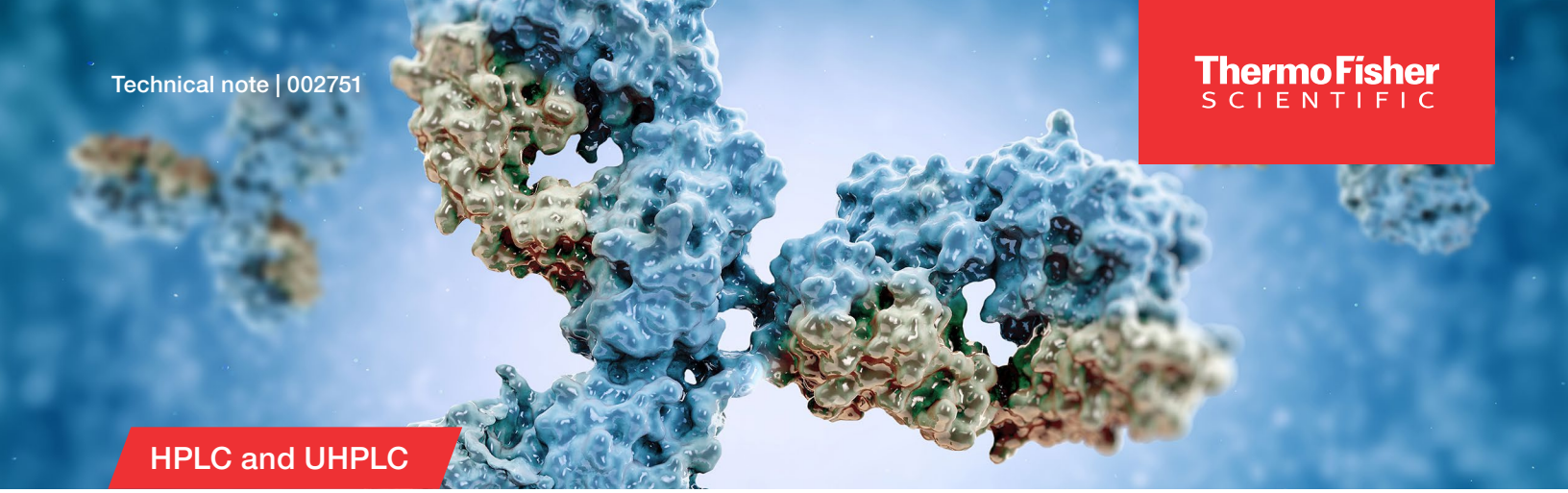

Technical note | 002751

HPLC and UHPLC

Setting the start position for flexible fraction collection by using custom variables in Chromeleon CDS

Authors

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Keywords

Vanquish UHPLC, fraction collector, collection start position, custom injection variables, Chromeleon CDS

Goals

To show that the use of custom variables, easily applied through the user-friendly Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), can greatly improve operation flexibility

Introduction

Driven recently by the vigorous growth of biopharmaceutical, pharmaceutical, and chemical industries, an urgent need for the extraction of active or rare components in complex matrixes has emerged.[1] The Thermo Scientific™ Vanquish™ Fraction Collector, which is fully integrated into the Thermo Scientific™ Vanquish™ Analytical Purification LC system, can achieve fully automated fraction collection from analytical to semi-preparative flow rates (up to 10 mL/min). The achievable fraction volume is extremely small with drop volume down to just 6 µL due to the fraction needle design. In addition, the fraction collection valve is designed so that residual fraction volume in the collection needle capillary and needle is pushed out into the fraction vessel by a “flush”, resulting in very low cross contamination rates of less than 0.15%.[2]

The highlights of the purification hardware performance can only be achieved through a robust and simple yet flexible and multi-faceted chromatography data system. From the instrument method wizard to the sequence control and the data processing, the Chromeleon CDS provides rich versatility for specific fraction collection needs to address seamless integration into the purification workflow. Where and how a compound is isolated in fractions can simplify further downstream processes or analytics.

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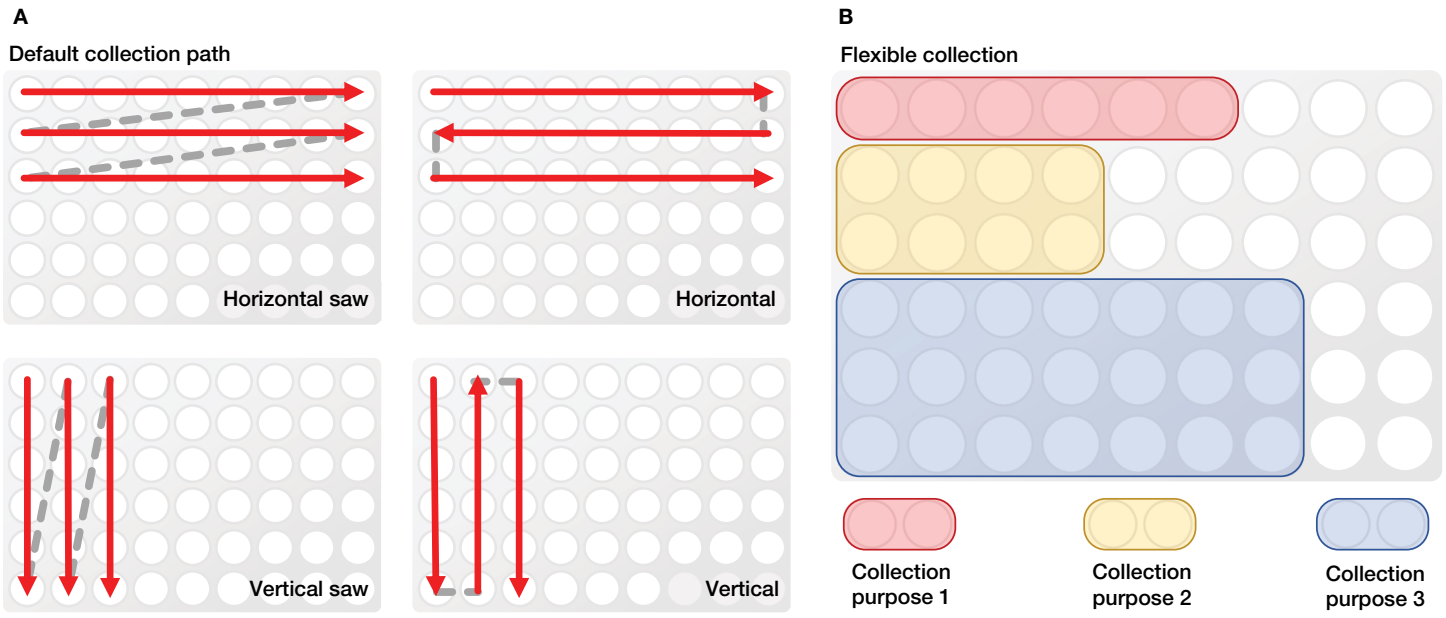


Figure 1. The default collection path (A) and customized flexible collection paths needed for different collection purposes (B)

Generally, the default path for automatic fraction collection is one of the four modes "HorizontalSaw, Horizontal, VerticalSaw, or Vertical," as shown in Figure 1A, which address most of the collection needs. However, some specific applications might require more customized collection paths/areas beyond the above-mentioned default modes, as shown in Figure 1B. As shown in this technical note, the system is extremely flexible. For example, the simple insertion of a custom variable into the injection list through the user-friendly interface can be used to customize the starting position of fraction collection. Monoclonal antibody (mAb) samples were used to validate multiple collection modes, including single-component, multi-component, and peak-based fraction collection. An operation protocol for setting custom-defined fraction start positions is provided, facilitating flexibility, ease of use, and efficiency in the purification process.

Experimental

Chemicals

The analytical grade reagents—disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), formic acid (FA) (99.7% in water)—were purchased from Sigma-Aldrich without further purification. Ultrapure water of 18.2 MΩ·cm was produced by a Milli-Q™ IQ 7000 ultrapure lab water system. A 20 mM phosphate buffered saline (PBS) was prepared in house. The mAb sample was produced by Shanghai OPM Biosciences, China.

Sample preparation

A stock solution of mAb sample at a concentration of 15 mg/mL in water was stored at -80 °C. After bringing to room temperature, it was diluted with water by a factor of 10 to 1.5 mg/mL for separation method development. Additionally, another 1.0 mL stock solution was transferred directly to a 2.0 mL vial for high concentration analysis and fraction collection.

Instrument configuration

The configuration of the Vanquish Analytical Purification LC system is described in Table 1 and includes a binary pump, autosampler, column compartment, UV detector, and fraction collector, and is controlled by the Chromeleon Chromatography Data System 7.3.1 and newer.

Chromatographic separation conditions and instrument settings

The mobile phase and typical gradient conditions are listed in Table 2. The critical instrument parameters are summarized in Table 3. The detailed fractionation method is summarized in Table 4.

Table 1. The configuration of the Vanquish Analytical Purification LC system

Description	Part number
Vanquish System Base Horizon/Flex	VF-S01-A-02
Vanquish Binary Pump F	VF-P10-A-01
Vanquish Split Sampler FT	VF-A10-A-02
Vanquish Column Compartment C	VC-C10-A-03
Vanquish Diode Array Detector CG	VC-D11-A-01
Vanquish Flow Cell, Bio, 2.5 µL, 7 mm, 120 bar	6083.0550
Column, Thermo Scientific™ ProPac™ WCX-10 4 × 250 mm, 10 µm	054993
Vanquish Integral Fraction Collector FT	VF-F20-A-01
Delay Capillary for Time-based Fractionation (0.10 × 350 mm)	6042.2340
Vial, 2 mL Screw 9 mm Transparent Glass	6PSV9-1PSS
Cap Screw, 9 mm, Blue PP with Silicone/Red PTFE Septa	6PSC9ST1R
Thermo Scientific™ SureSTART™ Intubation Vial, Flat Bottom, Transparent Glass 0.5 mL	6PME05F1

Table 2. Solvents and additives

Solvent	Composition		
Mobile phase A	20 mM aq. PBS, pH 6.0		
Mobile phase B	20 mM aq. PBS, pH 6.0, 300 mM NaCl		
Wash solvent	0.1% aq. Formic acid		
Flush solvent	Mobile phase B		
Typical gradient program	Time (min)	%A	%B
	-5.0	88.0	12.0
	0.0	88.0	12.0
	12.0	5.0	95.0
	16.5	5.0	95.0
	16.6	88.0	12.0

Table 3. Vanquish Analytical Purification system parameters

Module	Content	Parameters
Pump	Flow rate	1.0 mL/min
Autosampler	Temperature	15 °C
	Injection volume	10 µL
Column compartment	Temperature	35 °C
VWD	Data collection rate	5 Hz
	Wavelength	220 nm
Fraction collector	Temperature	10 °C
	Collection mode	By Time
	Needle positioning mode	InVial
	Flush	On
	Collection path mode	Horizontal
	Start position	By Custom Variable

Table 4. Typical fractionation parameters

Fractionation target	Total fractions in one injection	Collection general option settings			Start position
Single component	1	Timetable for collection			R: A1
		#	Start time	End time	
		F 1	9.590	9.890	
Multi component	4	Timetable for collection			R: B1
		#	Start time	End time	
		F 1	9.590	9.890	
		F 2	8.900	9.100	
		F 3	9.590	9.890	
		F 4	10.970	11.170	
Sliced fraction	8	Timetable for collection			R: D1
		#	Start time	End time	
		F 1–8	9.100	9.900	
		Collection period: 6 s			

Setting fraction start position

Tip: A prerequisite of this technical note is that a sequence list and instrument method including the fractionation step set up must already exist. Chromeleon CDS provides an intuitive process to generate a new sequence as well as an instrument method wizard to walk users through the various module parameters and fraction collection settings. A brief overview of the sequence and instrument method view can be found in the Vanquish Analytical Purification LC System Product Spotlight[3] and a comprehensive explanation of the fraction collection parameter settings can be found in the Principles of Fraction Collection Using the Vanquish HPLC and UHPLC Systems[2]. This section explains how to set a customized fraction start position.

Step 1. Insert fraction collection customer variables.

In Chromeleon CDS, right-click on the injection list header to insert a “Custom Variable” as shown in Figure 2. Enter the column name as “Frac_Start” and confirm the variable type is “Text” (Figure 3). After clicking the “Finish” button, a new column of “Frac_Start” will appear in the injection list (Figure 4).

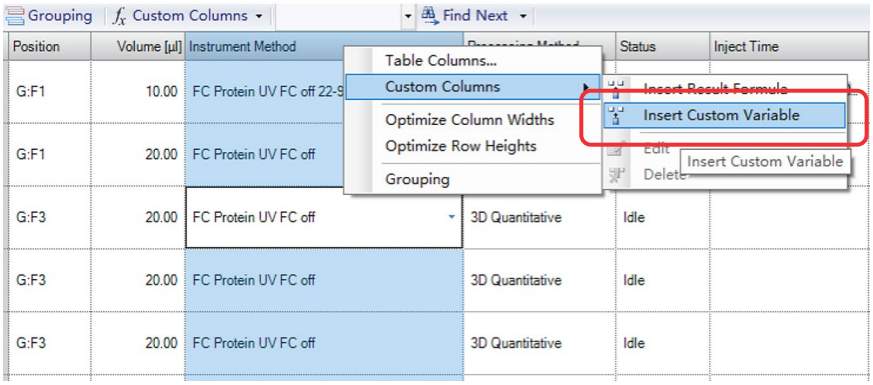


Figure 2. Insert a column of custom variable.

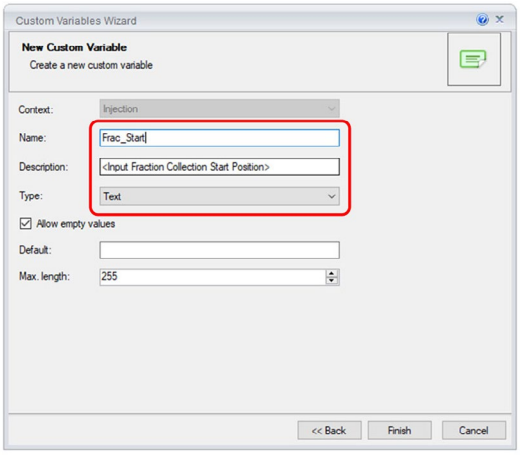


Figure 3. Select the variable type as “Text”.

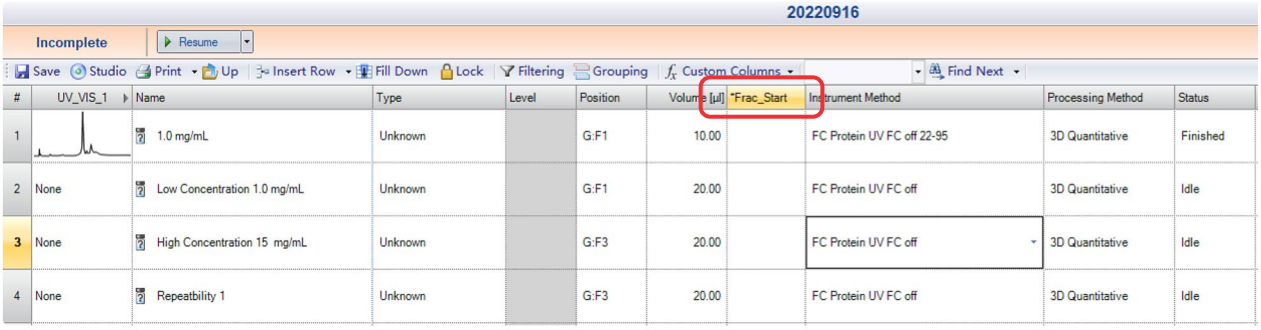
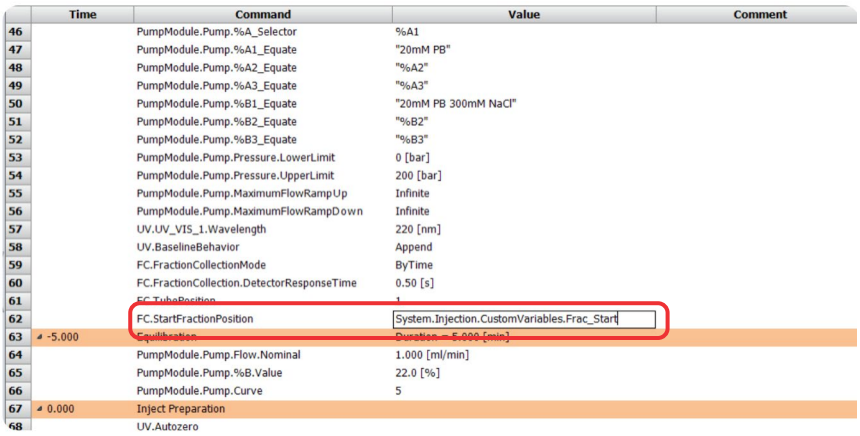


Figure 4. The column "Frac_Start" for entering the value by a custom variable is created.

Step 2. Edit script in the instrument method.

Open the “Instrument Method”, select the “Script Editor” to insert a command for fraction start position. Right-click at the command line, which is just before “Equilibration” and “Injection preparation” in the first section “{initial time}”. Type or choose the module to input the following command and value: “FC.StartFractionPosition” and “System.Injection.CustomVariables.Frac_Start” (Figure 5). (Note: the names of “Custom Variable” in the injection list and script editor should be same.)

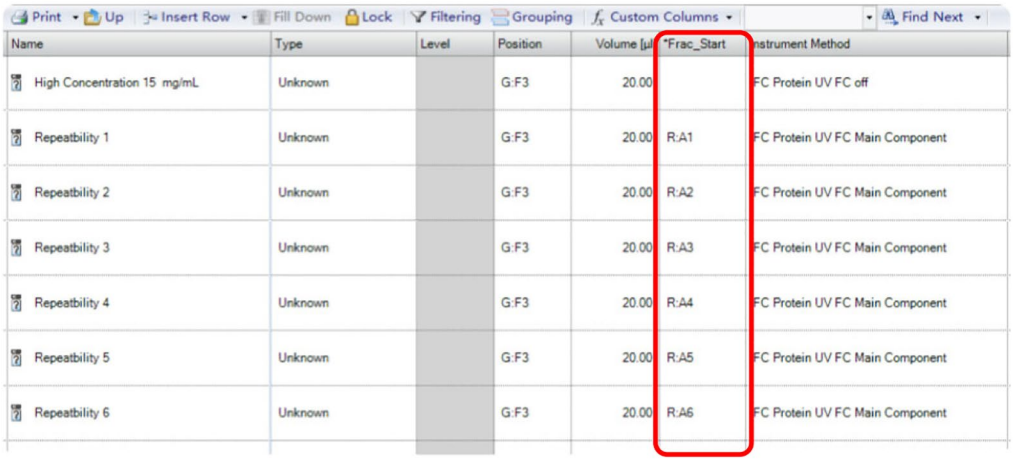


	Time	Command	Value	Comment
46		PumpModule.Pump.%A_Selector	%A1	
47		PumpModule.Pump.%A1_Equate	"20mM PB"	
48		PumpModule.Pump.%A2_Equate	"%A2"	
49		PumpModule.Pump.%A3_Equate	"%A3"	
50		PumpModule.Pump.%B1_Equate	"20mM PB 300mM NaCl"	
51		PumpModule.Pump.%B2_Equate	"%B2"	
52		PumpModule.Pump.%B3_Equate	"%B3"	
53		PumpModule.Pump.Pressure.LowerLimit	0 [bar]	
54		PumpModule.Pump.Pressure.UpperLimit	200 [bar]	
55		PumpModule.Pump.MaximumFlowRampUp	Infinite	
56		PumpModule.Pump.MaximumFlowRampDown	Infinite	
57		UV.UV_VIS_1.Wavelength	220 [nm]	
58		UV.BaselineBehavior	Append	
59		FC.FractionCollectionMode	ByTime	
60		FC.FractionCollection.DetectorResponseTime	0.50 [s]	
61		FC.TubePosition	1	
62		FC.StartFractionPosition	System.Injection.CustomVariables.Frac_Start	
63	-5.000	Equilibration	Duration = 5.000 [min]	
64		PumpModule.Pump.Flow.Nominal	1.000 [ml/min]	
65		PumpModule.Pump.%B.Value	22.0 [%]	
66		PumpModule.Pump.Curve	5	
67	0.000	Inject Preparation		
68		UV.Autozero		

Figure 5. Define the start position of the fraction collection in the method editor.

Step 3. Enter the start position of fraction collection.

Input the fraction collection position in the same format as the column “Position” for the injection into the “Frac_Start” column, as shown in Figure 6. Then, click the “Start” or “Resume” button to run the sequence.



Print | Up | Insert Row | Fill Down | Lock | Filtering | Grouping | Custom Columns | Find Next

Name	Type	Level	Position	Volume [µl	*Frac_Start	nstrument Method
High Concentration 15 mg/mL	Unknown		G:F3	20.00		FC Protein UV FC off
Repeatbility 1	Unknown		G:F3	20.00	R:A1	FC Protein UV FC Main Component
Repeatbility 2	Unknown		G:F3	20.00	R:A2	FC Protein UV FC Main Component
Repeatbility 3	Unknown		G:F3	20.00	R:A3	FC Protein UV FC Main Component
Repeatbility 4	Unknown		G:F3	20.00	R:A4	FC Protein UV FC Main Component
Repeatbility 5	Unknown		G:F3	20.00	R:A5	FC Protein UV FC Main Component
Repeatbility 6	Unknown		G:F3	20.00	R:A6	FC Protein UV FC Main Component

Figure 6. Input the collection vial position in the “Frac_Start” column, where the fractionation will start.

Results and discussion

UHPLC method development for separation

The optimized method showed that the crude mAb sample contains a main component presenting as a broad peak at 9.7 min (Figure 7A) as well as acidic (<7.0 min) and alkaline (>10.0 min) impurities. Although the sample concentration was increased 10-fold, with a maximum signal as high as 2,000 mAU, excellent resolution was still obtained as shown in Figure 7B. Thus, the Vanquish UHPLC system with ProPac WCX-10 column offers a reliable separation for protein samples even when high sample concentrations are analyzed.

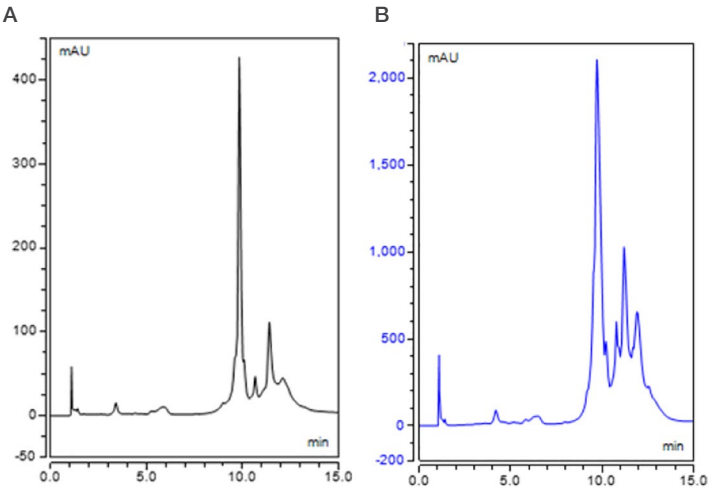


Figure 7. The typical IEX chromatograms of mAb protein sample at 1.5 mg/mL (A) and 15 mg/mL (B)

Repeatability of UHPLC and single-component collection

The repeatability of the chromatographic system and separation chemistry ensure the purity, precision, and reproducibility for fraction collection. Hence, the crude mAb sample was fractionated six times, successively (Figure 8).

The peak at 9.667 min is mAb with average peak area of 38.9%, and its retention time RSD of mAb was 0.06%, as listed in Table 5. The obtained fractions were then reanalyzed using the same condition used for validating the fractionating repeatability.

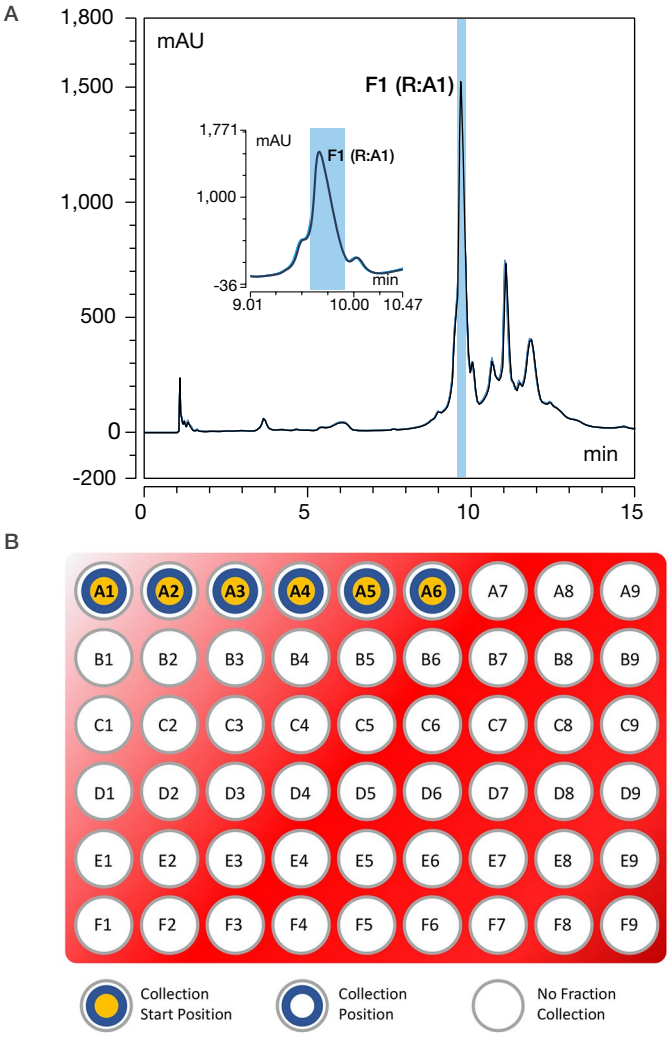


Figure 8. Overlaid chromatograms of six replicates (A) and setting of six different positions to start fraction collection (B)

Table 5. The repeatability of mAb chromatography on a Vanquish UHPLC (n=6)

Injection name	Ret. time (min)	Rel. area (%)	Area (mAU*min)	Height (mAU)
Iteration 1	9.667	39.19	336.63	1433.39
Iteration 2	9.665	39.04	334.82	1430.46
Iteration 3	9.663	39.14	335.22	1433.47
Iteration 4	9.662	38.98	334.61	1428.60
Iteration 5	9.655	38.64	334.50	1432.84
Iteration 6	9.653	38.58	334.18	1429.70
RSD	0.06%	0.43%	0.26%	0.15%

The re-analysis results for the collected main component are illustrated in Figure 9 and showed excellent repeatability. Table 6 summarizes the retention time and peak area of the re-analysis results (n=6). Taken together these data confirm that the obtained high purity mAb fraction can be used for further research.

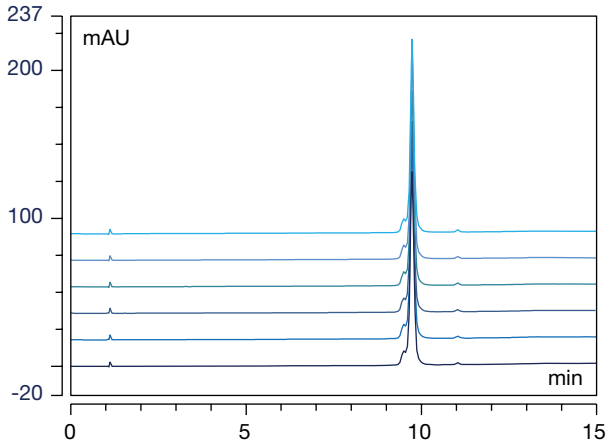


Figure 9. The repeatability of re-analysis of mAb (n=6)

Table 6. The repeatability of re-analysis of the collected main component (n=6)

Injection name	Ret. time (min)	Rel. area (%)	Area (mAU*min)	Height (mAU)
FC Reinjection 1	9.732	93.56	17.60	130.56
FC Reinjection 2	9.732	93.54	17.51	129.90
FC Reinjection 3	9.733	93.66	17.37	128.81
FC Reinjection 4	9.733	93.63	17.50	131.17
FC Reinjection 5	9.735	93.59	17.42	131.03
FC Reinjection 6	9.737	93.50	17.27	130.37
RSD	0.02%	0.06%	0.66%	0.66%

Multi-component collection

By setting the starting position of each injection, the multi-components collection can be easily repeated, as shown in Figure 10, making the operation more convenient, intuitive, and efficient.

It can be seen in Figure 11 that four pure compounds are successfully collected, with symmetrical peak shape and high purity.

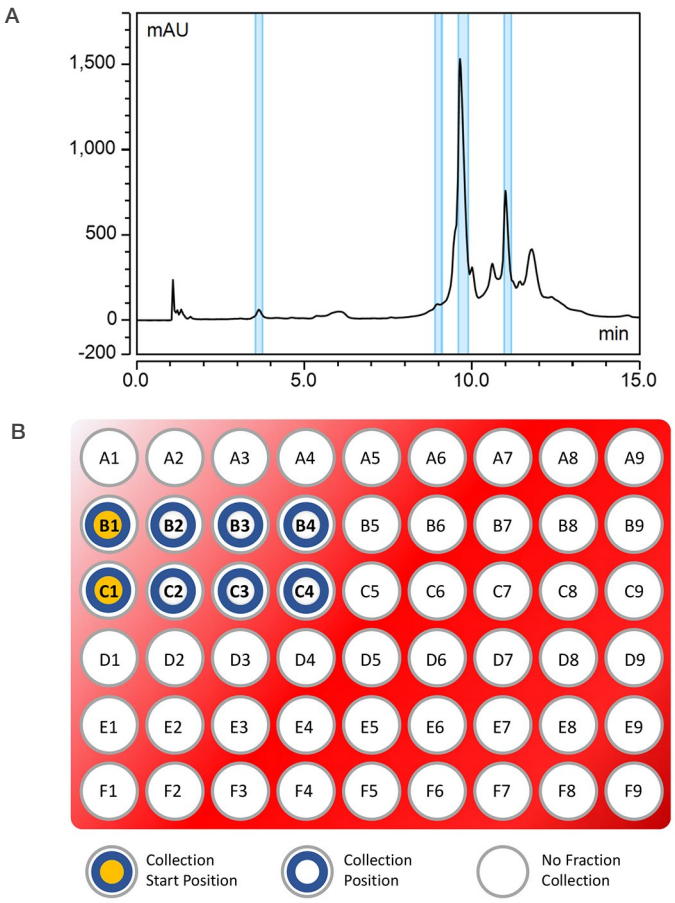


Figure 10. Multi-component fraction collection (A) and setting position to start fractionating (B)

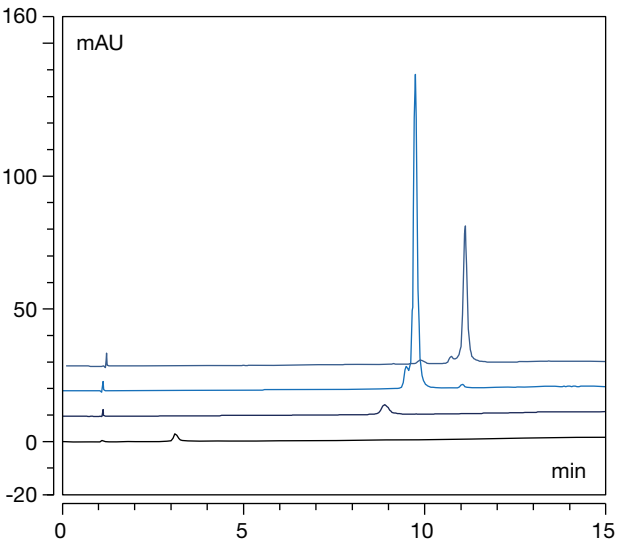


Figure 11. The re-analysis of four fractions in a multi-component collection

Sliced fraction collection

The strategy of slicing collection and re-analysis is used to verify the purity of a peak, determine the collection window, and obtain purer products. By setting the start position, collection window, and collection period easily in Chromeleon CDS, the Vanquish Fraction Collector automatically slices and collects the fraction. Figure 12A shows an example of slicing the main component (peak width 48 s) for a collection period of 6 s; the mAb peak is split into eight sections (Figure 12B) and repeated three times.

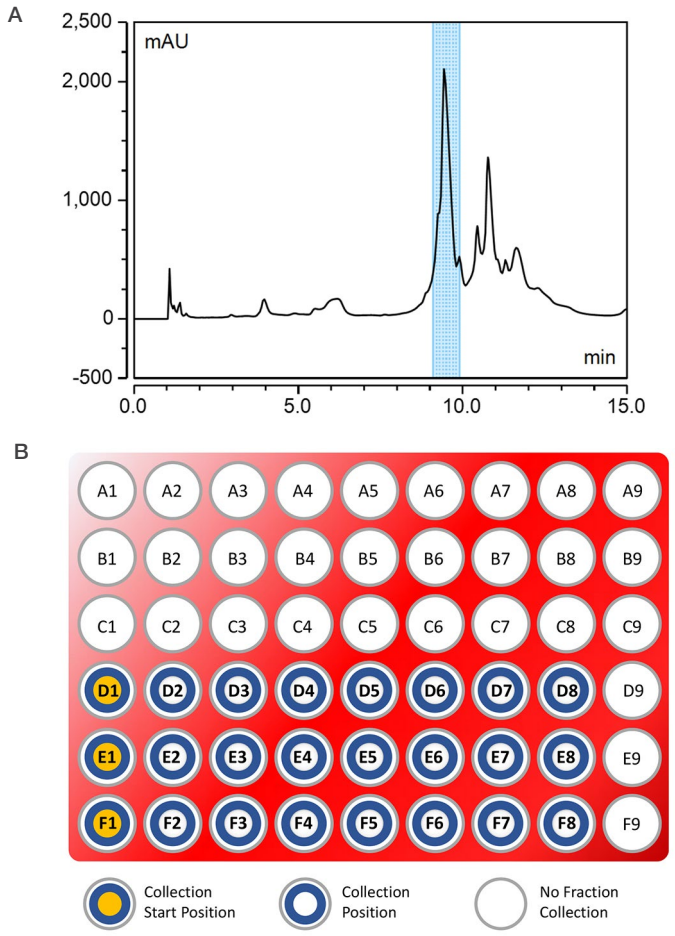


Figure 12. Slicing the main component (A) to eight sections and setting the fraction collection start position at R:D1 (B)

The re-analysis results presented in Figure 13 show that by setting the collection position, it is convenient to slice the main peak. The split peaks in the first and second sliced fractions indicate the presence of acidic peaks that were hidden in the original mAb main component peak. This flexible fractionation provides a simple method for peak purity validation.

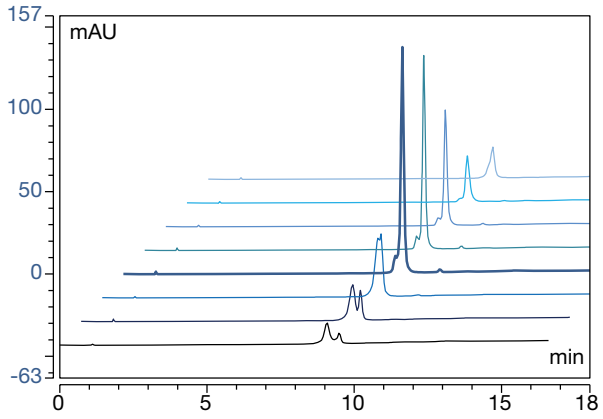


Figure 13. The reinjection of sliced collection of mAb

Conclusions

The Vanquish Fraction Collector controlled by Chromeleon CDS enables excellent purification combined with the flexibility of single-component, multi-component, and biocompatible collection. The use of the collection start position custom variable, combined with rapid and accurate flow control, lets scientists reliably obtain purer samples for further research.

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