope. When the software determines a condition has been met, the software sends a trigger to the fraction collector. During this short time window, the analyte has exited the flow cell and moves through the delay capillary until it reaches the collection needle tip. The time it takes the analyte to reach a fraction vessel from the flow cell is the delay time, which is defined by the delay volume and the specified flow rate (Figure 10). This value, as volume or time, is entered into Chromeleon CDS to trigger the fraction collection valve to switch from the position WASTE to COLLECT after the signal trigger is received and the delay has been reached. The combination of the Automated DVD, selected delay capillary, and choice of either time or peak trigger, is used by the CDS to automatically calculate the delay time, ensuring simple operation.

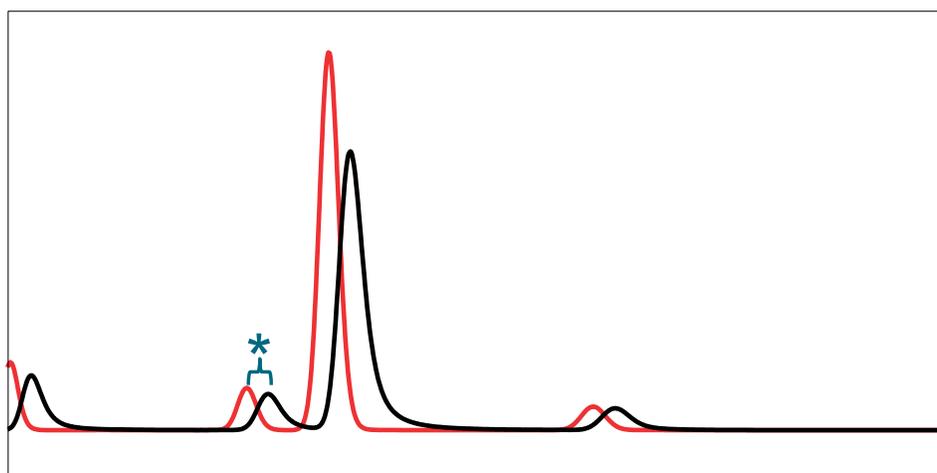


Figure 10. The delay time is the difference in time between the chromatogram from the detector (in red) and the resulting elution chromatogram delivered at the collection needle tip (in black) caused by the delay volume of needle, needle capillary, fraction collection valve, and delay capillary and the pump flow rate.

In addition to the delay time, which is defined by the fluidic delay volume and flow rate, an additional delay time is required to allow the detector to detect a peak and subsequently the CDS to react. This required minimum delay time is defined by the CDS version. Chromeleon CDS uses a specific formula to evaluate the delay volume or time compatibility in the sequence ready check and will provide an error if the delay volume or time is not compatible with the method. This is especially relevant for peak-based fraction collection, where the shortest possible delay time includes time for computational tasks of Chromeleon CDS and for the detector to respond to slopes and thresholds.

$$\text{Minimum delay time} = \text{OffsetTime} + \text{DerivativeStep} + \text{Max}(\text{PeakStartTrueTime or PeakEndTrueTime})$$

The *Delay Time* or corresponding *Delay Volume* must be greater than or equal to this computation (Figure 11) for Vanquish Fraction Collector FT under Chromeleon CDS version 7.3.1 or later. Earlier modules like the Fraction Collector F/FT require thorough setting of delay volume and delay time to fulfill the requirements for minimum delay time and passing the sequence ready check (see Appendix B for details).

Detection Property	Detection Channel 1
Channel Name	UV-VIS_1
Offset Time	0.0 [s]
Offset Volume	0 [µl]
Peak Start Conditions	
Peak Start Threshold	1.00 [signal]
Peak Start Slope	1.000 [signal/s]
Peak Start True Time	1.00 [s]
Peak End Conditions	
Peak End Threshold	1.00 [signal]
Peak End Slope	-1.000 [signal/s]
Peak End True Time	1.00 [s]
Time Step for Slope and	
Derivative Step	0.02 [s]
Special Peak End Threshold	
Threshold 'No Peak End'	Off

$$A + B + C = 1.02 \text{ s}$$

*Adjusted from 1.0 (s)

Figure 11. During peak-based fractionation, these parameters add to the total delay time or corresponding delay volume determined by fluidics.

Dispersion

Dispersion occurs immediately after the sample is taken into the autosampler needle; it adversely affects resolution and causes peak broadening. The capillaries, column, flow cell, and fraction collector are the largest contributors of dispersion. Specifically, Figure 12 shows the importance of measuring the impact of dispersion between detector and fraction collector. More specifically, the components between the detector and collection vessel that contribute to dispersion include the detector flow cell volume, delay capillary (length and diameter), fraction collection valve, needle capillary, and collection needle.

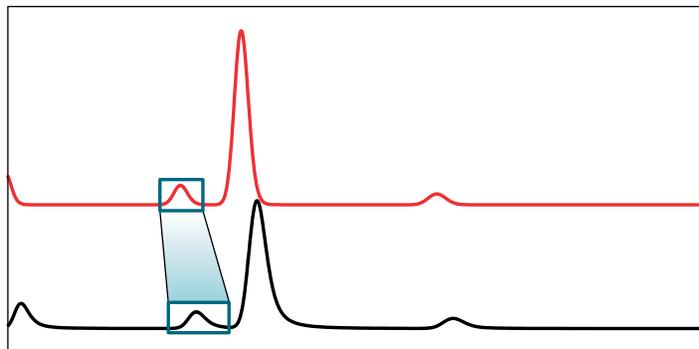


Figure 12. Dispersion sources influence the original chromatogram (in red) resulting in the elucidating chromatogram (in black). This exemplifies peak broadening due to the fluidic components from flow cell to drop formation.

Flow cell: This is selected based on the analyte concentrations and peak volumes, but once selected, the influence the flow cell has on dispersion is constant.

Needle capillary: These can have a 0.18 mm I.D. or a 0.25 mm I.D. Below 5 mL/min, the 0.18 mm I.D. needle capillary is recommended to minimize dispersion and carry-over.

Fraction collection valve: Its contribution to dispersion is constant.

C* Delay capillary: The largest impact the user has on the dispersion between detector and fraction collector is the choice of delay capillary length and inner diameter.⁴ Since an increase in the inner diameter of the capillary system increases the diffusion exponentially, the best results are obtained using the smallest I.D. delay capillary possible, as described by the Taylor-Aris equation.⁵ The limitation of the delay capillary I.D. on the system is its effect on back pressure, which exponentially increases as the I.D. decreases. This, in turn, will dictate which backpressure-resistant flow cell can be used. Therefore, the backpressure caused by the delay capillary for a given flow rate should be taken into consideration to prevent damage to an up-stream

fluidic component such as the detector flow cell. Given these influences, Table 1 outlines the match between application flow rate and corresponding specific delay capillary. The delay capillaries for time-base fractionation are available in the ship kit of the VFC, whereas the delay capillaries for peak-based fractionation can be ordered separately.⁶

If contamination from previous fractions is observed when collecting fractions by the time-based approach, this may be caused by dispersion or improper delay settings, and not by carry-over. Contamination can also be observed when the

detecting chromatogram displays baseline, and the re-analyzed fractions contain trace amounts of the previous peak. This results from the dispersion principle and is shown in Figure 13. Here, Fraction 7 exhibits a very slight presence of the compound found in Fraction 2. This exemplifies the diffusion effects, resulting in increased tailing after the detector due to the fluidic components from the detector flow cell to the tip of the needle. Optimized fluidic connections as implemented in the integrated VFC minimize the dispersion effect. Therefore, an adequate delay capillary for the set flow rate should be selected according to Table 1.

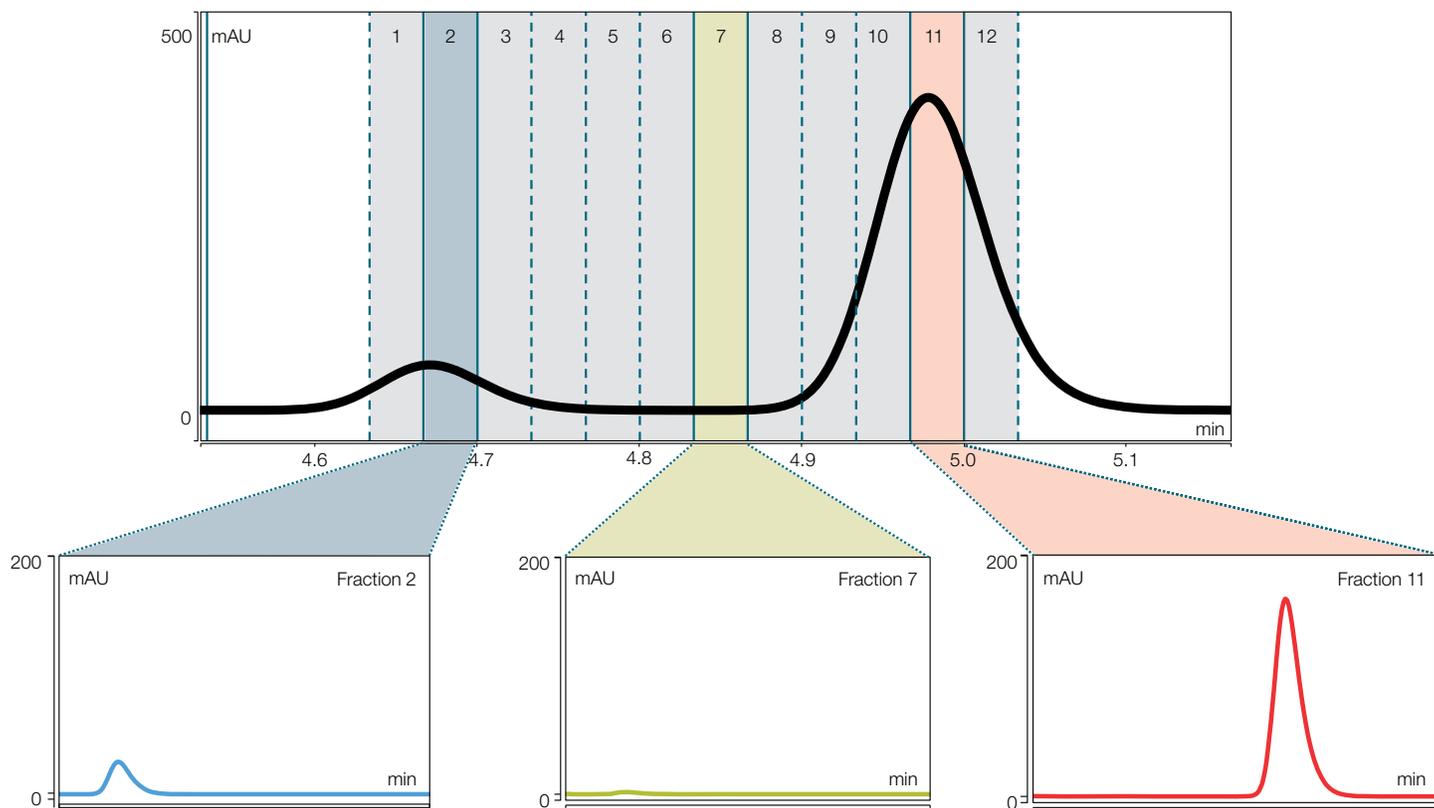


Figure 13. The above time-base fractionation was subsequently re-analyzed showing that in fraction 7 (green) a tailing effect is caused by diffusion. The re-analysis of Fraction 2 (blue) shows a pure compound. In the source chromatogram, one observes a baseline in Fraction 7 though in the resulting re-analysis, there is a trace amount of the compound seen in Fraction 2.

Carry-over

There is an inherent flaw in all fraction collectors—how the volume of liquid between the tip of the collection needle (or drop former) and the collection valve (or diverter valve) exits the fraction collector. This volume is excluded from the fluidic pathway when the valve is switched to waste. If this needle and needle capillary volume contain some analyte from a tailing peak and the fraction collection valve is then switched to waste, this will lead to contamination of the next sample. This carry-over is experienced only when collecting by peak-based methods or time-based collection with multiple individual collection time frames and contributes to poorer resolution between fractions. This effect can be observed in the elution and re-analysis shown in Figure 14. Carry-over can be minimized by activating the flush function. Additionally, an internal needle rinse and external needle wash option, as discussed above, eliminates carry-over effects between separate collection runs (see *Flush, rinse, and wash functions* section)

Recovery

Recovery is determined by the amount of product collected. As discussed above, many factors affect the ability to collect pure fractions with acceptable recovery, such as detector settings, delay volume, fraction collector valve volume and switch time, and peak level threshold and slope. A practical example using the fractionation of oligonucleotides (ON) is presented in the next section.

Experimental

To demonstrate the required settings for a typical fractionation, an ON mixture was first separated by reversed-phase chromatography and then fractionated using either a time-based trigger or a peak-based trigger. Fractions were then reanalyzed using the same chromatographic conditions to evaluate fractionation performance.

Chemicals

- Deionized (DI) water, 18.2 M Ω -cm resistivity
- Fisher Scientific™ Optima™ Acetonitrile, LC-MS grade (P/N A955-4)
- Applied Biosystems™ Triethylamine acetate (TEAA) 2.0 M (P/N 400613)
- Fisher Scientific™ Optima™ Acetic acid, LC-MS grade (P/N A11350)
- Integrated DNA Technologies, Inc. Oligonucleotide standard. 8-combo DNA containing: 12mer (GACT)₃; 16mer (GACT)₄; 20mer (GACT)₅; 24mer (GACT)₆; 28mer (GACT)₇; 32mer (GACT)₈; 36mer (GACT)₉; and 40mer (GACT)₁₀

Sample handling

Vial and closures: Polypropylene, 0.3 mL vials (Thermo Scientific™, P/N 055428)

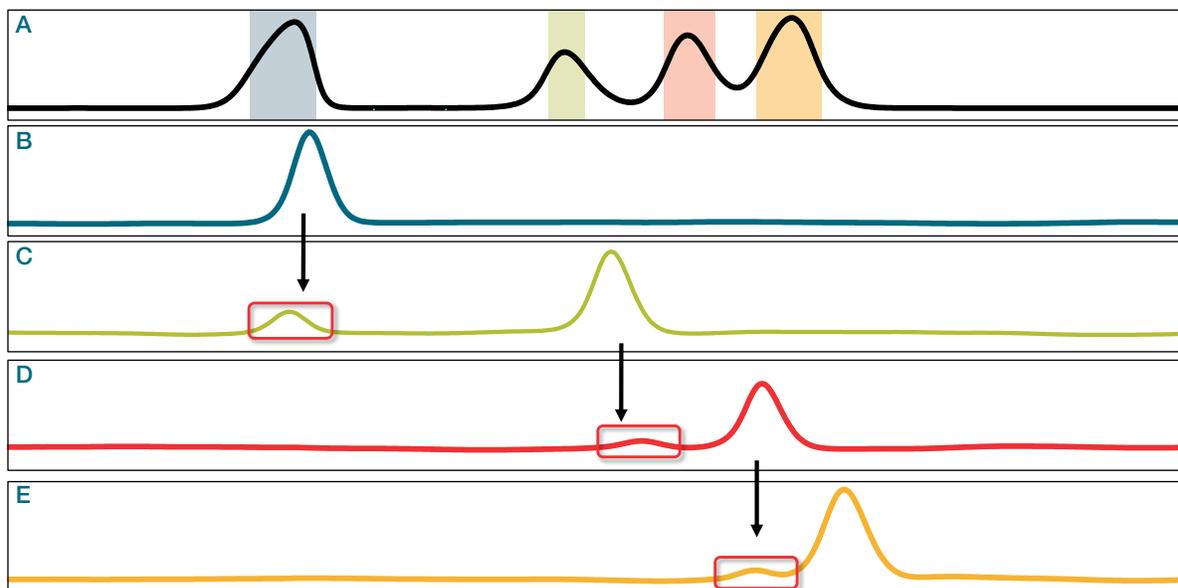


Figure 14. Carry-over between fractions occurs with fraction collector modules without flush function. The source chromatogram (A) is fractionated by a Thermo Scientific™ Fraction Collector F with 0.4 mm drop former kit. Fractions subsequently re-analyzed in (B), (C), and (D), showing prior peak carry-over due to the volume between the fraction collection valve and drop former tip, which cannot be flushed between peaks. This is only applicable during peak based fractionation.

Sample preparation

Stock solutions were prepared by dissolving the ONs to 200 μM with deionized water (DI) and stored at 5 $^{\circ}\text{C}$. Equivalent amounts of each ON were mixed to prepare 25 μM for each ON. The sample was further diluted five-fold with DI water to make 5 μM mixed standard working solution.

Instrumentation

Thermo Scientific™ Vanquish™ Flex Analytical Purification LC system consisting of:

- System Base Vanquish Horizon/Flex (VF-S01-A-02)
- Vanquish Quaternary Pump F (VF-P20-A)
- Vanquish Split Sampler FT (VF-A10-A-02)
- Vanquish Column Compartment H (VH-C10-A-02)
- Vanquish Variable Wavelength Detector F (VF-D40-A) with Semi-Micro Flow cell, 2.5 μL (7 mm), SST (P/N 6077.0360)
- Vanquish Integral Fraction Collector FT (VF-F20-A-01) with delay capillary depending on fractionation trigger:
 - for time-based fractionation: 0.1 mm \times 350 mm (P/N 6042.2340)
 - for peak-based fractionation: 0.25 mm \times 1500 mm (P/N 6706.1110)

Software

- Chromeleon CDS, 7.3.1

Method parameters

Time-based fractionation

The ON mixed standard working solution was analyzed using the LC conditions described in Table 5 and fractionated in a time-based fashion as described in Table 6.

Peak-based fractionation

The ON mixed working solution was separated analyzed using the LC conditions described in Table 5 and fractionated in a peak-based fashion as described in Table 7.

Table 5. LC method for separation ON mixture and re-analysis of fractions

Parameter	Value		
Column	Thermo Scientific™ DNAPac™ RP, 2.1 \times 50 mm, 4 μm (P/N 088924)		
Eluent	A—100 mM TEAA, pH 7.0 B—100 mM TEAA, pH 7.0 / acetonitrile (75:25 v/v)		
Flow rate	0.6 mL/min		
Gradient	Time (min)	%A	%B
	-3.0	85	15
	0	85	15
	1.0	85	15
	5.0	65	35
	5.1	10	90
	6.4	10	90
	6.5	85	15
7.0	85	15	
Autosampler temp.	5 $^{\circ}\text{C}$		
Column temp.	60 $^{\circ}\text{C}$ forced air 60 $^{\circ}\text{C}$ active pre-heater		
Injection volume	10 μL		
Injection wash solvent	10% methanol in water		
Detection	260 nm, 20 Hz, 0.2 s response time, 4 nm bandwidth, slit width Wide		

Table 6. Fraction collector instrument methods settings for time-based fractionation

Parameter	Value
Wash solvent	10% methanol in water
Flush solvent	Eluent A: 100 mM TEAA, pH 7.0
Fraction collector compartment temp.	5 $^{\circ}\text{C}$
Collect by time collection period	3.0 s
Collection time frame	3.0–5.2 min
Flush	Inactive
Max. tube volume	0.125 mL
Minimum time for tube change	2 s
Delay volume	7.7 μL —determined by Automated DVD with delay capillary 0.10 \times 350 mm (P/N 6042.2340)
Collection path mode	Horizontal
Collection valve mode	Continue
Needle positioning mode	AboveVial
Use safe needle height	Active
Wash mode	Both
Wash speed	100.0 $\mu\text{L/s}$
Wash time	3.0 s
Rinse mode	Both
Puncture offset	0.0 mm

Table 7. Fraction collector instrument methods settings for peak-based fractionation

Parameter	Value	
Wash solvent	10% methanol in water	
Flush solvent	Eluent A—100 mM TEAA, pH 7.0	
Fraction collector compartment temperature	5 °C	
Collection time frame	3.0–5.3 min	
Flush	Active	
Max. tube volume	0.125 mL	
Minimum time for tube change	2 s	
Delay volume	35.5 µL determined by Automated DVD with delay capillary 0.25 × 1500 mm (P/N 6706.1110)	
Collection path mode	Horizontal	
Collection valve mode	Continue	
Needle positioning mode	AboveVial	
Use safe needle height	Active	
Wash mode	Both	
Wash speed	100.0 µL/s	
Wash time	3.0 s	
Rinse mode	Both	
Puncture offset	0.0 mm	
Peak detection options	Show advanced settings	
	Peak start threshold	0.00 mAU
	Peak start slope	6.000 mAU/s
	Peak start true time	0.20 s
	Peak end threshold	2.00 mAU
	Peak end slope	-5.000 mAU/s
	Peak end true time	0.2 s
	Derivative step	0.02 s

Results and discussion

Time-based fractionation

The chromatogram of the ON separation and the visualization of time-based fractionation with the settings as described in Table 6 is shown in Figure 15. The fractions were then re-analyzed by injecting the individual fractions (Figure 16).

Based on the time window settings, the valve switches with a delay reflecting the previously determined accurate delay volume with the optimized delay capillary installed. When comparing the individual chromatograms of the re-analyzed fraction, the purity of the fractions is revealed, which was not detectable in the initial separation. For instance, the overlay of the re-injected fractions for the 36mer and 40mer (Figure 16, zoomed box) shows that the single peak detected out of the separation of the full mixture (Figure 15) contains side compounds (see #1, black trace in the zoomed box of Figure 16). The subsequent fractions #2 and #3 show a high purity, whereas in fraction #4, one can already see a rider of the main peak. With this information, it is possible to optimize the time windows to separate even the lowest levels of impurities from the main peak. This is supported by the low dispersion of the fraction collector in combination with the optimized fluidics of the delay capillary, e.g., the asymmetry of 36mer peak with an impurity in the source chromatogram results from the mixture of the individual ON and impurities. Depending on the need, one could do a re-injection of fraction #1 with fraction collection and repeat this several times to enrich the impurity by pooling to have enough material for further impurity analysis. Overall, fraction #2 and #3 for the 36mer and fraction #5 and #6 for the 40mer are the best choice, if high yield fractions at good purity are needed.

When comparing the individual chromatograms of the re-analyzed fractions with the source chromatogram, one can draw a few conclusions regarding the efficacy of the fraction collector.

- One can isolate impurities for further analysis due to the low dispersion and efficient fractionation resolution.
- One can pool pure fractions of target analytes to utilize for further workflow applications with the confidence that impurities will not cause reduction of interference with target analyte activity.

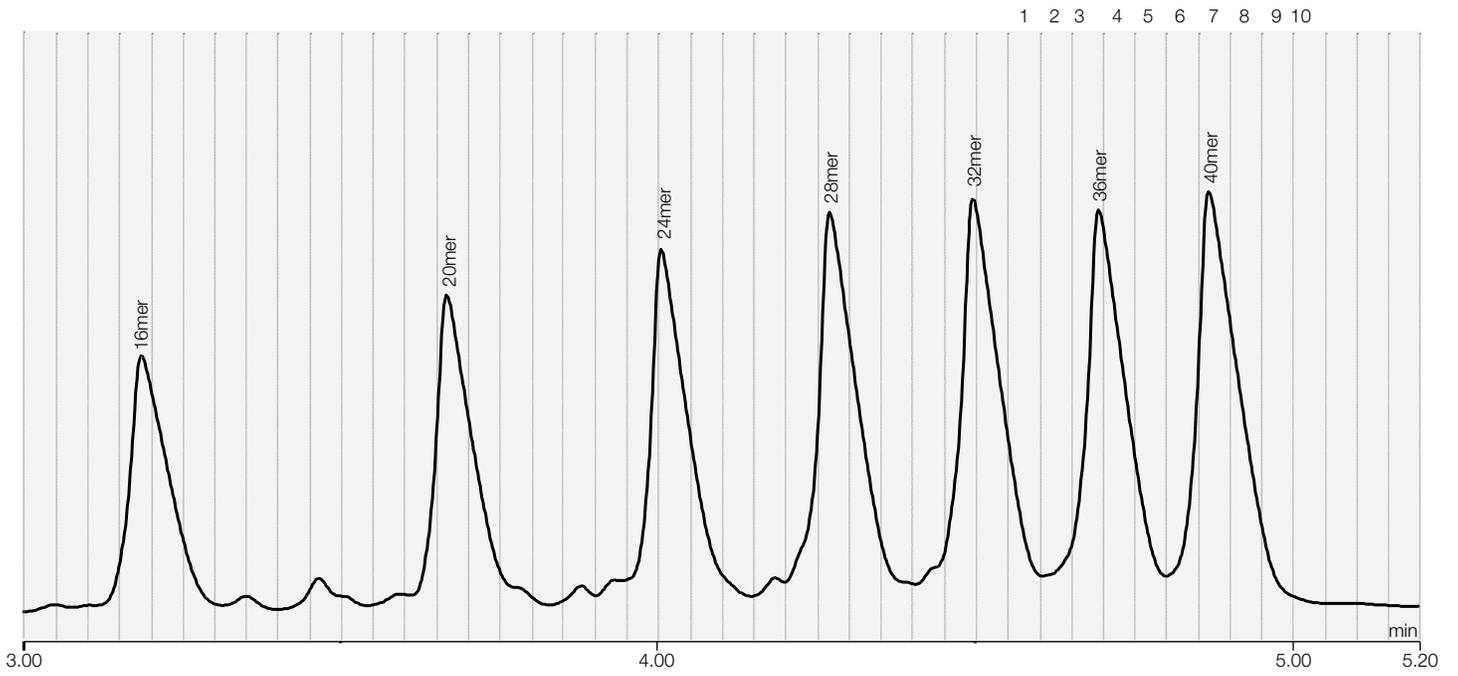


Figure 15. Time-based fractionation of ON mixture with visualization of fraction collection based on software parameters

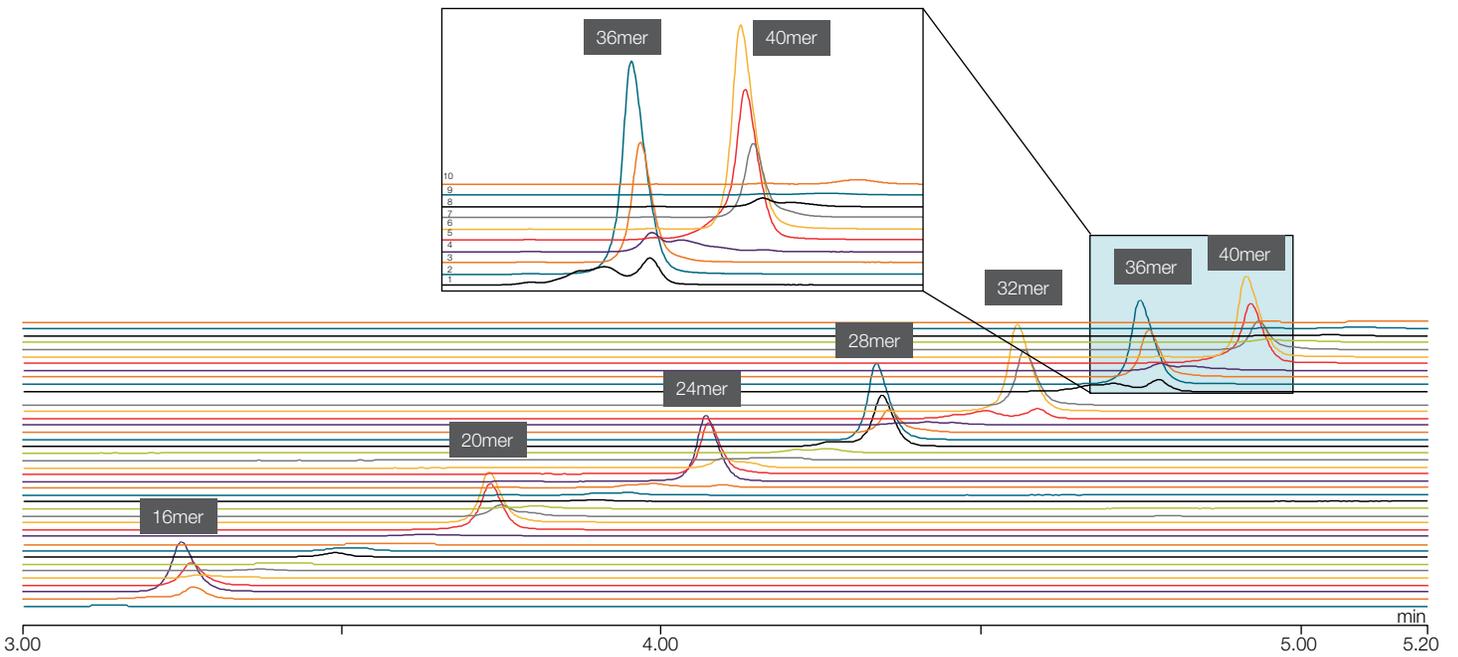


Figure 16. Time-based fractionation of ON mixture and re-analysis of collected fractions

Peak-based fractionation

The same ON mixture was fractionated in a peak-based fashion as described in Table 7. The resulting fractionation of the source chromatogram and subsequent re-analysis is shown in Figure 17.

The re-analysis of the top chromatogram's fractions from Figure 17 shows efficient purification of the individual peaks based on ON length. The high resolution of the ON mixture and efficient fractionation resulted in collection and detection of a single ON peak only. This is achieved by the properties of the VFC such as low dispersion by low delay volume, which has been exactly determined, as well as by the flush, rinse, and wash functions, which eliminated carry-over from previous fractions and has a high impact on purity and recovery. The peak-based collection mode is recommended to be used for separations showing well resolved peaks or for doing the initial method development.

Recovery

The recovery is the ratio between injected amount versus the resulting amount in the fraction. For example, when an absolutely pure sample is injected with a known concentration and is fractionated into one single vial, it is expected that the resulting fraction in the vial contains all of the amount injected from the sample. While travelling through the fluidic pathway of the HPLC, inherently the injected sample volume is diluted through the diffusion effect. Therefore, one needs to account for this dilution effect in terms of the resulting peak volume in the chromatogram calculated by the peak width and flow rate. The simplest method to calculate the recovery is to compare the peak area of the fractionation chromatogram to the area of the fraction

re-analysis. Briefly, one compares the source chromatogram peak area with the resulting re-analysis peak area in combination with the dilution effect. When using the flush function, there is an additional 22 μL dilution that needs to be addressed. Since one normally does not re-inject all of the resulting fraction, the volume of the fractionated sample re-injected versus the source injection volume needs to be considered. All these factors result in the equation shown below using the 16mer of the oligonucleotide purification as an example.

$$V_0 = F \cdot \Delta t = 600 \mu\text{L} \cdot (3.3135 - 3.1468) = 100.0 \mu\text{L}$$

$$\% \text{ Recovery} = \frac{\frac{V_0 + V_f}{V_2} \times A_2 \times 100(\%)}{A_1} = \frac{\frac{100 + 22}{10} \times 1.4743 \times 100(\%)}{18.5923} = 96.74\%$$

Variable	Description	Value
F	Flow rate (mL/min)	0.6
Δt	Collection end time (min) – Collection start time (min)	3.3135–3.1468
V_0	Collection Volume (μL)	100.0 (see below)
V_1	Injection volume for the source fractionation (μL)	20.0
V_2	Injection volume for the re-analysis of the fraction (μL)	10.0
V_f	Flush volume (μL)	22
A_1	Peak area for the source fractionation (mAU*min)	18.5923
A_2	Peak area for the re-analysis of the fraction (mAU*min)	1.4743

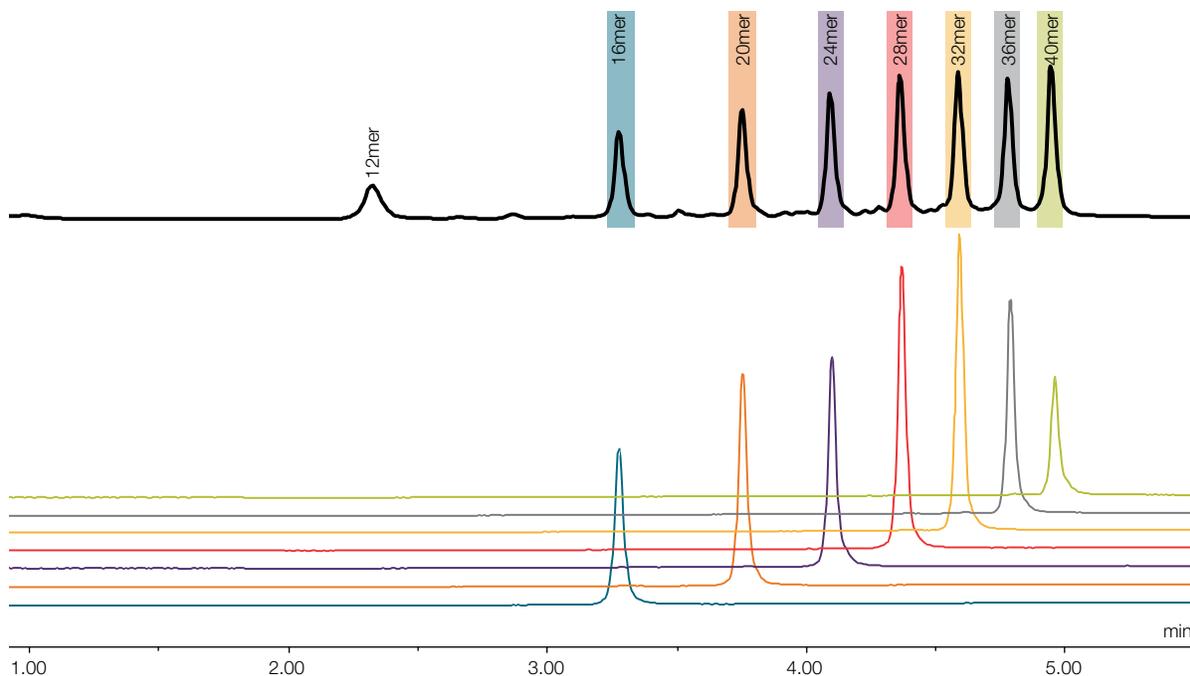


Figure 17. Peak-based fractionation of ON mixture and re-analysis of collected fractions

Conclusion

The efficacy of fractionation can be determined by comparing the sample chromatogram at the detector with the resulting elution profile achieved by re-analyzing the collected fractions. High-performance fraction collectors like the Vanquish Fraction Collector enable fractionation with such high resolution that the resulting elution profile matches exactly the corresponding peaks of the sample chromatogram. This is achieved by optimizing the fluidics, such as implementing low-dispersion capillaries and a fraction collection valve, as well as precise determination of delay times based on delay volume and flow rate by empirical experiments. The exactly determined delay time triggers an ultra-fast fraction collection valve switch. Automated tools to measure the delay volume in an exact way, as well as flush and wash options, allow the separation power of the UHPLC instrument to be transferred to pure fractions with high yield.

References

1. Gamache, P.H. Nebulization. In *Charged Aerosol Detection for Liquid Chromatography and Related Separation Techniques*; Paul H. Gamache/John Wiley & Sons, Inc., 2017; pp. 12–14.
2. Thermo Scientific Vanquish Integral Fraction Collector Operating Manual, version 1.0, July 2022.
3. [Thermo Scientific Chromatography Consumables Catalog - Sample Handling](#)
4. Kromidas, S.; Steiner, F.; Lamotte, S.; Detection Limit, Peak Capacity, Resolution: Possibilities for Gradient Optimization. In *The HPLC Expert: Possibilities and Limitations of Modern High Performance Liquid Chromatography*; Stavros Kromidas/Wiley-VCH, 2016; pp. 156–158.
5. Dittmann, M. The Issue of External Band Broadening in HPLC/UHPLC Devices. In *The HPLC Expert II Find and Optimize the Benefits of Your HPLC/UHPLC*; Stavros Kromidas/Wiley-VCH, 2017; pp. 89–94.
6. Thermo Scientific Vanquish Fraction Collector Product Specification Sheet (PS000922)

Appendix A: Chromeleon CDS fraction collection wizard

1. Begin by creating a new instrument method and enter the pertinent information for the pump, autosampler, column compartment, and detector.
2. General options for fraction collection:
 - a. **Off (do not collect at all):** This will defer all eluting solvent from the LC system through the waste line on the fraction collection valve/diverter valve.
 - b. **Collect by peak:** This will trigger the collection based on chromatographic peak recognition parameters such as threshold, peak slope, etc. defined in the *Peak Detection* section.
 - c. **Collect outside of peaks:** This is selected only in combination with *Collect by Peak* to collect the eluent in fractions before and after recognized peaks. There is the *Collecting Options* section to define either how much volume these between-peak fractions can possess or how much time for each fraction is allotted.
 - d. **Collect by time:** This setting forces the user to set a time slice for each fraction. This parameter will override the *Max Tube Volume* in the *Collecting Options*.
 - e. **Collection time frame:** The default condition for *Start Time* is *Start Run* to begin collection at the time of injection. If a time (in minutes) is specified, all eluent before this entered time goes to waste over the fraction collection valve/diverter valve. Subsequently, the default value for *End Time* is *End Run* and an entered time after the *Start Time* will eliminate any fraction collection after this specified time. *Append Range* adds a new range to the last entry row. *Insert Range* inserts a range above the selected row. *Remove Range* deletes the range. The option to *Flush* can be selected independently for every range for high recovery and low carry-over of fractions.
 - f. **Select injection:** This button will open a dialog box prompting the user to select an example chromatogram on which a simulated fractionation pattern will be presented. In the dialog box, one can navigate into other sequences, select different channels, and injection types.
3. Collection options
 - a. **Pump device:** This parameter defines which module is responsible for determining the flow and thus the corresponding volume in the collection vessels.
 - b. **Max tube volume:** This parameter is used in conjunction with *Collect by Peak* in the *General Options* and defines the maximum volume a collection vessel can contain.
 - c. **Max number of tubes per fraction:** This parameter is used in conjunction with *Collect by Peak* in the *General Options*. After the number of vessels entered in this field is reached for a specific peak being collected, the valve switches to drain and no further fractions of that specific peak will be collected.
 - d. **Total number of tubes:** This parameter defines the absolute number of tubes in the rack(s). After the total number of tubes has been reached in the unfinished run, the needle moves back to the first collection position and continues to collect at that initial position.

- e. **Minimum time for tube change:** This parameter is used in conjunction with *Collect by Peak* in the *General Options* and overrides a tube change triggered by the *Peak Detection* options. This minimum time needs to be reached in order for the needle to move on to the next fraction, whether or not the peak detection options trigger a tube change (Figure 18). This setting can also be used to avoid filling tubes with an insufficient volume when collecting fractions.

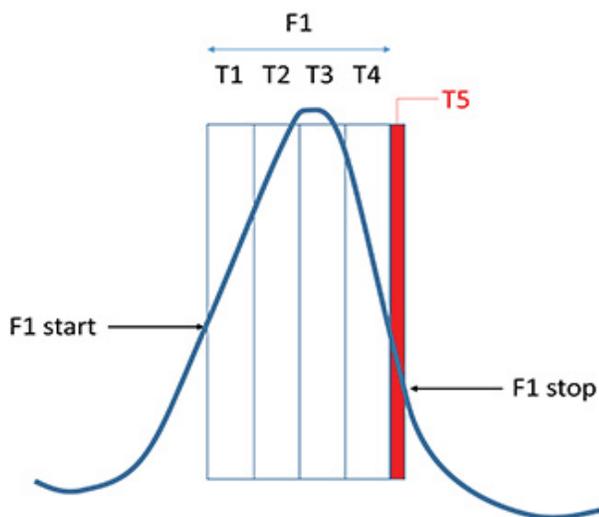


Figure 18. Avoid illogically small fractions by defining a minimum volume a fraction needs to have to proceed to the next fraction. F1 refers to Fraction 1 or the entire peak and T1-T5 refers to the individual tubes or fractions. One wants to avoid a small fraction due to Collect-by-Peak parameters represented by T5. To avoid this small fraction T5, set the Minimum time for tube change to a larger volume.

- f. **Tube wrapping:** When this option is activated, the needle will move back to position 1 in a run after the end of the available vessels has been reached.
- g. **Fraction pooling (Reset the tube position for each run):** When this option is activated, the needle will move always to position 1 for subsequent runs. If this option is not selected, the subsequent run will continue to the position right after the last collected vessel from the prior run.
- h. **Delay time:** This is the difference in time (seconds) when a peak elutes through the detector and arrives at the needle tip or drop former for other models. Hence, it is the difference in time between which the CDS recognizes a collection trigger based on time or peak, and when the fraction collection valve/diverter valve switches from WASTE to COLLECT. It can either be specified in *Delay Time* or *Delay Volume* fields.

- i. **Delay volume:** This is the difference in volume (μL) represented by the delay capillary, fraction collection valve, needle capillary and collection needle volumes. The delay volume is automatically determined for the VFC by the DVD procedure during qualification of the system. For non-integrated FC it needs to be manually calculated and set.
4. Peak detection options – The *Peak Detection Options* page is only available when *Collect by Peak* option is selected.
- a. **Show advanced settings** (located in the bottom left corner): when this box is checked, there are 11 further peak detection options available.
 - b. **Preview—Select injection:** select a chromatogram on which a simulated fraction collection will be portrayed.
 - c. **Channel evaluation:** When in the Instrument Configuration, the fraction collector’s number of detection channels can be set here. The default value is one (1) channel; therefore, the Channel Evaluation cannot be changed. When in the instrument configuration and the number of channels is 2 or more, one can select the channel to be used to trigger the fraction collector here.
 - d. **Channel name:** Select in this field the specific wavelength at which the peak detection is to take place defined by the *Channel Name*. The listed *Channel Name* correlates to the defined channel in the *General Detections Options* of the instrument wizard.
 - e. **Offset time:** *Advanced setting.* This entered time will be added to the total delay and is simply an additional delay factor in seconds between the *Peak Detection* trigger and the switch of the fraction collection valve from WASTE to COLLECT. This option is only available if there is a *Delay time* specified in *Collection Options* and not a *Delay volume*.
 - f. **Offset volume:** *Advanced setting.* This entered volume will be added to the total delay volume and is simply an additional delay factor in μL between the *Peak Detection* trigger and the switch of the fraction collection valve from WASTE to COLLECT. This option is only available if there is a *Delay volume* specified in *Collection Options* and not a *Delay time*.
 - g. **Peak start threshold:** The peak start triggers when the signal value exceeds this entered value and when the *Peak Start Slope* condition has been met. This value can span the range of the signal axis. This value can also be turned off to specify that peaks will be collected by the *Peak Start Slope* only.

- h. **Peak start slope:** The peak start triggers when this slope value is exceeded, and the *Peak Start Threshold* condition has been met. The slope evaluation proceeds after the *Peak Start Threshold* signal has been reached. This value can also be turned off such that the peak start trigger will depend only on the *Peak Start Threshold*.
- i. **Peak start true time:** *Advanced setting.* This parameter sets a window of time in the chromatogram. During this time window, the relevant peak start conditions must be met so that the peak start can be triggered. This time window needs to be met to fully evaluate the peak start. The range is 0.0–4.0 seconds and directly influences the delay time. It accordingly influences the delay volume with the corresponding flow rate. A smaller value would be selected for smaller peak volumes.
- j. **Peak end threshold:** The peak end triggers when the signal value drops below this entered value after a peak maximum has been met. Unlike the peak start conditions, this value is independent of the *Peak End Slope*.
- k. **Peak end slope:** The peak end triggers when the slope value in (-) signal/s, exceeds the value entered. This peak evaluation occurs only above the signal defined by the *Peak End Threshold* and after a peak maximum.
- l. **Peak end true time:** *Advanced setting.* This parameter sets a window of time during the chromatography after a peak maximum condition has been met. During this time window, the relevant peak end conditions must be met so that the peak end can be triggered. The range for the time window is 0.0–4.0 seconds and directly influences the delay time. This time window needs to be met to fully evaluate the peak end. It accordingly influences the delay volume with the corresponding flow rate. A smaller value would be selected for smaller peak volumes.
- m. **Derivative step:** *Advanced setting.* During this interval, the difference in signal is evaluated so that its slope can be determined i.e., Peak Start Slope & Peak End Slope. The larger this value is the more noise will be filtered out and not collected. This range is 0.02–60.00 seconds and directly influences the delay time. It accordingly influences the delay volume with the corresponding flow rate.
- n. **Threshold 'No Peak End':** *Advanced setting.* This value defines the end of a peak based on the precondition that a peak maximum has been met and the signal has fallen below the entered value. This value overrides any *Peak End Slope* trigger after a peak maximum has been reached. This function is used when a detector signal becomes saturated. The noise at saturation generates numerous *Peak End Slope* triggers and the goal is to continue the collection as one peak. Note: when the *Max Tube Volume* is reached, a new fraction vessel is chosen, otherwise the peak is collected in one vessel until the *Threshold 'No Peak End'* is met (Figure 19).

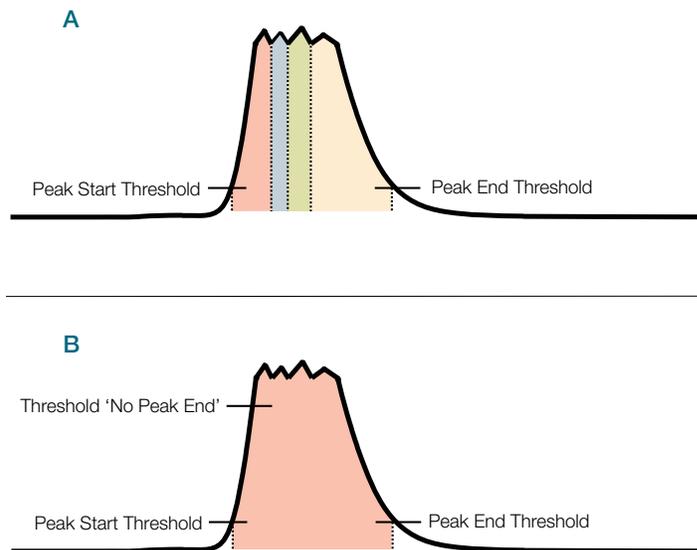


Figure 19. Set the Threshold 'No Peak End' to eliminate subdivisions of an oversaturated peak. (A) The slight valleys subdivide the peak at its saturation point into multiple fractions. (B) Set the Threshold 'NoPeakEnd' below the saturation point, and the plateaued peak will be collected as one peak versus numerous slices. This is only applicable during peak based fractionation.

- o. **Threshold 'Do Not Resolve':** *Advanced setting.* This feature overrides the *Peak End Slope*. It is used when the valley between two peaks does not reach the baseline and one wants to collect everything between two peaks. When this entered signal value is met after a peak maximum is detected, the next fraction will be selected only after the slope reaches 0.0 (Figure 20).

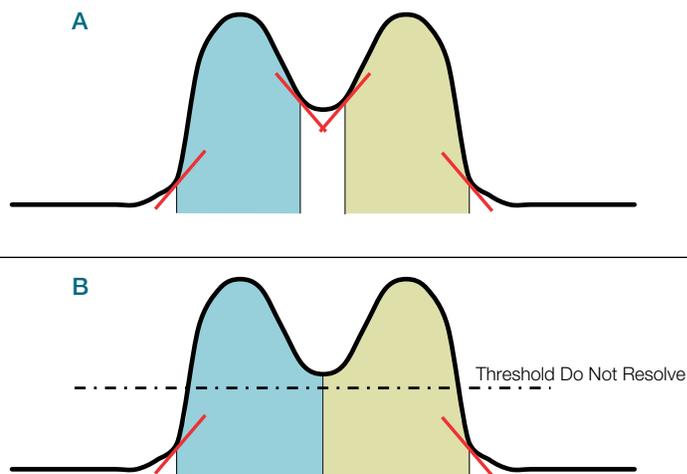


Figure 20. When two fractions do not resolve, there are two options. (A) Two fractions generated with the standard begin slope and end slope settings. This generates a section between the unresolved peaks which does not collect due to the slope settings indicated in red. (B) Set the Threshold 'Do Not Resolve' parameter below the valley between two unresolved peaks to avoid an undesired subdivision in the valley. This is only applicable during peak-based fractionation.

p. **Peak max slope:** *Advanced setting.* When this value is entered in (-) signal/s, the peak maximum will only be associated with a peak whose exit slope is less than the entered value. This avoids the splitting of peaks which have shouldering or long tailing. The greater the negative slope setting, the sharper the peaks need to fall off to have a defined peak maximum condition. The default value is “off”. Note: for a peak that does not meet peak maximum conditions, there can be no peak end trigger. Thus, the peak will continue to be collected until the next peak start trigger has been issued.

q. **Peak max true time:** *Advanced setting.* This defines a time window (0.00–4.00 s) in which the peak maximum conditions must be met for there to be a peak maximum.

r. **Peak start curve:** *Advanced setting.* This threshold is used to detect shoulders on the front side of peaks whose unit is (signal/s²). The condition can only be met after a peak start has been triggered but before a peak maximum has been defined. The higher the value entered the sharper the shoulder needs to be to be recognized as a separate fraction.

s. **Peak end curve:** *Advanced setting.* This threshold is used to detect shoulders on the tailing side of peaks whose unit is (signal/s²). The condition can only be met after a peak start and a peak maximum have been triggered but before a peak end has been defined. The higher the value entered the sharper the shoulder needs to be to be recognized as a separate fraction.

t. **Baseline drift:** The *Baseline Drift* is used in combination with the *Baseline Offset* to compensate the signal value for drift and is a function of time with the unit signal/s. A calculated signal value is extrapolated from the equation: Calculated Value = Signal Value – (BaselineOffset + Δt*BaselineDrift)

u. **Baseline offset:** The *Baseline Offset* is used together with the *Baseline Drift* to correct the signal value in order to compensate for drift. A calculated signal value is extrapolated from the equation: Calculated Value = Signal Value – (BaselineOffset + Δt*BaselineDrift)

5. General settings

a. **Collect mode:** This setting determines how the fraction collector meanders when it reaches the end of a row. The choice is between *Vertical* or *SawVertical*, *Horizontal* or *SawHorizontal* (Figure 21).

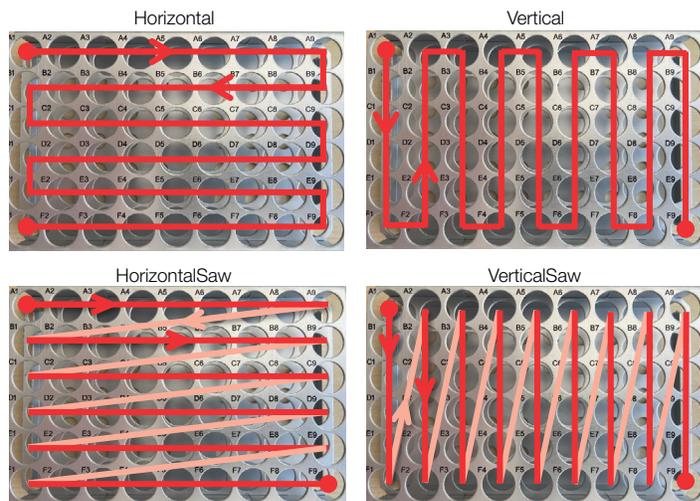


Figure 21. There is a choice between Horizontal or Vertical, SawHorizontal and SawVertical meandering schemes.

- b. **Movement mode:** This setting determines if the fraction collection valve is switched to the WASTE position when moving from one collection position to the next. *Interrupt* switches the fraction collection valve to WASTE between fractions and *Continue* does not. This is especially important to keep in mind when collecting in the *SawVertical* or *SawHorizontal* movement mode, that the setting *Interrupt* avoids contamination while the needle moves the long distance.
- c. **Needle height for vials:** This setting defines the distance between the vial bottom and the needle tip during fraction collection. The recommended setting is *Automatic*, which refers to 10 mm.
- d. **Needle height for well plates:** This setting defines the distance between the well plate bottom and the needle tip during fraction collection. The recommended setting is *Automatic*, which refers to 3 mm.
- e. **Wash mode:** Choose between *NoWash*, *BeforeCollection*, *EndOfCollection*, or *Both* to wash the outer surface of the needle by moving the needle tip into the wash port and switching the fraction collection valve to WASTE.
- f. **Wash speed:** This parameter defines the speed of the wash liquid.
- g. **Wash time:** This parameter defines the speed of the wash liquid.
- h. **Rinse mode:** Choose between *NoWash*, *BeforeCollection*, *EndOfCollection*, or *Both* to wash the inner surface of the needle by moving the needle tip into the wash port and switching the fraction collection valve to COLLECT.
- i. **Use temperature control:** The temperature control can be switched on or off.

Appendix B: Specific properties for non-integrated Thermo Scientific Fraction Collector F/FT

In addition to the Vanquish Integral Fraction Collector FT (P/N VF-F20-A-01, VFC), two non-integrated fraction collector modules are available: The Thermo Scientific™ Fraction Collector F (P/N VF-F11-A-01) and Fraction Collector FT (P/N VF-F10-A-01).

Within this section, details specific to this fraction collector design will be addressed.

Note: Another fraction collector option is only available with the Thermo Scientific™ UltiMate™ 3000 portfolio but will not be discussed in this document.

When reading this Technical Note, readers should be aware that there are some design and use differences between Fraction Collectors F/FT and the VFC.

Differences between Fraction Collector F/FT and Vanquish Fraction Collector

Fraction Collectors F/FT:

- Use a 3 port/2 position diverter valve. This diverter valve switching time is slower and has a larger internal volume than the fraction collection valve of the VFC. These factors impact the delay volume and thus performance (see below).
- Use a drop former and not a needle.
 - Fraction Collector F (P/N [VF-F11-A-01](#)) is equipped with a 1.0 mm ID drop former.
 - Fraction Collector FT (P/N [VF-F10-A-01](#)) is equipped with a 0.4 mm ID drop former.
 - Both fraction collectors can have their drop former kits modified post-purchase.
 - Movement is only possible in the XY-plane.
- Use a bridge Capillary to connect the detector to the diverter valve “sample inlet” port.
- The Fraction Collector FT has Peltier cooling elements to maintain fractions at 4 °C.

Delay

The bridge capillary, diverter valve, and drop former define the delay volume, which needs to be accounted for in the CDS for achieving accurate fractionation. The time needed for diverter valve switching, arm movement and computational time contributes as delay time to the delay, which translates with the actual flow rate into a delay volume. All these parameters are discussed in detail in the following paragraphs.

Bridge capillary

The bridge capillary needs can be optimized for the required flow rate range, especially when working with peak-based triggered fractionation, to allow for a minimum delay time needed for CDS computational time, which is described in Software settings.

Diverter valve

The diverter valve is solely responsible for redirecting the flow between a waste position and a collection position. The diverter valve contains an L-joint, which connects sample inlet and waste or sample inlet and drop former.

Drop former

The Fraction Collector F/FT has the option to choose between two drop formers depending on the intended fraction volume, which can vary from a few microliters to several milliliters. The volume of the drop formers is listed below and contributes to the overall delay volume.

- 0.4 mm I.D. drop former = 1.3 µL
- 1.0 mm I.D. drop former = 4.8 µL

Arm movement

The arm delivers the fluidics to an XY-plane. The user can decide between *Vertical* or *SawVertical* Movement mode. The delay time in moving the arm is depending on necessary movement steps depending on vessel positions.

Software settings

The delay time is a combination of the physical delay from detector to FC, as well as computational time of the Chromeleon CDS (i.e., the time taken for the CDS to react to a peak or trigger). The software automatically adds a 3 s delay to compensate for the computational time and the time taken for the diverter valve to position itself correctly in space and switch from DRAIN to COLLECT. It can either be specified in the Delay Time or Delay Volume fields.

A minimum delay time is needed to allow the instrument method ready check to pass, which is calculated by:

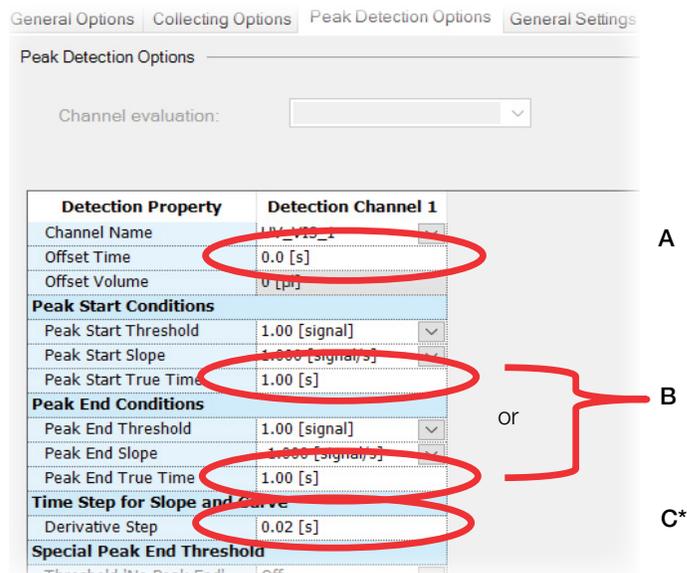
$$\text{Minimum delay time} = \text{OffsetTime} + \text{DerivativeStep} + \text{Max}(\text{PeakStartTrueTime or PeakEndTrueTime}) + 3 \text{ seconds}$$

The Delay Time or corresponding Delay Volume must be greater than or equal to this calculation. The length of the bridge capillary is matched to reflect the required delay time determined by the equation above. If the minimum delay time is not achieved, the ready check of the instrument method will fail, and an adoption of times according to Figure 22 is needed.

Recommendations for analytical fractionation bridge capillaries with the corresponding Chromeleon CDS *Delay Volume* settings, as well as the *Derivative Step* for peak recognition, are important for optimized setup and can be found in the [Fraction Collector F/FT Operating manual](#) and Table 8.

Table 8. Recommended capillaries and Chromeleon CDS settings for given application flow rate ranges with the Fraction Collector F/FT (P/N VF-F11-A-01 and VF-F10-A-01).

Capillary color	Capillary ordered length	Actual length (± 0.5 cm)	Compatible flow rate range (mL/min)	Derivative step value (s)	Chromeleon 7.2 CDS delay volume (µL)
Red (125 µm I.D.)	152.4 cm (5 ft)	157.5 cm (5.17 ft)	0.5–0.8	0.02	55
Blue (250 µm I.D.)	152.4 cm (5 ft)	157.5 cm (5.17 ft)	0.5–1.2	0.02	86
Orange (500 µm I.D.)	152.4 cm (5 ft)	157.5 cm (5.17 ft)	1.2–5.0	0.02	334



$$A + B + C + 3 = 4.1s$$

*Adjusted from 1.0 (s)

Figure 22. The Delay Time is the sum of OffsetTime + DerivativeStep + Max(PeakStartTrueTime or PeakEndTrueTime) + 3 s.

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