Application note | 003192

Biopharma

Method transfer case study for instrument and LC column migration of the purification and analysis workflow for synthetic oligonucleotides

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Keywords

Oligonucleotides, fraction collection, semi-preparative purification, Vanquish Flex Binary UHPLC system Vanquish Fraction Collector, Hypersil GOLD reversed phase column, Chromeleon Chromatography Data System (CDS)

Application benefits

- A method transfer of semi-preparative purification and QC from a non-Thermo Fisher Scientific liquid chromatographic system to the Thermo Scientific™ Vanquish™ LC platform
- Purification and quality assurance columns to facilitate a Thermo Fisher workflow solution
- A simple method to purify oligonucleotides via the Vanquish Analytical Purification LC system and perform quality control using UHPLC high-resolution mass spectrometry (HRMS)

Goal

Demonstrate the complete method transfer of oligonucleotide purification and qualification from a non-Thermo Fisher LC and column workflow to a Thermo Fisher solution using high-performance liquid chromatography (HPLC) for purification and HPLC-HRMS for quality control, all managed under Thermo Scientific™ Chromeleon™ Chromatography Data Solution CDS

Introduction

Nucleic acids play a vital role in the existence of life. In recent years, there has been a growing interest in oligonucleotides within the fields of biochemical research, diagnostics, and pharmaceuticals.[1] As a result, significant efforts have been made to optimize and automate their synthesis. However, the process of oligonucleotide synthesis involves multiple reactions, leading to the accumulation of impurities, such as truncated nucleotide sequences, partial deprotection, and fluorophore/quencher degradation. Therefore, it is crucial to purify the desired oligonucleotides effectively and with high purity for downstream applications, such as quantitative polymerase chain reactions (qPCR), integrated human identification (HID) solutions for forensics, and gene transfer agents (GTA).

Since the 1970s, various chromatographic methods have been utilized for the analysis and purification of synthetic oligonucleotides.[2] In recent years, significant advancements have been achieved in terms of instrument performance and stationary phase.[3] Reversed-phase high-performance liquid chromatography (RP-HPLC) is the widely employed technique for high-resolution separation of nucleic acids.[4] However, the purity specification for oligonucleotides is on the rise. For example, the 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] For other applications, oligonucleotide purity needs to exceed 90%. The increasing demand for oligonucleotides as therapeutic agents necessitates the development of a HPLC purification scheme that satisfies a high purity specification.

When developing methods to separate and purify oligonucleotides, it is essential to consider their unique characteristics. These include the length of the oligo, specific sequence, fluorophore/quencher combination, and ability to form secondary structures. Other factors that influence an oligonucleotide's interaction with the stationary phase, and therefore retention time, include buffer (pH and salt concentration), reversed-phase column, and LC system selection.[2]

Additionally, column selection, LC hardware/software, and automation can also affect purity. As a result, the ability to seamlessly transfer oligonucleotide purification methods from one vendor instrumentation and columns to another while meeting or exceeding the expected quality criteria is a valuable tool.

This work demonstrates the effective method transfer of the semi-preparative RP-HPLC purification of two different dual-labeled 15mer oligonucleotides from a source HPLC instrument and column to an alternate vendor column and HPLC. By-products of the DNA synthesis are resolved, and the target oligonucleotides are successfully isolated and collected. LC-HRMS and HPLC analyses were used to confirm oligonucleotide purity, resulting in passing QC standards for the yield and purity, and suggesting a successful method transfer for the preparation of the high-purity oligonucleotides for downstream application (Figure 1).

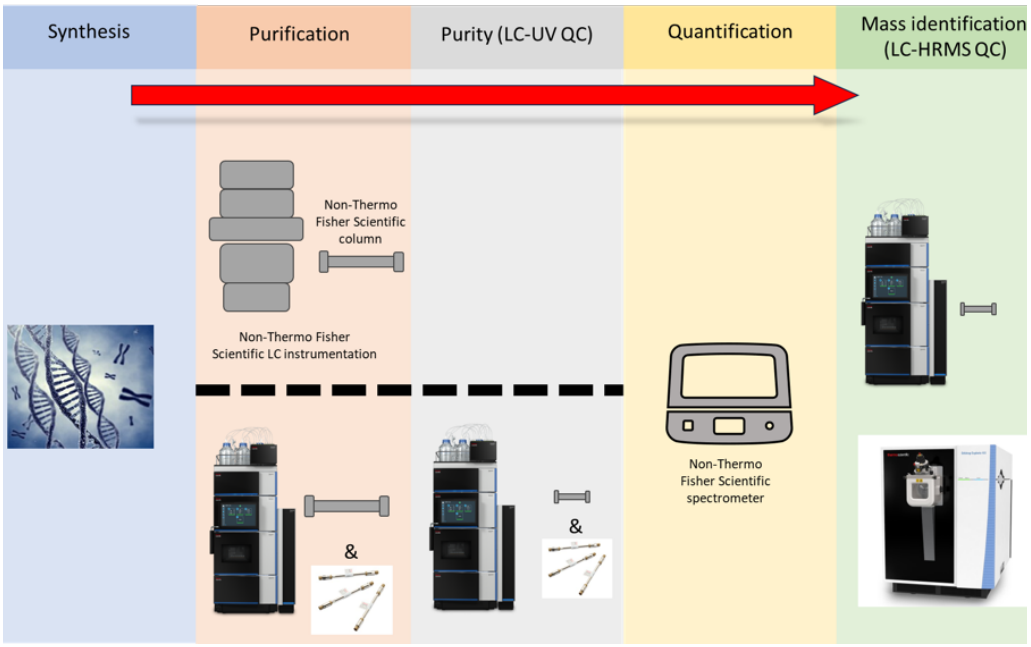


Figure 1. The workflow method transfer for the purification, quantification, and qualification of dual-labeled 15mer oligonucleotides

Experimental

Reagents and consumables

- Oligonucleotide samples, crude synthesis product, see Table 1 for sequence information (Genetic Sciences Division, Thermo Fisher Scientific, Inc.)
- Triethylammonium acetate (TEAA), 2 M solution (**P/N 400613**)
- Thermo Scientific™ Hypersil GOLD™ C18 RP HPLC column, 2.1 × 50 mm, 1.9 µm (**P/N 25002-052130**)
- Thermo Scientific™ Hypersil GOLD™ C18 Prep HPLC column, 10 × 150 mm, 5 µm (**P/N 25005-159070A**)
- Deionized water, 18.2 MΩ.cm resistivity or higher by Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus UV/UF-TOC
- Acetonitrile (MeCN), Optima™ LC/MS grade, Fisher Chemical™ (**P/N A955-4**)
- Methanol (MeOH), Optima™ LC/MS grade, Fisher Chemical™ (**P/N A456-212**)
- Thermo Scientific™ 96 Deepwell-Plate, square wells, with barcode (**P/N 60180-P105B**)
- Thermo Scientific™ WebSeal™ CLR Silicone Mat, 96 square wells (**P/N 60180-M121**)
- Eppendorf Research™ plus pipette, 100–1,000 µL
- Daisogel semi-preparative column: Daisogel™ SP-100 ODS-P; 5 µm, 100 Å 10 x 150 mm
- Waters analytical column: Waters™ ACQUITY UPLC™ BEH C18 column, 130 Å, 1.7 µm, 2.1 x 50 m

Solvent preparation

- **Solvent A**: 250 mL of 2.0 M TEAA was measured and brought to 5 L using ultra-pure water. The solution was mixed thoroughly.
- **Solvent B**: 2.5 L of methanol was added to 2.5 L acetonitrile to make a 1:1 solution. The solution was mixed thoroughly.

Oligonucleotide sample preparation

Samples were prepared separately.

- 500 µL of Solvent A was pipetted into a crude dual-labeled oligonucleotide sample tube. No dissolution was initially observed.
- 300 µL of Solvent B was pipetted into the tube. Dissolution was observed.
- The sample was vortexed for 1 min, then sonicated at 40°C for 5 min, and vortexed for 1 min.
- 200 µL Solvent A was pipetted into the sample tube.
- The sample was vortexed for 1 min, then sonicated at 40°C for 5 min, and vortexed for 1 min.

Table 1. Oligonucleotide sample sequences, chemical formula, and monoisotopic mass.
The monoisotopic mass was manually calculated.

Oligonucleotide name	Sequence	Monoisotopic mass
ABY-MGB	[ABY]-TTGGTCTCTATCTGC-[MGB]	6357.2
JUN-MGB	[JUN]-TTGGTCTCTATCTGC-[MGB]	6437.3

Instrumentation
Purification

Module	Part number
Thermo Scientific™ Vanquish™ Analytical Purification LC System consisting of:	
System Base Thermo Scientific™ Vanquish™ Horizon/Flex UHPLC system	VF-S01-A-02
Vanquish Binary Pump F Thermo Scientific™ Viper™ capillary, 0.18 × 350 mm MP35N, from pump to autosampler*	**VF-P10-A-01** **6042.2337**
Vanquish Split Sampler FT 1,000 µL Viper MP35N sample loop*	**VF-A10-A-02** 6850.1980
Vanquish Column Compartment H Active Pre-Column Heater Viper capillary, 0.18 × 450 mm MP35N, from column outlet to detector inlet*	**VH-C10-A-03** **6732.0110** **6042.2365**
Vanquish Diode Array Detector FG Standard biocompatible flow cell, 13 µL, PEEK, 10 mm	**VF-D11-A-01** **6083.0540**
Vanquish Fraction Collector Delay capillary for peak-based fractionation, 0.25 × 1,500 mm, MP35N, Viper* Needle capillary, 0.18 × 415 mm, partially PEEK-shielded MP35N, Viper (default) Flush buffer loop, 50 µL, PEEK, Viper (default)	**VF-F20-A-01** **6706.1110** 6706.1010

*These components are not standard and need to be ordered separately

An oligo-purification method used on an Agilent™ 1260 Infinity II Preparative-Scale LC Purification system was transferred to the Vanquish Analytical Purification LC system proving the suitability.

LC-UV QC

Module	Part number
Thermo Scientific™ Vanquish™ Flex Binary UHPLC System consisting of:	
System Base Vanquish 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 H Active Pre-Column Heater	**VH-C10-A-03** **6732.0110**
Vanquish Diode Array Detector FG Standard biocompatible flow cell, 11 µL, PEEK, 10 mm	**VF-D11-A-01** **6083.0540**

To concentrate the purified fractions, a concentrator was used:
Thermo Scientific™ Savant™ SpeedVac™ SPD1010 Concentrator

To calculate yield, a spectrophotometer was used:
Molecular Devices™ SpectraMax™ 384+ UV-Vis Spectrophotometer

LC-HRMS QC

Module	Part number
Vanquish Flex Binary UHPLC System consisting of:	
System Base Vanquish 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 H Active Pre-Column Heater	**VH-C10-A-03** **6732.0110**
Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer	**BRE725539**

Chromatography

Step 1 - Purification

The Vanquish Analytical Purification LC system was used to isolate the pure oligonucleotides. This was performed using a Daisogel semi-preparative column, and then the exact method was transferred to the Hypersil GOLD semi-preparative column to compare the column performance.

Table 2. The LC-UV chromatographic conditions for the purification of the oligonucleotides

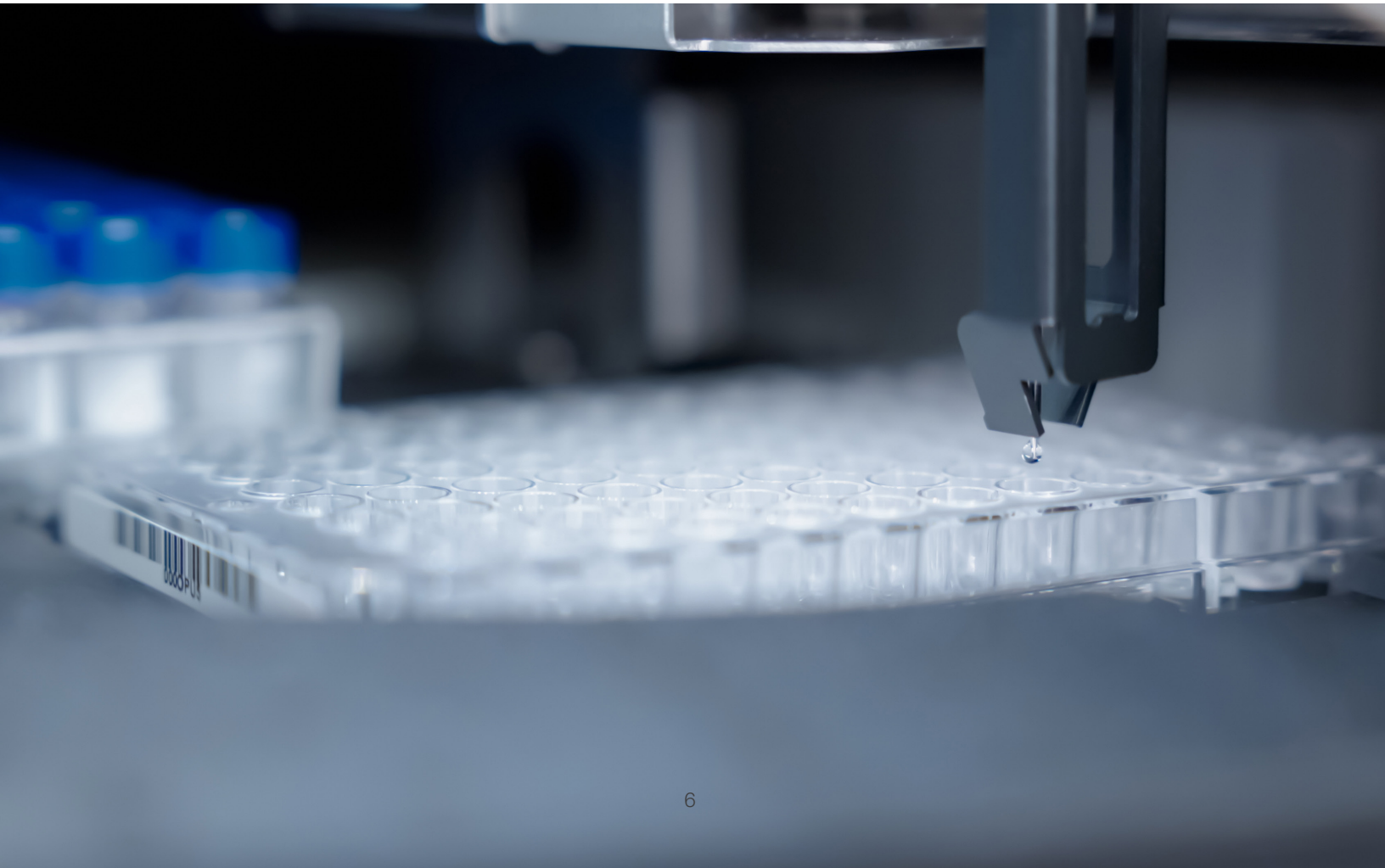
Parameter	Value	
Column	Daisogel SP-100 ODS-P; 5 µm, 100 Å 10 x 150 mm Hypersil GOLD, 5 µm, 175 Å, 10 x 150 mm	
Mobile phase	Solvent A: 100 mM TEAA Solvent B: 1:1 MeCN:MeOH	
Gradient		
Time (min)	A	B
-10	65	35
0.0	60	40
15	33	67
15.01	5	95
18.0	5	95
18.01	65	35
21	65	35
Flow rate	4.5 mL/min	
Column temperature	50°C, forced air mode 50°C, active pre-heater	
Autosampler temperature	4°C	
Autosampler wash solvent **Fraction collection flush solvent** **Fraction collection wash solvent**	10% MeOH in water 1:1 Solvent A:Solvent B 10% MeOH in water	
Injection volume	900 µL	
UV detector settings	λ^1 = 260 nm λ^2 = 350 nm λ^3 = 560 nm DCR: 10 Hz	

Table 3. The fraction collection settings for purification of the oligonucleotides

Fraction collection settings	
Parameter	**Value**
Collection	Collect by peak
Time range	6.0–17.0 min
Flush	Active
Max. tube volume	1.7 mL
Max. number of tubes per fraction	Unlimited
Minimum time for tube change	0.0 s
Delay volume	99.3 µL*
Collection path mode	SawHorizontal
Collection valve mode	Interrupt
Needle positioning mode	InVial
Needle height for well plates	30.0 mm
Wash mode	Both
Wash speed	100.0 µL/s
Wash time	5.0 s
Rinse mode	Both
Temperature	4 °C
Puncture offset	2.0 mm

*The delay volume was determined by the Delay Volume Determination Calculator.

Peak detection options	
Parameter	**Value**
Channel name	UV_VIS_1 (260 nm)
Peak start threshold	2,845 mAU
Peak start slope	Off
Peak start true time	0.1 s
Peak end threshold	2,911 mAU
Peak end slope	Off
Peak end true time	0.1 s
Derivative step	0.02 s
Threshold 'No peak end'	2,928 mAU
Peak max true time	0.01 s



Step 2 - LC-UV QC

Immediately after the purification was completed, the fractions were analyzed by LC-UV to ascertain the performance of the semi-preparatory purification columns and to compare the analytical columns. The aim was to determine if similar conclusions could be made with a Waters ACQUITY UPLC BEH C18 column and subsequent transfer of the exact method to the Hypersil GOLD analytical column. The samples were injected directly out of the 96 deep well plate taken from the Vanquish Fraction Collector and placed into the Vanquish Split Sampler.

The collected purified fractions were then placed in the Savant SpeedVac SPD1010 concentrator without any additional heat to evaporate the solvent, requiring a minimum of 8 hours concentration time to fully remove all solvent from the samples.

Table 4. LC-UV QC conditions

Parameter	Value	
Column	Waters ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 x 50 mm Hypersil GOLD, 175 Å, 1.9 µm, 2.1 x 50 mm	
Mobile phase	Solvent A: 100 mM TEAA Solvent B: 1:1 MeCN:MeOH	
Gradient		
Time (min)	A	B
-5	65	35
0.0	60	35
8	35	65
8.01	5	95
9.0	5	95
9.01	65	35
10	65	35
Flow rate	0.6 mL/min	
Column temperature	50°C, forced air mode 50°C, active pre-heater	
Autosampler temperature	4°C	
Autosampler wash solvent	10% MeOH in water	
Injection volume	1 µL – crude 10 µL – pure fraction	
UV detector settings	λ^1 = 260 nm λ^2 = 350 nm λ^3 = 560 nm DCR: 10 Hz	

Mass spectrometry

Step 3 – LC-HRMS QC

In addition to measuring sample purity using LC-UV, this work used LC-HRMS to confirm the presence of the desired oligonucleotide and the absence of critical impurities, such as dye/quencher degradation and truncated sequences. Samples were introduced into the Orbitrap Exploris 480 mass spectrometer via the Vanquish Flex UHPLC system. The raw mass spectrometry data was acquired using Thermo Scientific™ Xcalibur™ software and analyzed using the Thermo Scientific™ FreeStyle™ application. Within the FreeStyle application, the Xtract tool was used to deconvolute the mass spectra.

Table 5. Chromatographic LC-HRMS QC conditions

Parameter	Value	
Column	Waters™ Atlantis™ T3 3 µm 2.1 x 50 mm	
Mobile phase	Solvent A: Proprietary aqueous solvent Solvent B: Proprietary organic solvent	
Gradient		
Time (min)	A	B
0.0	99	1
4.00	62	38
4.01	1	99
5.00	1	99
5.01	99	1
6.00	99	1
Flow rate	0.8 mL/min	
Column temperature	30°C	
Autosampler temperature	5°C	
Autosampler wash solvent	30% methanol in water	
Injection volume	5 µL	
UV detector settings	H-ESI	

Table 6. FreeStyle application Xtract tool and deconvolution parameters

Xtract tool parameters	Value
***m/z* range**	469.67–3,055.55
Deconvolution parameters	**Value**
Output mass	M
Adduct element	H+
Charge range	2–50
Analyzer type	OT
Relative abundance threshold (%)	0
Isotope table	Nucleotide
Negative charge	Checked
Minimum number detected charge	2

Step 4 – Quantification

After drying, samples were dissolved in 100 µL of 1xTE buffer and quantified by measuring absorbance at 260 nm using a UV-Vis spectrophotometer. The concentration/yield of each sample was manually calculated by applying Beer-Lambert's Law.

Results and discussion

Step 1 – Purification

Initially, both 200 nmol crude samples were purified using both the Daisogel Prep and the Hypersil GOLD prep columns. The ABY-MGB crude was purified, and the differing retention and selectivity of the column performances can be observed in Figure 2. Although the target oligonucleotide elutes at an earlier time, the Hypersil GOLD prep column resolves neighboring peaks with higher resolution than the Daisogel column. This difference in elution time is likely due to the differences in pore size and carbon load between the Hypersil and Daisogel columns. The Daisogel column has a shoulder peak at 10.9 minutes, which makes for a more challenging purification versus the Hypersil GOLD prep column. In Figure 3, the JUN-MGB crude was purified. This sample purification proved more challenging than that of the ABY-MGB sample due to neighboring peaks prior to the target JUN-MGB oligo and a co-eluting shoulder after the target peak. By focusing the collection window in the UV saturation region, one can increase the purity of the JUN-MGB oligo where the later eluting impurity shoulder is sharper using the Hypersil GOLD semi-preparative column. Furthermore, the peak volume for the Hypersil GOLD column provides a smaller collection volume compared to that of the Daisogel column (Table 7), thus increasing the convenience of having less volume to evaporate after collection.

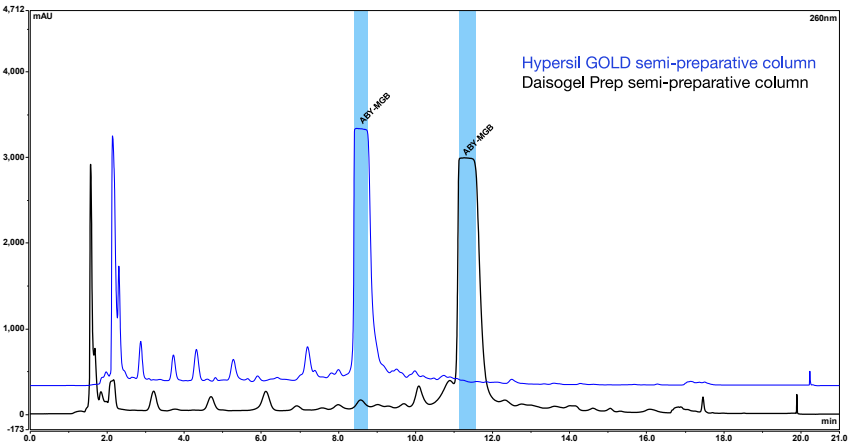


Figure 2. Column performance comparison between the Daisogel and Hypersil GOLD prep columns for the ABY-MGB crude oligo synthesis purification

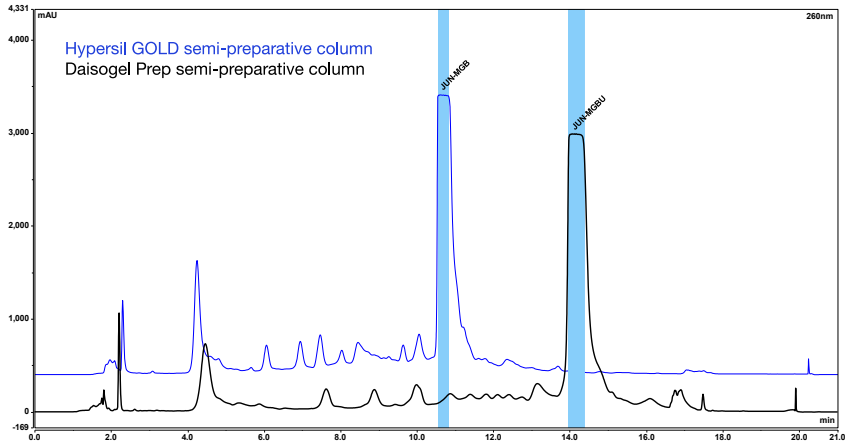


Figure 3. Column performance comparison between the Daisogel and Hypersil GOLD prep columns for the JUN-MGB crude oligo synthesis purification

Table 7. Collection volume comparison between the Daisogel and Hypersil Gold semi-preparative columns

Sample name	Prep column	Fraction volume (µL)
ABY-MGB	Hypersil GOLD	**1,635**
ABY-MGB	Daisogel	**1,972**
JUN-MGB	Hypersil GOLD	**1,440**
JUN-MGB	Daisogel	**1,777**

Step 2 - LC-UV QC

Prior to evaporation of the solvent, the LC-UV was used to control the quality and determine if the Hypersil GOLD analytical column could be used as a substitute for the Waters analytical column. A successful transfer of the analytical method would be represented by comparable purity of the purified fractions. The crude sample chosen was that of the ABY-MGB purified on the Hypersil GOLD prep column shown in Figure 4. As shown in Table 8, the Hypersil GOLD analytical column can perform equivalent to the Waters analytical column.

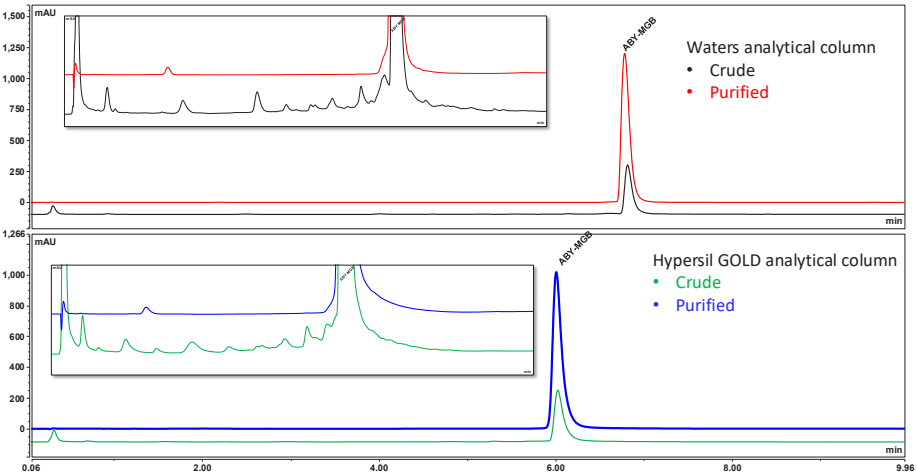


Figure 4. The crude ABY-MGB sample was purified using the Hypersil GOLD prep column, and the chromatograms show the crude ABY-MGB sample analyzed by the Waters and Hypersil GOLD analytical columns as well as the re-analysis of the purified ABY-MGB sample using both analytical columns. The zoomed plots focus on the baseline showing highly pure result from the purification prior to the evaporation.

Table 8. Represented is the analysis of the crude ABY-MGB and JUN-MGB samples analyzed using the LC-UV QC method on both the Waters analytical and Hypersil GOLD columns. After the purification using the Hypersil GOLD prep column, the purified fractions were re-analyzed on both the Waters analytical and Hypersil GOLD columns.

	Hypersil GOLD purification			
	Waters LC-UV analytical column		Hypersil GOLD LC-UV analytical column	
	Crude	Purified	Crude	Purified
ABY-MGB	85.84%	98.62%	86.61%	99.70%
JUN-MGB	81.99%	99.68%	82.67%	99.92%

Given that the Hypersil GOLD analytical column is suited for the QC for the purity of the isolated target oligonucleotides, the comparison of the Hypersil GOLD prep column versus the Daisogel prep column performance on the purification of the crude oligonucleotides can be made. From Table 9, one can conclude that the Hypersil GOLD semi-preparative column is an appropriate column to transfer this LC method from the Daisogel semi-preparative column for the purification of short-chain dual-labeled oligonucleotides.

Table 9. Using the Hypersil GOLD analytical column to analyze the purity of the oligonucleotides.
This compares the performance of the Hypersil GOLD semi-preparative versus the Daisogel semi-preparative columns.

Sample	Daisogel semi-preparative column	Hypersil GOLD semi-preparative column
ABY-MGB	98.41%	**99.70%**
JUN-MGB	98.76%	**99.92%**

Important: At this point, the purified oligonucleotides needed to be shipped internationally for the LC-HRMS QC and therefore were dried using a concentrator. This method of evaporating the solvents was determined by subsequent LC-UV-HRMS as being sub-optimal due to the time it took to eliminate the solvents (8 hours) and the internal ambient temperature of the concentrator to be 35°C. A superior method would have been to lyophilize the oligonucleotides preserving their integrity and thus, purity.

Step 3 – LC-HRMS QC

The mass spectrum of each sample was measured to confirm the presence of the full-length oligonucleotide product and identify any impurities. The expected monoisotopic mass was observed (± 0.2 Da) for each sample and no critical impurities were detected.

Step 4 – Quantification

The last step of the synthesis to purification workflow (aside from the final product) is quantification. This was performed by measuring the absorbance via the spectrophotometer shown in Figure 5.

Summary

The experiment of method transfer is to compare the purity, yield, and mass identification from the source instrumentation to the Vanquish LC platform to provide evidence of a successful method transfer. This was proven via the purities and yields of the Thermo Fisher Scientific workflow solution where the yields and purities are comparable to that of the non-Thermo Fisher Scientific workflow.

- In Figure 5, the LC instrument-to-instrument comparison was made, and the result showed comparable yields and purities for both oligonucleotide samples. This experiment uses the non-Thermo Fisher Scientific columns and only compares the source LC and Vanquish LC instrumentation showing a straightforward method transfer.
- In Table 9, the semi-preparative column comparison was made. The result illustrates the capabilities to purify the oligonucleotide samples with passing R&D QC requirements on the Hypersil GOLD semi-preparative column.
- In Table 8, the analytical columns were compared to show that the Hypersil GOLD analytical column gives results similar to the source column. This provides users with the option to apply the Hypersil GOLD analytical column for LC-UV QC purity analysis.

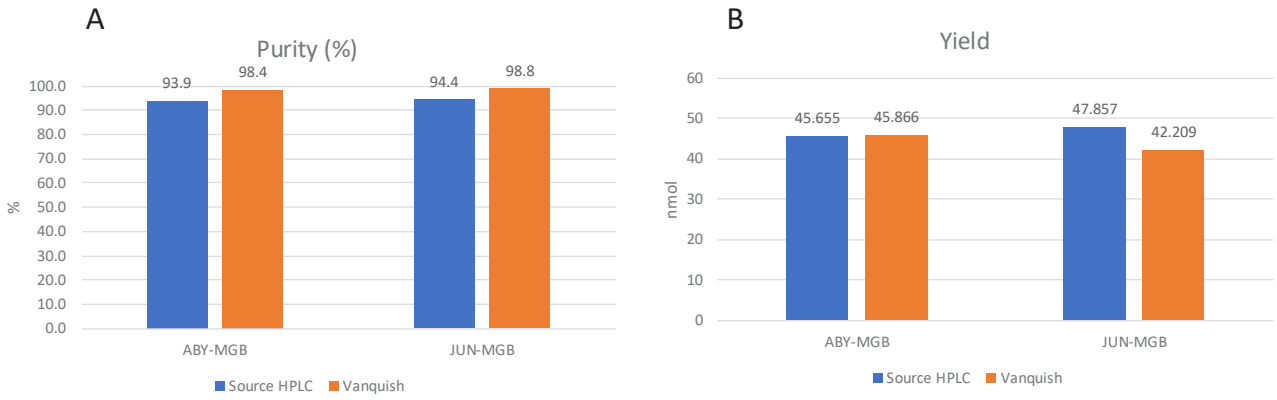


Figure 5. The LC instrument-to-instrument comparison. Using the source method semi-preparative and analytical columns, the LC purification systems were compared to show that the method can be transferred as-is from an Agilent 1260 Infinity II Preparative-Scale LC Purification system to the Vanquish Analytical Purification LC system where (A) the purities of the oligonucleotide samples were compared as well as (B) the yields were also comparable from the source LC instrument solution to the Vanquish LC instrument solution.

Conclusions

- A simple method transfer was successfully shown for the purification of the dual-labeled oligonucleotide samples from a non-Thermo Fisher Scientific LC purification system to the Vanquish Analytical Purification LC system.
- The Vanquish system purification yields quantitation/QC data comparable to the Agilent system purification for the ABY/JUN-MGB probes (Figure 5).
- The Hypersil GOLD semi-preparative column yields similar quantification and purification performance to that of the non-Thermo Fisher Scientific semi-preparative column when used to isolate the short-chain dual-labeled oligonucleotide samples. This has the added benefit that the fraction volume is reduced making for less solvent to be evaporated from the purified products.
- For QC, the Hypersil GOLD analytical column successfully passed the criteria demonstrating to be a suitable substitute of the non-Thermo Fisher Scientific analytical column.

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