

Pharma

Semi-preparative reversed-phase liquid chromatographic purification of oligonucleotides

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Application benefits

- Incorporation of the Thermo Scientific™ Vanquish™ LC platform into the production workflow of an industry-leading pharma company, Eurofins Genomics Europe Pharma and Diagnostic Products & Services Synthesis GmbH, Ebersberg, Germany, for reversed phase purification of customer-defined oligonucleotides.
- Highlighting the flexibility of the Thermo Scientific™ Vanquish™ UHPLC and HPLC systems, which can run not only under UHPLC analytical conditions but are also covering semi-preparative LC purification applications using the integrated Thermo Scientific™ Vanquish™ Fraction Collector.
- Highly precise isolation of target synthetic products using the Thermo Scientific™ Vanquish™ Analytical Purification LC system.

Goal

- To provide an easy-to-follow outline to purify synthetic oligonucleotides and re-analyze the resulting purification by reversed phase semi-preparative liquid chromatography (RP-LC).
- To exemplify the seamless integration of the Vanquish Analytical Purification LC system into the development of oligonucleotide purification and analysis.

Introduction

Nucleic acids are the key to life. Deoxyribonucleic acid (DNA) carries the genetic information, and ribonucleic acid (RNA) plays an important role in general cellular processes, such as transmitting genetic information, regulating gene expression, and catalyzing chemical reactions. During the last few years, oligonucleotides have gained increased interest in the field of biochemical research and diagnostics and as pharmaceuticals,¹ which is why great effort was made to optimize and automate their synthesis. Oligonucleotide synthesis involves a large number of individual reactions, which leads unavoidably to the accumulation of impurities such as truncated sequences. Therefore, the purification of the desired oligonucleotides is a crucial step. Since the 1970s, several chromatographic approaches have been used to analyze and purify synthetic oligonucleotides.² Significant developments in terms of instrument performance and stationary phase have been made during the past years.³ High-performance liquid chromatography (HPLC) is the method of choice if high-purity products are desired; among the separation techniques commonly used for nucleic acid separation is reversed-phase (RP-HPLC).⁴ When developing the methods to separate and purify oligonucleotides, some of the unique features of these molecules need to be considered such as failure sequences of similar length, secondary structures (e.g., hairpin loops formation), pH and salt concentration (ionic strength), as well as other additives and variables. All these factors can additionally affect retention time and altering the interaction with the stationary phase.²

Eurofins (<https://www.eurofinsgenomics.eu/>) is an internationally leading provider of DNA sequencing services, genotyping services, DNA and RNA synthesis products, as well as bioinformatics services for pharma, diagnostics, food, agriculture, biotechnological, and research markets. The company's strength is its high-quality services in industrial scale for the life science industries and institutions around the world. Eurofins offers in its production facilities in Germany oligonucleotide and gene synthesis services with ISO 13485 certification for industrial customers using semi-preparative and preparative RP-HPLC. The increasing significance of oligonucleotides as therapeutic agents elevates the level of quality control. In such protocols, chromatographic analysis of active pharmaceutical ingredients (API) is required to ensure the detection of contaminants at concentration levels down to trace amounts relative to the drug.⁵ Highly automated workflows for high-throughput production allow shipping of key products in less than 24 hours. Eurofins has also recently invested heavily in R&D activities, with a focus on developing new methods and implementing new technology and higher degrees of automation.

This work demonstrates the semi-preparative reversed-phase liquid chromatographic purification of three different dual-labeled 25mer oligonucleotides. Dual-labeled oligonucleotides are common in markets such as:

- quantitative polymerase chain reactions (qPCR), a technology used for measuring DNA using PCR,
- integrated human identification (HID) solutions for forensics,
- gene transfer agents (GTA) that make horizontal and lateral DNA transfer possible.

The goal is to resolve byproducts of the DNA synthesis, isolate and capture the target oligonucleotides, and determine the effectivity of the purification via re-analysis of the collected fractions all on the Vanquish Analytical Purification LC system facilitated by the integrated Vanquish Fraction Collector.

Experimental

Chemicals

- Deionized water, 18.2 M Ω -cm resistivity or higher by Thermo Scientific™ Barnstead™ GenPure™ x CAD Plus UV/UF-TOC (P/N 50136146)
- Triethylamine Acetate (TEAA) 2.0 M, Applied Biosystems™ (P/N 40-061-3)
- Acetic acid, Optima™ LC/MS, Fisher Chemical™ (P/N A11350)
- Acetonitrile, Optima™ LC/MS Grade, Fisher Chemical™ (P/N A955-4)
- Methanol, Optima™ LC/MS Grade, Thermo Scientific™ (P/N A456-212)
- Custom DNA synthesis supplied by Eurofins Genomics Germany GmbH
 - [CY5]-CAGGTGGAACCTCATCAGGAGATGC-[BHQ2] (Cy5-BHQ2), 1 μ mol
 - [AT647]-CGTGGTTGACCTACACAGGTGCCATCA-[BHQ2] (AT647-BHQ2), 1 μ mol
 - [FAM]-ACCCCGCATTACGTTTGGTGGACC-[BHQ1] (FAM-BHQ1), 1 μ mol

Sample handling

- Pipette, 100–1,000 µL
- Thermo Scientific™ WebSeal™ 96 Deepwell Plate, square wells, with barcode (P/N 60180-P105B)
- Thermo Scientific™ MicroMat™ CLR Silicone Mat, 96 square wells (P/N 60180M121)
- Thermo Scientific™ Convenience Kit, 2 mL Certified 9 mm Wide Opening SureStop Vial (P/N CERT5000-575W)
- Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)

Sample preparation

Each of the three dual-labeled oligonucleotide sequences were synthesized and lyophilized by Eurofins Genomics Germany GmbH. Each sample was dissolved by pipetting 2.5 mL deionized water to make each a 400 µM solution. The samples were vortexed to aid dissolution.

Instrumentation

Vanquish Flex Analytical Purification LC system comprising:

- Vanquish System Base Horizon/Flex (P/N VF-S01-A)
- Vanquish Binary Pump F (P/N VF-P10-A-01)
- Vanquish Split Sampler FT (P/N VF-A10-A)
- 1000 µL sample loop (P/N 6850.1980)
- Vanquish Column Compartment H with active pre-heater (P/N VH-C10-A-02)
- Vanquish Diode Array Detector FG (P/N VF-D11-A-01)
- Semi-Micro Biocompatible Flow Cell (P/N 6083.0550)
- Integral Vanquish Fraction Collector (P/N VF-F20-A-01)
- Delay capillary for peak-based fractionation, 0.25 × 1500 mm, MP35N, Viper (P/N 6706.1110)
- Needle capillary, 0.18 × 415 mm, partially PEEK-shielded MP35N, Viper (P/N 6706.1010)
- Flush buffer loop, 50 µL, PEEK, Viper (P/N 6706.1070)

Chromatography Data System

The Thermo Scientific™ Chromeleon™ Chromatography Data System version 7.3.1 (CDS) was used for data acquisition and analysis.

Table 1. LC method

Parameter	Value		
Column	C18 prep column, 130 Å, 5 µm, 10 mm × 100 mm		
Mobile phase A	50 mM TEAA, pH 7 with 5% acetonitrile (v/v)		
Mobile phase B	Acetonitrile		
Run time	40.0 min		
Flow rate	5.0 mL/min		
Gradient	Time (min)	%A	%B
	0.0	100	0
	10.0	85	15
	31.0	50	50
	32.0	5	95
	34.0	5	95
	35.0	100	0
40.0	100	0	
Column temperature	60 °C, forced air		
Active pre-column heater temperature	60 °C		
Autosampler temperature	5 °C		
Autosampler wash solvent	10% methanol in water		
Injection volume	500 µL		
Detection	260 nm, 4 nm bandwidth, 10 Hz DCR, 0.5 RT, 190–800 nm 3D field collected		

Table 2. Fraction collection parameters

Parameter	Value
FC wash solvent	10% methanol in water
FC flush solvent	Eluent A—50 mM TEAA, pH7 with 5% acetonitrile (v/v)
Fraction collector compartment temperature	5 °C
Collection time frame	Start to end
Flush	Active
Maximum tube volume	1.600 mL
Minimum time for tube change	0 s
Delay volume	81.3 µL determined by automated delay volume determination with delay capillary 0.25 × 1500 mm (P/N 6706.1110)
Collection path mode	Horizontal
Collection valve mode	Interrupt
Needle positioning mode	InVial
Needle height for well plates	30.0 mm

Table 2 (cont). Fraction collection parameters

Parameter	Value	
Wash mode	Both	
Wash speed	100.0 $\mu\text{L/s}$	
Wash time	3.0 s	
Rinse mode	Both	
Puncture offset	2.0 mm	
Peak detection options	UV wavelength	260 nm
	Offset time	0.0 s
	Offset volume	0.0 μL
	Peak start true time	0.20 s
	Peak end true time	0.20 s
	Derivative step	0.02 s
	Threshold 'Do Not Resolve'	Off
	Peak max slope	Off
	Peak max true time	1.0 s
	Peak start curve	Off
	Peak end curve	Off
	Baseline drift	0.0 (mAU/s)
	Baseline offset	0.0 (mAU)

Results and discussion

The three different oligonucleotide sequences were individually purified using the instrumentation, configuration, instrument method, and parameter settings described above. The actual liquid chromatographic separation is universally applicable for all three samples as listed in the LC method details. Each of the provided samples were initially analyzed for purity to evaluate the extent of impurities present in the samples. Each sample analyzed is represented in Figures 1–3 showing the overview of the entire chromatographic runs and a zoomed section focusing on the small near-baseline impurities as well as shoulders.

Each of the synthesized oligonucleotides presents unique challenges for their respective purifications. The primary focus must be on the individual peak-based fraction collection settings tailored to each specific sequence. These individually tailored peak-based fraction collection parameters are shown in Table 3. There is a special case for the AT647-BHQ2 where the dye-label isomers are chromatographically separated but are treated as one product (Figure 2). To treat the separated isomers as one peak, the parameter 'Threshold No Peak End' is implemented. The 200 mAU value is selected to indicate that the UV absorbance needs to fall below 200 mAU to trigger a new peak. Since the valley between the two isomerized product peaks lies above 300 mAU, the two isomer peaks are treated as one peak.

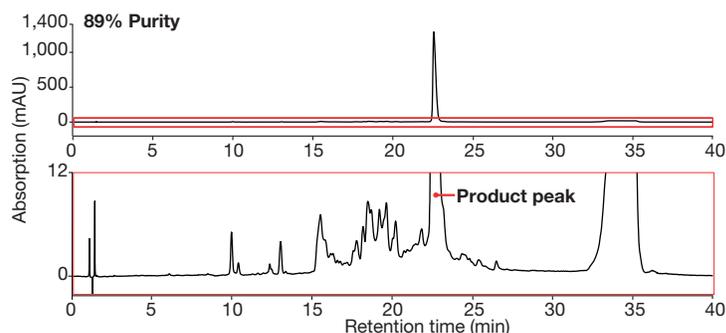


Figure 1. The CY5-BHQ2 oligonucleotide sample analyzed for purity giving initially an 89% purity. Observed in the baseline are numerous byproducts from the synthesis that need to be separated while isolating the main product peak via offline fraction collection.

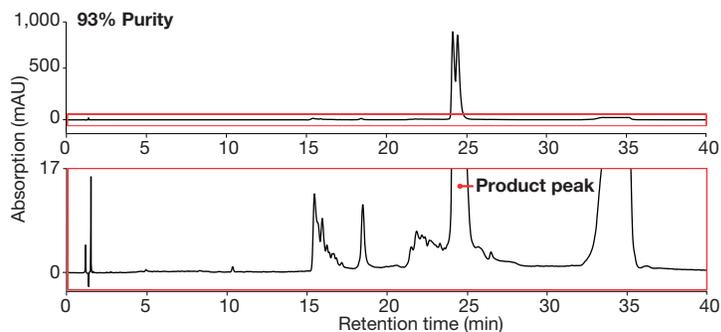


Figure 2. The AT647-BHQ2 oligonucleotide sample was analyzed providing a purity of 93%. The AT647 dye label has inherently two isomers that can be chromatographically separated, but chromatographic separation of these isomers is not required for this application. Therefore, both isomers of the dye label are treated as one target product.

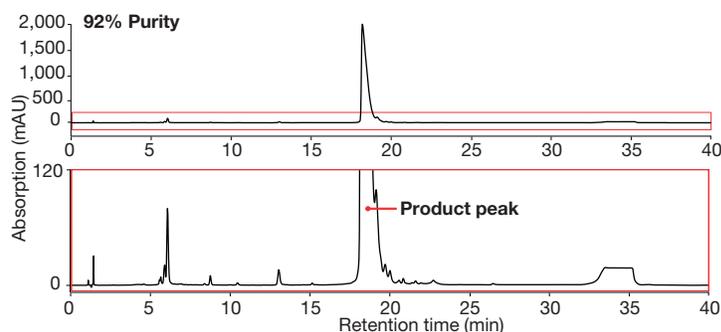


Figure 3. The FAM-BHQ1 oligonucleotide sample was analyzed yielding a purity of 92%. Like the previous oligonucleotide samples, there are several impurities that need to be separated. Most importantly to separate is the shoulder peak seen in the zoomed window, exemplifying the need to have a very precise fractionation cutting the shoulder out of the purified main product peak.

Table 3. Sample specific peak detection parameters for the UV triggered fraction collection

	CY5-BHQ2	AT647-BHQ2	FAM-BHQ1
UV channel (nm)	260	260	260
Peak start threshold (mAU)	75	20	250
Peak start slope (mAU/s)	1.0	2.0	2.0
Peak end threshold (mAU)	60.0	38.0	400.0
Peak end slope (mAU/s)	-1.0	-1.4	-2.0
Threshold no peak end (mAU)	off	200.0	off

To further secure the integrity of each collected fraction, a combination of the 96 deep-well plates and silicone mats were used. This has the advantage that the process can be more automated when collecting fractions overnight without the possible evaporation of the eluted solvents. The default setting 'Automatic' needle height in the instrument method dictates that the needle moves into the collection position down to 3 mm above the bottom of the collection position. This concept is known as *fill from bottom*. This is not the desired effect for this specific application. For this application, the desired effect is to fill into the collection vessel, in this case a deep well plate, above the collect eluent fill point. For example, the 96 deep well plate selected at 1.6 mL fill level of the total 2.2 mL makes for a potential ~75% fill level in each collected fraction position in the well plate. Using the 'Move Needle' function in the Chromeleon FC ePanel, the needle height was tested prior to the purification and the finding that

30 mm is the optimal needle height for the *fill from top* concept. The *fill from top* concept lowers the needle inside the collection vessel or well and through the septum but still above of the level of the total collected fraction volume. The last important parameter is the 'Puncture Offset' controlling the Y-axis after the needle has pierced the septum. For this case, it was required that the fractionation process includes the 'Puncture Offset'. This feature moves the carousel holding the sample trays by the specified millimeters after the needle has punctured the septum. This creates a small opening in the septum to allow displaced air to escape and prevent the accumulation of pressure inside the closed collection vessel. This is especially important when collecting in closed vessels where the collected volume is >10% of the closed collection vessel. In this case, 2 mm puncture offset was selected. These parameters are shown in Figure 4.

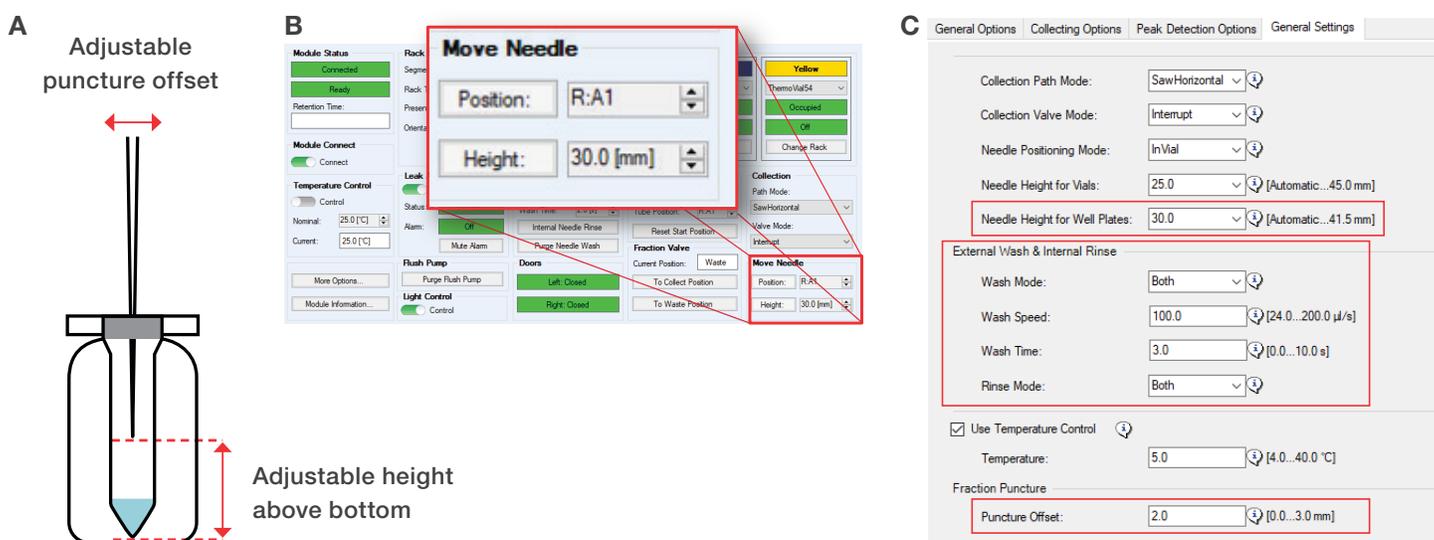


Figure 4. Needle height and puncture offset. (A) The needle height positioning and puncture offset are fully customizable. (B) The testing of the needle height by adjusting parameters in the Chromeleon FC ePanel and simultaneously observing the physical change of needle height. (C) Parameter setting for in-vial fraction collection with septum puncturing while still collecting above the collected eluent fill level. Additionally, the external needle wash and internal needle rinse functions are activated both prior to run and post collection. For more information, refer to the technical note: *Principles of fraction collection using the Vanquish HPLC and UHPLC systems.*⁶

The final step in the fraction collection instrument method setup is the definition of external needle wash, the internal needle rinse, and the flush function. The external needle wash function takes a separately selected solvent, fills the wash port with this solvent, and dips the needle into the wash solvent to clean the outer surface of the needle. This can be performed either prior to the beginning of each run and at the end of each run, or both prior and at the end of the run. It can also be deactivated. Here, 'Both' was selected to achieve best cleaning results. The internal needle rinse applies the HPLC eluent, by guiding the eluent flow through the collection needle into the wash port in turn rinsing the inside of the fraction collection needle. Its timing is selected similar to the

external needle wash ('Both'). This avoids any run-to-run cross contamination (Figure 4c). Finally, the activated flush function places the volume of the fraction collection needle capillary and needle into the collection vessel after a peak collection is completed. This solvent is also individually selectable. In this case, the flush solvent was selected as mobile phase A from the HPLC mobile phases. This eliminates the peak-to-peak cross contamination. Further details on choosing the best suited solvents for the wash and flush options, as well as using other specific features of the integrated Vanquish Fraction Collector can be found in the technical note: *Principles of fraction collection using the Vanquish HPLC and UHPLC systems.*⁶

After each oligonucleotide sample was purified, each fraction was re-analyzed using the same HPLC method settings but without the fraction collection option activated, i.e., detect only (Figures 5–7).

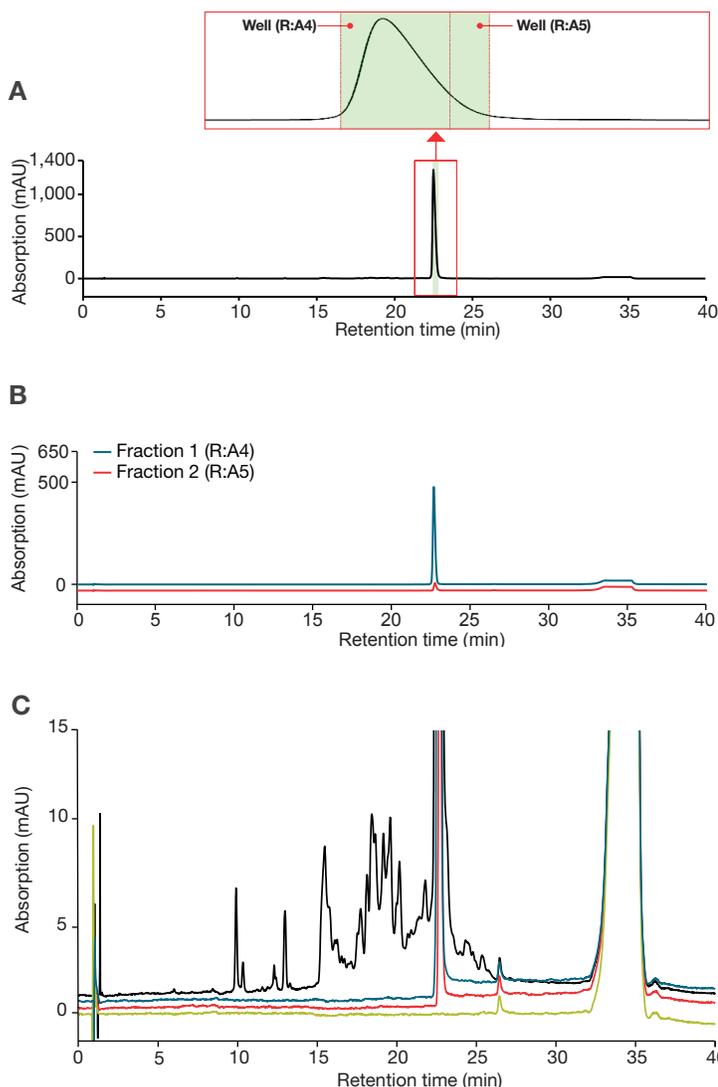


Figure 5. CY5-BHQ2 oligonucleotide sample. (A) Overview of the peak-based fractionation. (B) Re-analysis of the collected fractions. (C) Zoomed chromatogram comparison of the baseline from the original unpurified sample (black) with the re-analysis of the fractions (blue and red traces). One distinctly observes the elimination of impurities from the original (black) chromatogram in the purified fractions (blue and red traces). The green trace is a blank injection.

For each of the re-analyzed fractions, measurable UV absorbing impurities were found giving a 100% relative purity.

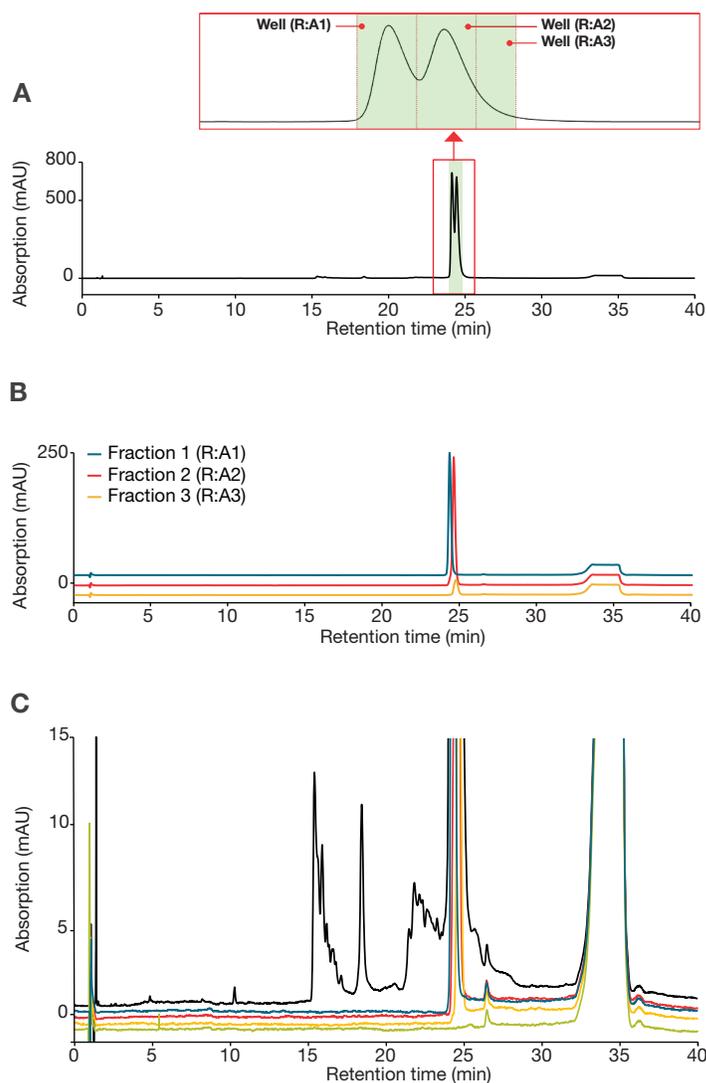


Figure 6. AT647-BHQ2 oligonucleotide sample. (A) Overview of fractionation. The maximum fraction volume of 1.6 mL is the trigger to move from fraction 1 to fraction 2 which, only by coincidence, is located in the valley between the two isomers. (B) Re-analysis of all collected fractions. One can even note that the optimized LC fluidics and fractionation performance resulted in the additional finding that both of the AT647 isomers could be isolated. The isolation of the isomers was not necessary as both isomers are treated as one product but highlights the resolving power of the integrated Vanquish Fraction Collector. The parameter "Threshold No Peak End" set at 300 mAU was used to treat these two peaks as one. (C) Again, the baseline impurity peaks in the black trace are eliminated via the intended purification, which cannot be seen in fraction 1 (blue), fraction 2 (red), or fraction 3 (yellow), proving the high-performance and effective isolation of the target product. The green trace is a blank injection.

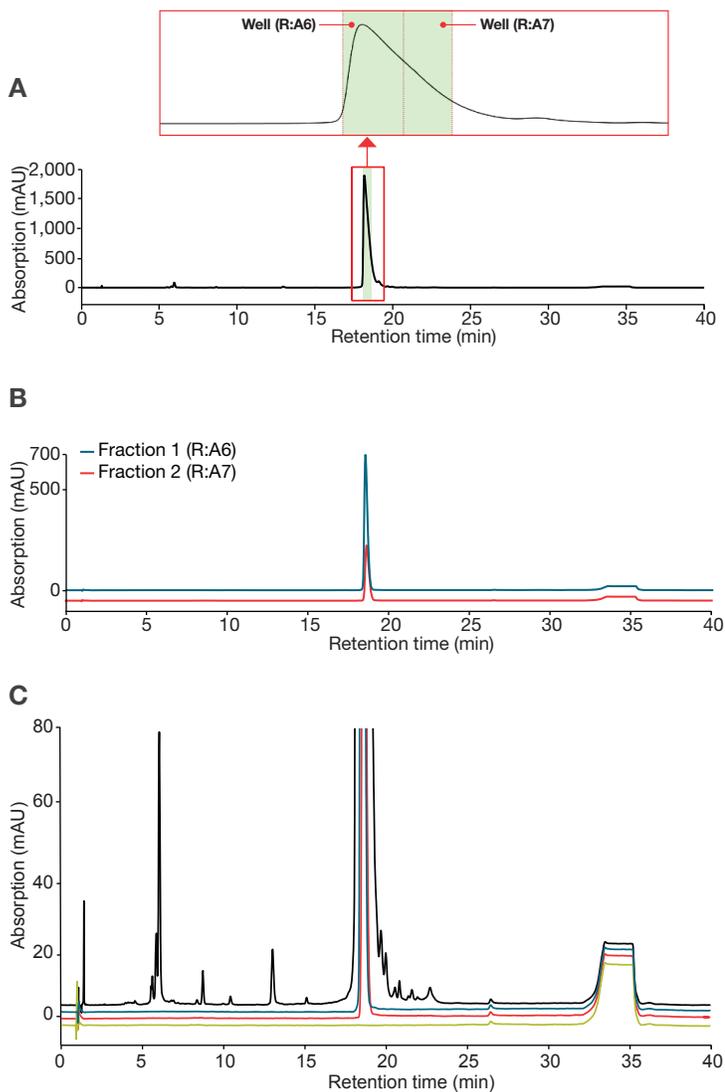


Figure 7. FAM-BHQ1 oligonucleotide sample. (A) Overview of fractionation using the sequence specific peak-based fraction collection parameters to eliminate the undesired impurity shoulder showing reproducibly the superb resolving power of the low dispersion fluidics and precise fraction collection. (B) Re-analysis of all collected fractions. (C) The original sample for the FAM-BHQ1 contains significantly more impurities and at higher concentrations as seen in the black trace. The subsequent fraction 1 (blue) and fraction 2 (red) re-analyses support the consistent purification performance as seen in the baseline magnification with the blank injection (green) baseline as the benchmark.

Conclusion

The integration of the Vanquish Analytical LC Purification system with the Chromeleon eCDS (Figure 8) into the semi-preparative RP-LC oligonucleotide purification workflow proved to be a great advantage with the resulting purity. The fluidics of the Vanquish Flex UHPLC gives an excellent benefit towards the resolving power of the chromatographic system compared to general semi-preparative to preparative LC instrumentation. The optimized biocompatible fluidics of the fully integrated Vanquish Fraction Collector in the Vanquish analytical product portfolio complement the excellent purification workflow required in the field of oligonucleotide analysis.



Figure 8. The integrated Vanquish Fraction Collector in the Vanquish Analytical Purification LC system with the Chromeleon eCDS

The cooperation between Eurofins and Thermo Fisher Scientific ensures that the Eurofins customers requesting custom oligonucleotides receive unparalleled product purity.

- Purification of three synthetic oligonucleotides was successfully implemented yielding no UV measurable impurities.
- Optimized fluidics reduce the band broadening effects on the separation in the LC system. The well-designed liquid pathway from detector through the fraction collector into the collection vessel gives confidence that the detector trace reflects exactly what is transferred to the fraction collector. Minimized peak dispersion, exact delay volume information (using the automated delay volume determination program), and precise needle positioning are the key factors for reaching these excellent purity levels.
- The external needle wash and internal needle rinse provide assurance that carry-over is avoided from one run to the next, allowing automated purification of multiple oligonucleotide samples in succession.
- The puncture offset is a key feature when collecting larger volumes in capped vials or matted well plates and preserves the integrity of each collected fraction.
- The flush function ensures that any component from a single peak is emptied into the collection well reducing potential cross-contamination of subsequent fractionated peaks.
- All features are easily customizable in the Chromeleon eCDS to meet the specific customer application needs.
- The Vanquish Analytical Purification LC system can be applied as both purification tool and analytical HPLC system. This semi-preparative oligonucleotide purification workflow exhibits versatility and performance, even for applications operating at high flow rates.

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