LC purification

Semi-preparative reversed-phase liquid chromatographic purification of oligonucleotides

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Abstract

Purpose: This work demonstrates the semi-preparative RP-HPLC purification of three different dual-labeled 25mer oligonucleotides, which are applied in quantitative polymerase chain reactions (qPCR), integrated human identification (HID) solutions for forensics, and gene transfer agents (GTA) that make horizontal and lateral DNA transfer possible. By-products of the DNA synthesis are resolved, the target oligonucleotides are successfully isolated and captured.

Methods: Using a Triethylamine acetate(TEAA)/acetonitrile gradient and a highly precise fraction collector integrated in the HPLC system, the oligonucleotides were purified via reversed phase chromatography.

Results: The effectivity of the purification is proven by re-analysis of the collected fractions and analysis of UV absorbing impurities. A 100% relative purity for all re-analyzed fractions was found, showing the precise isolation of target compounds by high resolution fractionation.

Introduction

Nucleic acids are the key to life. 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 many 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]. Reversed-phase high-performance liquid chromatography (RP- HPLC) is commonly used for high-resolution nucleic acid separations [4]. When developing the methods to separate and purify oligonucleotides, some of their unique features need to be considered, such as failure sequences of similar length, secondary structures (e.g., hairpin loops formation), as well as pH and salt concentration (ionic strength), as they affect retention time and alter the interaction with the stationary phase [2].

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, which is of high relevance for international service providers as Eurofins.

Materials and methods

Sample preparation and samples

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.

Sample information

- [CY5]-CAGGTGGAACCTCATCAGGAGATGC-[BHQ2]
- [AT647]-CGTGGTTGACCTACACAGGTGCCATCA-[BHQ2]
- [FAM]-ACCCCGCATTACGTTTGGTGGACC-[BHQ1]

Instrumentation

Thermo Scientific™ Vanquish™ Flex Analytical Purification LC system including:

- Thermo Scientific™ Vanquish™ Binary Pump F
- Thermo Scientific™ Vanquish™ Split Sampler FT with 1000 µL sample loop
- Thermo Scientific™ Vanquish™ Column Compartment H with active pre-heater
- Thermo Scientific™ Vanquish™ Diode Array Detector FG with semi-Micro Biocompatible Flow Cell
- Thermo Scientific™ Integral Vanquish™ Fraction Collector
- Delay capillary for peak-based fractionation, 0.25 × 1500 mm,
- MP35N, with Thermo Scientific™ Viper™ Fingertight Fittings

Chromatography data system

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

Column	Waters Xbridge® BEH C18 OBD™ Prep, 130Å, 5 µm, 10 mm x 100 mm		
Flow rate:	5.0 mL/min		
Mobile phase A	50 mM TEAA, pH 7 with 5% acetonitrile (v/v)		
Mobile phase B	Acetonitrile		
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 incl. active eluent pre-heating)		
Injection volume:	500 µL		
UV detector parameters:	260 nm, 4 nm Bandwidth, 10 Hz DCR, 0.5 RT, 190 – 800 nm 3D field collected		

Table 1: LC method

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
Max. tube volume	1.600 mL
Minimum time for tube change	0 s
Delay volume	81.3 µL determined by automated Delay Volume Determination
Collection Path Mode	Horizontal
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	3.0 s
Rinse Mode	Both
Puncture Offset	2.0 mm
Peak Detection Options	UV 260 nm

Table 2: Fraction collection parameters

Figure 1. Vanquish Flex Analytical Purification LC system

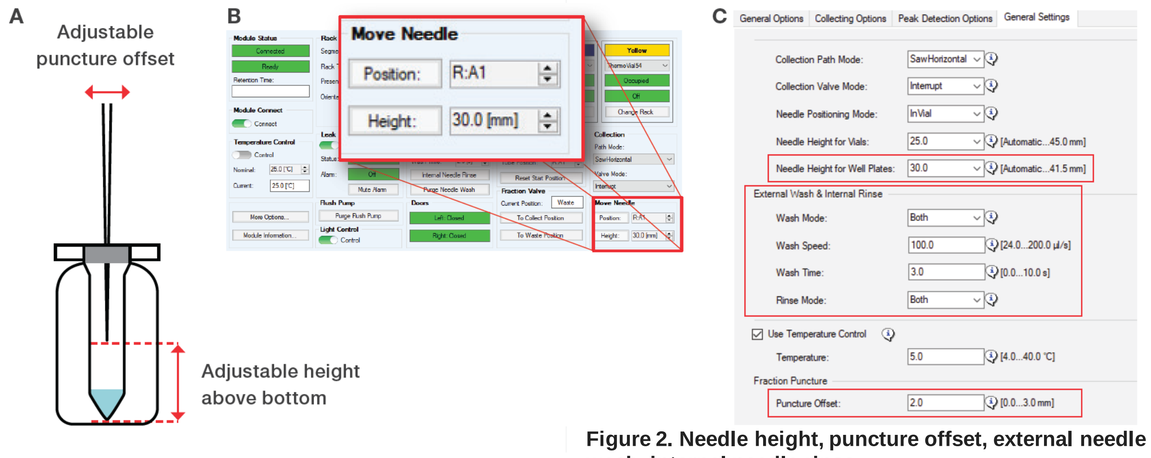

Figure 2. Needle height, puncture offset, external needle wash, internal needle rinse

Results

Analytical step

Each of the provided samples were initially analyzed for purity to evaluate the extent of impurities present in the samples. Each sample analyzed are represented in Figure 1, Figure 2, and Figure 3 showing the overview of the entire chromatographic runs and a zoomed section focusing on the small near-baseline impurities as well as shoulders.

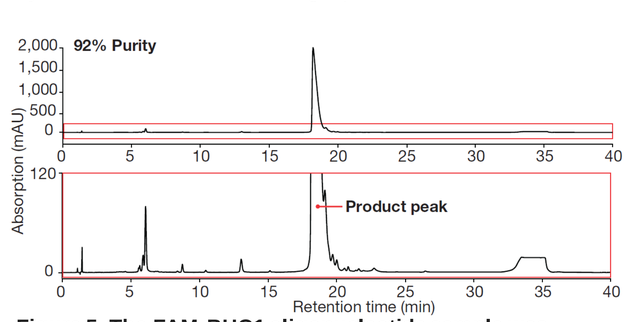

Figure 3. The CY5-BHQ2 oligonucleotide sample analyzed for purity giving initially an 89% purity. Observed in the baseline are numerous byproducts from the synthesis that need to be separated while isolating the main product peak via offline fraction collection.

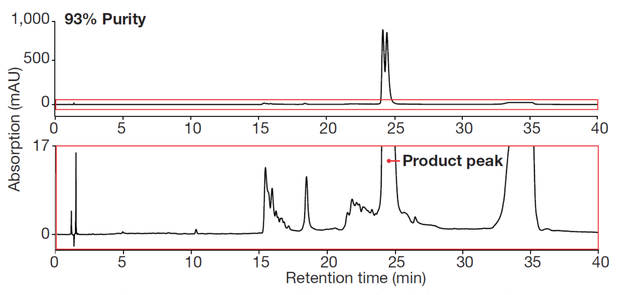

Figure 4. The AT647-BHQ2 oligonucleotide sample was analyzed providing a purity of 93%. The AT647 dye label has inherently two isomers that can be chromatographically separated, but chromatographic separation of these isomers is not required for this application. Therefore, both isomers of the dye label are treated as one target product.

Figure 5. 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**.**

Purification step

Each of the provided samples were initially analyzed for purity to evaluate the extent of impurities present in the samples. Each analyzed sample is represented in a) an overview of sample injected with subsequent fractionation incl. zoom, b) re-analysis of fractions, and c) overlay pre-and post-fractionation.

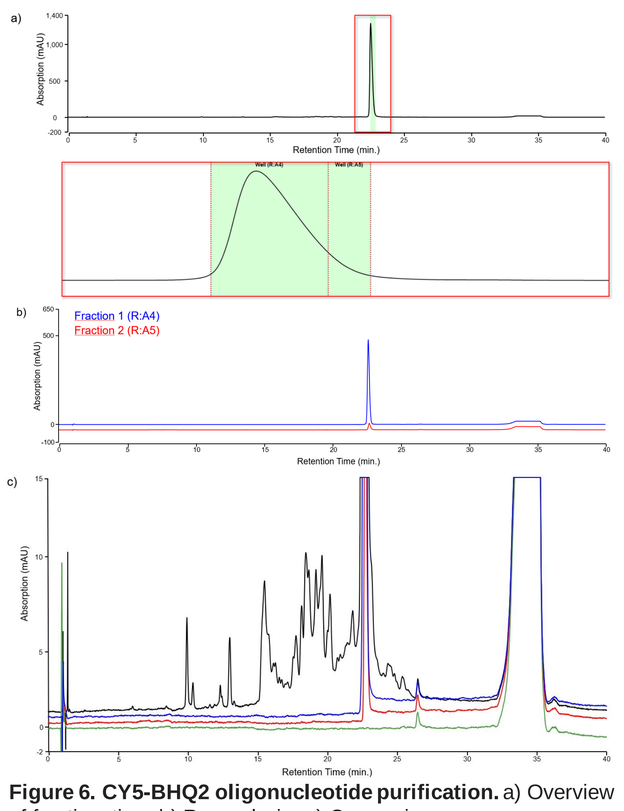

Figure 6. CY5-BHQ2 oligonucleotide purification. a) Overview of fractionation. b) Re-analysis. c) Comparison

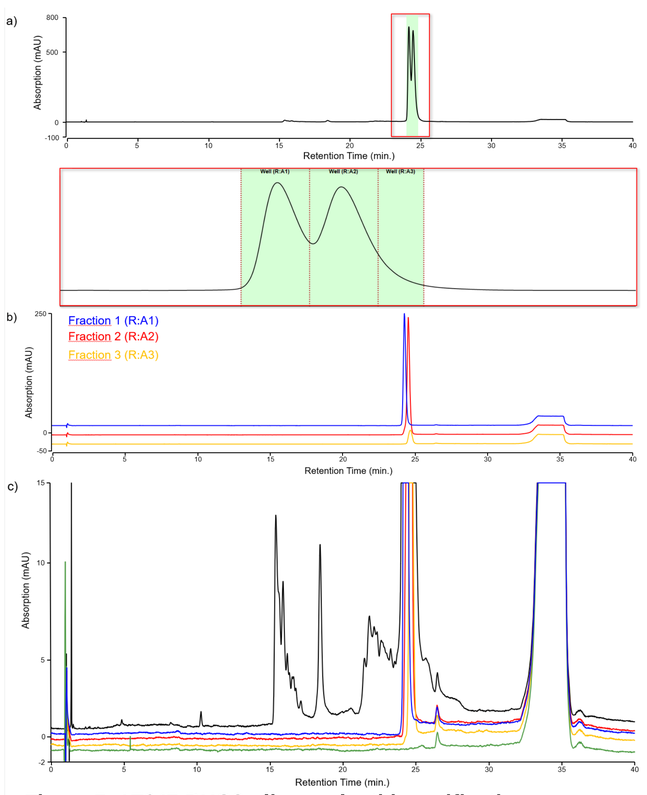

Figure 7. AT647-BHQ2 oligonucleotide purification.
a) Overview of fractionation. b) Re-analysis. c) Comparison

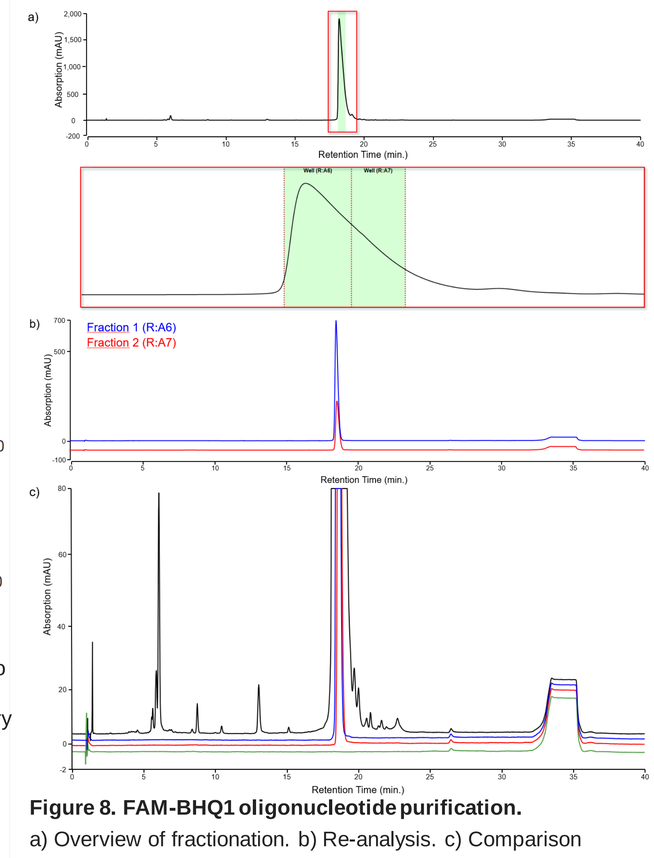

Figure 8. FAM-BHQ1 oligonucleotide purification.
a) Overview of fractionation. b) Re-analysis. c) Comparison

Conclusions

The integration of the Vanquish Flex Analytical Purification LC system [Figure 1] with the Chromeleon CDS into the semi-preparative RP-LC oligonucleotide purification workflow proved to be a great advantage with the resulting purity. The fluidics of the UHPLC system with high resolution performance gives an excellent benefit compared to general semi-preparative to preparative LC instrumentation. The optimized fluidics of the fully integrated Vanquish Fraction Collector in the Vanquish analytical product portfolio complements the excellent purification workflow required in the field of oligonucleotide analysis.

References

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