Oligonucleotide purification

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

Dennis Köhler[1], Talia Hill[2], and Susanne Fabel[1]
[1]Thermo Fisher Scientific, Dornierstrasse 4, 82110 Germering, Germany
[2]Thermo Fisher Scientific, 6055 Sunol Blvd., Pleasanton, CA 94566, US

Abstract

Purpose: Demonstrate the complete method transfer of oligonucleotide purification and qualification from a non-Thermo Fisher Scientific LC and column workflow to a Thermo Fisher Scientific solution using HPLC to purify along with HPLC-HRMS quality control under Chromeleon CDS.

Methods: A method transfer of **semi-preparative reversed phase purification** and **RP-LC quality control** was performed from a non-Thermo Fisher Scientific liquid chromatographic system to the Thermo Scientific™ Vanquish™ LC platform. This included the use of purification and quality assurance columns to facilitate a Thermo Fisher Scientific workflow solution. A straightforward method was employed to purify **oligonucleotides** using the Thermo Scientific™ Vanquish™ Analytical Purification LC system and to perform quality control with UHPLC high-resolution mass spectrometry (HRMS).

Results: The method transfer for purifying dual-labelled oligonucleotide samples from a non-Thermo Fisher LC system to the Vanquish Analytical Purification LC System was successful. Vanquish yielded comparable quantitation/QC data to Agilent for ABY/JUN-MGB probes. The Thermo Scientific™ Hypersil GOLD™ column matched previous purification performance and reduced fraction volume. For QC, it met all criteria as a suitable substitute.

Introduction

In recent years, there has been a growing interest in oligonucleotides within the fields of biochemical research, diagnostics, and pharmaceuticals.[1] 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 biproducts, and fluorophore/quencher degradation impurities. Therefore, it is crucial to purify the desired oligonucleotides effectively and with high purity for downstream applications.

Since the 1970s, various chromatographic methods have been utilized for the analysis and purification of synthetic oligonucleotides.[2] Reversed-phase high-performance liquid chromatography (RP-HPLC) is the widely employed technique for high-resolution separation of nucleic acids.[3] 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, in some cases, sub-ppb amounts relative to the drug.[4] 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 the wetted parts of the HPLC system.[2] 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 critical aspect.

This work demonstrates the successful method transfer of the semi-preparative RP-HPLC purification of two different dual-labeled 15mer oligonucleotides from an Agilent™ 1260 Infinity™ II Preparative-Scale LC Purification system and various third-party columns to the Vanquish Analytical LC Purification system and Thermo Scientific columns. [Figure 1]

Materials and methods

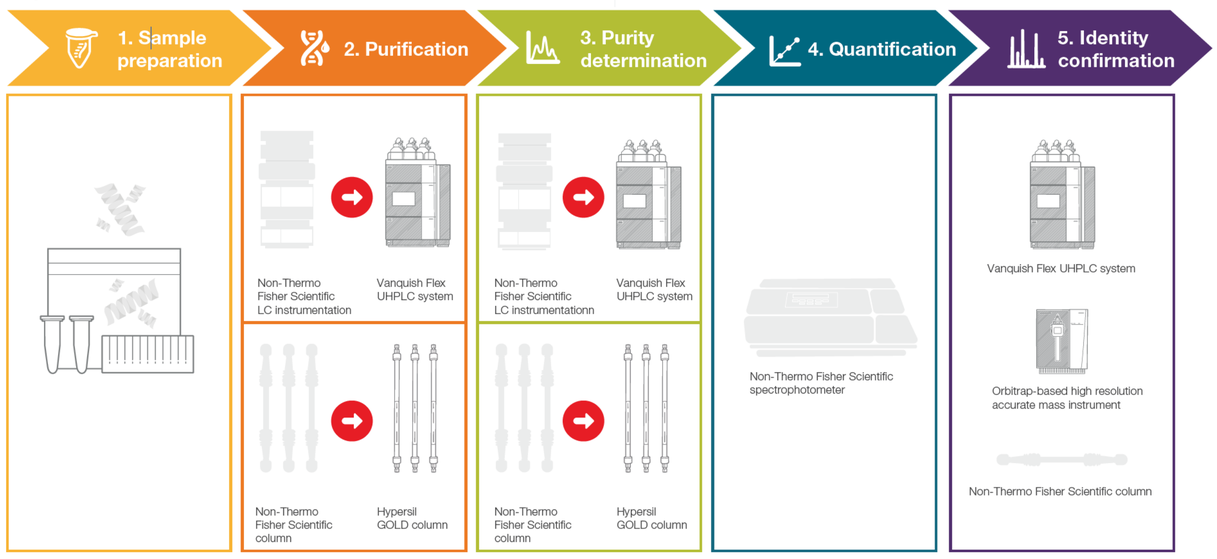
Step 1 - Sample Preparation

Oligonucleotide samples, crude synthesis product, see Table 1 for sequence information (Genetic Sciences Division, Thermo Fisher Scientific, Inc.). The 200nmol crude dual-labeled oligonucleotide samples in 700µL 100mM TEAA, 150µL methanol, and 150µL acetonitrile.

Table 1. Oligonucleotide sample sequences

Oligonucleotide name	Sequence
ABY-MGB	[ABY]-TTGGTCTCTATCTGC-[MGB]
JUN-MGB	[JUN]-TTGGTCTCTATCTGC-[MGB]

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



Step 2 - Purification

The Vanquish Analytical Purification LC system was used to isolate the pure oligonucleotides (Table 2). 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 with the settings in Table 3.

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

Parameter	Value	
Columns	• Daisogel semi-preparative column: Daisogel™ SP-100 ODS-P; 5 µm, 100 Å 10 × 150 mm • Thermo Scientific™ Hypersil GOLD™ C18 Prep HPLC column,10 × 150 mm, 5 µm (P/N 25005-159070A)	
Solvent A	100 mM TEAA	
Solvent B	1:1 MeCN:MeOH	
Gradient	Time (min)	%B
	-10	35
	0	40
	15	67
	15.01	95
	18	95
	18.01	35
	21	35
Flow rate	4.5 mL/min	
Column temperature	50° C active preheater 50° C forced air mode, fan speed 5	
Autosampler temperature	4° C	
Autosampler wash solution Fraction collection flush solvent Fraction collection wash solvent	90/10 water/ methanol (v/v) 1:1 Solvent A:Solvent B 90/10 water/ methanol (v/v)	
Needle wash mode	Both (before and after)	
Injection volume	900 µL	
UV detector settings	Data collection rate: 10 Hz	
	λ^1=260nm	
	λ^2=350nm	
	λ^3=560nm	

Table 3. Fraction collection and peak detection settings for purification of the oligonucleotides

Fraction Collection Settings	
Collection	Collect by peak
Time range	6.0 – 17.0 min
Flush	Active
Max. tube volume	1.7 mL
Delay volume	99.3 µL
Collection valve mode	Interrupt
Needle positioning mode	Invial
Needle height for well plates	30.0 mm
Wash mode	Both
Rinse mode	Both
Temperature	4° C
Puncture offset	2.0 mm

Peak detection options	
Channel name	Collect by peak
Peak start threshold	6.0 – 17.0 min
Peak start slope	Active
Peak end slope	1.7 mL
Peak end true time	99.3 µL
Derivative step	Interrupt
Threshold 'No peak end'	Invial
Peak max true time	30.0 mm

Step 3 - LC-UV QC

Immediately after the purification was completed, the fractions were analyzed by LC-UV on the Thermo Scientific™ Vanquish™ Flex Binary UHPLC System to ascertain the performance of the semi-preparatory purification columns and to compare the analytical columns [Table 4].

Table 4. LC-UV QC conditions

Parameter	Value	
Columns	• Waters analytical column: Waters™ ACQUITY UPLC™ BEH C18 column, 130 Å, 1.7 µm, 2.1 × 50 mm • Thermo Scientific™ Hypersil GOLD™ C18 RP HPLC column, 2.1 × 50 mm, 1.9 µm (P/N 25002-052130)	
Solvent A	100 mM TEAA	
Solvent B	1:1 MeCN:MeOH	
Gradient	Time (min)	%B
	-5	35
	0	35
	8	65
	8.01	95
	9	95
	9.01	35
	10	35
Flow rate	0.6 mL/min	
Column temperature	50° C active preheater 50° C forced air mode, fan speed 5	
Autosampler temperature	4° C	
Autosampler wash solution	90/10 water/ methanol (v/v)	
Needle wash mode	Both (before and after)	
Injection volume	1 µL – crude 10 µL – pure fraction	
UV detector settings	Data collection rate: 10 Hz	
	λ^1=260nm	
	λ^2=350nm	
	λ^3=560nm	

Step 4 – Quantification

The next step of the synthesis-to-purification workflow (aside from the final product) is quantification. This was performed by measuring the absorbance via the spectrophotometer.

Step 5 – LC-HRAM MS identity confirmation

In addition to measuring sample purity using LC-UV, LC-HRAM MS was used to confirm the presence of the desired oligonucleotide and the absence of critical impurities. The identity confirmation results are not in focus in this work.

Data Analysis

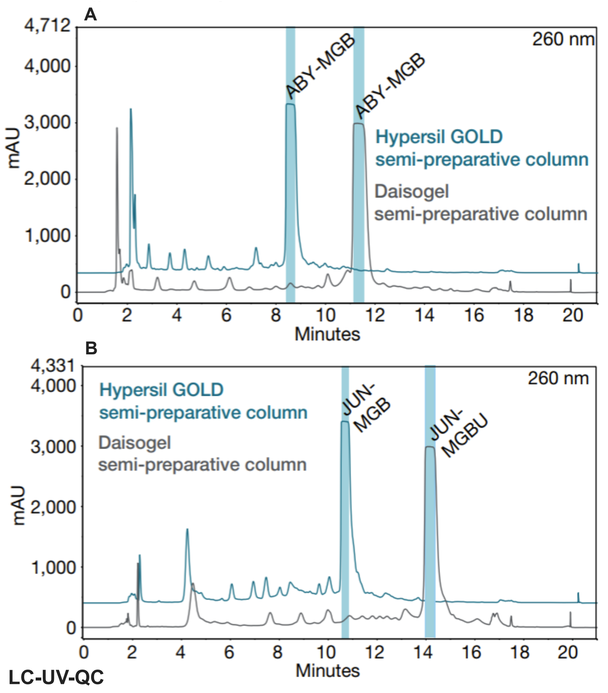
Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.3.2 was used for data acquisition and processing.

Results

Purification

The ABY-MGB & JUN-MGB crude samples were purified. For the ABY-MGB sample, the differing retention and selectivity of the column performances can be observed in Figure 2a. Although the target oligonucleotide elutes at an earlier time, the Hypersil GOLD prep column resolves neighboring peaks with higher resolution than the Daisogel column. The JUN-MGB crude sample purified on both the Daisogel and Hypersil GOLD semi-preparative columns 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 [Figure 2b]. By focusing the collection window on 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.

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



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 in focus was that of the ABY-MGB purified on the Hypersil GOLD prep column shown in Figure 3. As shown in Table 5, the Hypersil GOLD analytical column can perform equivalently to the Waters analytical column in the objective of isolating a target oligonucleotide with similarly high purity and yield.

Figure 3. 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 showcasing the difference in purity between the crude and purified samples.

Table 5. Summary of the purity 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.

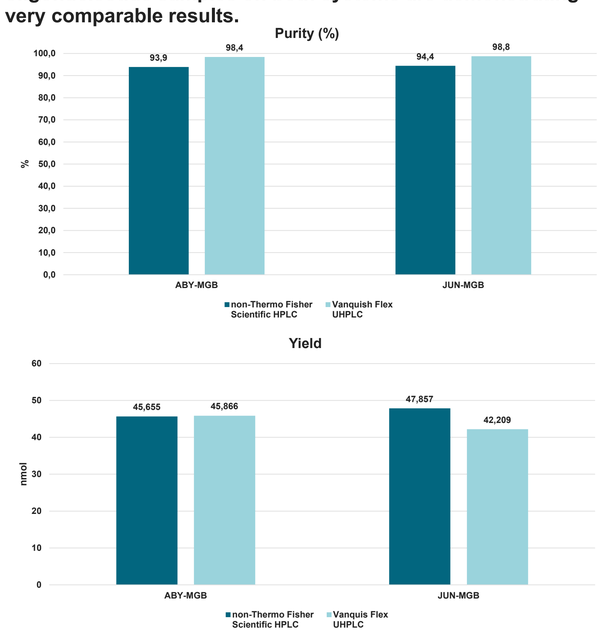
	Waters analytical column		Hypersil GOLD analytical column	
	Crude	Purified	Crude	Purified
ABY-MGB	85.84%	98.62%	86.61%	99.70%
JUN-MGB	81.99%	99.68%	82.67%	99.92%

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 6, 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 6. Performance comparison of the Hypersil GOLD semi-preparative versus the Daisogel semi-preparative columns summarizing achieved purities of the two oligonucleotide samples ABY-MGB and JUN-MGB.

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

Figure 5. Comparison of results regarding purity and yield obtained from the third-party HPLC and the Vanquish Flex UHPLC. Using the non-Thermo Fisher Scientific method, semi-preparative column, and analytical column, the LC purification systems were compared to demonstrate that the method can be transferred as-is from a non-Thermo Fisher Scientific LC purification system to the Vanquish Analytical Purification LC system. Comparison of (A) the purities and (B) the yields of the oligonucleotide samples on both systems are demonstrating very comparable results.



Conclusions

The focus of this study was 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 derived 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 5, 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.
- In Table 6, the semi-preparative column comparison was made. The result illustrates the capabilities to purify the oligonucleotide samples with passing R&D requirements on the Hypersil GOLD semi-preparative column.

References

1. Roberts *et al.* (2020). Advances in oligonucleotide drug delivery. Nature Reviews Drug Discovery, 19, 673–694.
2. Zhang *et al.* (2016 Dec 18). Recent Methods for Purification and Structure Determination of Oligonucleotides. International Journal of Molecular Sciences, 17(12), 2134.
3. Catani, M et al. (2020). Oligonucleotides: Current Trends and Innovative Applications in the Synthesis, Characterization, and Purification. Biotechnology Journal, 15(8), 1900226.
4. Kanwal et al. (2018). Large-Scale in Vitro Transcription, RNA Purification and Chemical Probing Analysis. Cellular Physiology and Biochemistry, 1915–1927.

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